Examination for platelet-activating factor production by preimplantation mouse embryos using a specific radioimmunoassay*

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Summary. A specific and highly sensitive radioimmunoassay was used to measure platelet-activating factor (PAF) production by preimplantation mouse embryos *in vitro*. Levels of PAF greater than 1 pg per embryo were not observed in 24-h culture medium from 2-cell embryos, compacted morulae or blastocysts, or in extracts from these embryos. Synthetic PAF added to embryos at the start of culture could be almost totally recovered after the incubation period, indicating negligible degradation of PAF during culture. PAF was also not detected in embryo samples using a washed rabbit-platelet aggregation assay. It can be concluded that mouse embryos do not produce substantial levels of PAF, or any of the biologically active analogues of PAF detected by the assay.

Keywords: platelet-activating factor; mouse embryos; radioimmunoassay

Introduction

Platelet-activating factor (PAF), a phospholipid with the structure 1-0-alkyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (Hanahan *et al.*, 1980), is a potent mediator of inflammation. A role for PAF in mediating uterine vascular permeability changes at sites of embryo implantation has been proposed, and is supported by a number of studies. Both human and mouse preimplantation embryos have been shown to secrete a substance with PAF-like activity which appears to be homologous to synthetic PAF (Collier *et al.*, 1988; Ryan *et al.*, 1989). The level of production by human embryos appears to correlate with their potential for implantation and establishment of pregnancy (O'Neill *et al.*, 1987). It has also been demonstrated that direct injection of PAF into the uteri of pseudo-pregnant rats induces a decidua-like response which is similar to that observed after embryo implantation (Acker *et al.*, 1989). The implantation of mouse and rat embryos is inhibited by specific PAF antagonists (Spinks & O'Neill, 1988; Acker *et al.*, 1988), and in the mouse this inhibition is due to a direct or indirect effect of the antagonists on the embryo (Spinks *et al.*, 1990). In addition, O'Neill *et al.* (1989) have shown that supplementation of human embryo culture medium with PAF results in increased pregnancy rates, possibly by stimulation of embryo metabolism. Taken together, all these data suggest that PAF has positive autocrine effects on the early preimplantation mammalian embryo, as well as being an early embryonic signal which mediates the uterine decidual reaction at implantation. However, other workers have been unable to demonstrate PAF activity by rabbit blastocysts (Angle *et al.*, 1988b), or inhibition of an implantation response in mice with PAF antagonists (Milligan & Finn, 1990). A role for embryo-derived

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PAF in early pregnancy therefore remains controversial. It still remains to be determined whether embryos produce PAF, or molecules which have some of the biological properties of PAF but which are distinct structural entities and have different responses in the various assay systems.

The detection and measurement of PAF has traditionally been based on various bioassays involving the action of PAF on platelets which either measure the extent of platelet aggregation, or the release of [^3H]serotonin (Hanahan & Weintraub, 1985). PAF production by human embryos has been assayed using the splenectomized mouse platelet bioassay (O'Neill *et al.*, 1987). However, all these biological assays have problems of large variability within and between assays, and the need for careful control of non-specific interferences which cause platelet activation. The development of a specific radioimmunoassay (RIA) for PAF (Smal *et al.*, 1990b) overcomes some of these problems, and allows accurate quantitation of low levels of PAF in biological samples. In the present study we have applied this RIA in an attempt to detect PAF production by preimplantation mouse embryos.

Materials and Methods

Animals. The 4–5-week-old female F_1 mice (C57BL/6J $\times$ CBA/H) were superovulated with 5 i.u. pregnant mares' serum gonadotrophin (PMSG; Folligon, Intervet, Sydney, Australia) followed 48 h later with 5 i.u. human chorionic gonadotrophin (hCG; A.P.L., Ayerst, Parramatta, NSW, Australia). The females were caged overnight with F_1 males of proven fertility, and examined for copulation plugs the following morning (Day 1 of pregnancy).

Embryo culture. Two-cell embryos were flushed from the oviducts of mice on Day 2 of pregnancy, into M2 medium (Quinn *et al.*, 1982). Compacted morulae were obtained after 24–30 h of culture in drops of M16 medium (Whittingham, 1971) under light-weight paraffin oil (BDH Chemicals, Kilsyth, Victoria, Australia) pre-equilibrated at 37°C in 5% CO_2 in air. To obtain blastocyst stages, embryos were left in culture for an additional 24 h. For analysis of PAF production, 2-cell, compacted morulae, and blastocysts were cultured in fresh M16 medium in 12 $\times$ 75 mm polystyrene round-bottom tubes (Labco, Braeside, Victoria, Australia), without paraffin oil. Between 25 and 45 embryos were cultured in 250 μl of M16 medium for 24 h. Control tubes contained no embryos. In some experiments known concentrations of C_{18} -PAF (see below) were added to the culture tubes at the start of the 24-h incubation time. After the culture period, tubes were centrifuged at 1250 g and the supernatant was transferred to Eppendorf tubes containing 25 μl 1 N-HCl. Acidified samples were immediately frozen and kept at -80°C until assayed. Embryo pellets were frozen directly without acidification.

Extraction of samples. Frozen samples were thawed, sonicated and 240 μl were added to 900 μl chloroform/methanol (1:2 v/v). Embryos alone were suspended in 240 μl 0.1% BSA/PBS before proceeding with extraction. The extraction mixture was vortexed and sonicated extensively before chloroform (300 μl) and water (300 μl) were added to effect phase separation. The upper phase was discarded and the lower chloroform phase was evaporated. The residue was then dissolved in ethanol, this was separated into samples and each sample was evaporated.

Quantitation by RIA. Each sample was reconstituted, with sonication, in 5 μl ethanol and 210 μl assay buffer. The PAF standard solution (0.1 mg/ml in aqueous ethanol, consisting of equal parts C_{16} -PAF and C_{18} -PAF) was diluted in assay buffer to give standard solutions in the range 0.1 to 25 ng/ml. Sera were acid-treated (Smal *et al.*, 1989) to inactivate the enzyme PAF-acetylhydrolase, which hydrolyses PAF to lyso-PAF, and is known to be present in serum (Farr *et al.*, 1980). Acid-treated anti-PAF antiserum was diluted to 1 in 8000 in assay buffer containing acid-treated normal sheep serum (1 in 2000). Donkey anti-sheep immunoglobulin (the second antibody) was diluted 1 in 250 in assay buffer containing 6% polyethylene glycol and [^{125}I]PAF was added to give approximately 40 000 c.p.m. per 100 μl . Into duplicate polystyrene RIA tubes (Disposable Products, Technology Park, SA, Australia) was placed 100 μl of each of the following: sample or PAF standard solution, anti-PAF antiserum, and anti-sheep/tracer. The B_0 tubes contained no PAF, while the non-specific binding (NSB) tubes contained only anti-sheep/tracer. The tubes were incubated for 16 h at room temperature, and 4 ml assay buffer were added. The tubes were centrifuged at 1900 g for 25 min and the supernatants decanted. The remaining radioactivity was counted and the percentage tracer bound to the precipitate ($\% \text{B}/\text{B}_0$) was calculated from the formula $(\text{B} - \text{NSB})/(\text{B}_0 - \text{NSB}) \times 100$. The amount of PAF was determined from the standard curve obtained by plotting PAF concentration against $\% \text{B}/\text{B}_0$.

Platelet aggregation studies. Extracted embryo samples were reconstituted in ethanol (2 $\times$ concentrated compared to original sample) and the mixed PAF standard solution was diluted in ethanol or 0.25% BSA/PBS. Rabbit platelets were isolated and washed as described previously (Pinckard *et al.*, 1979) and to 500 μl stirred (900 rev./min) platelets, suspended in Tyrode's-gelatin buffer pH 7.2 containing 1.3 mM- CaCl_2 , 5 μl of test sample or PAF standard were added. Changes in optical transmission were monitored by a Payton aggregometer (Buffalo, NY, USA). To determine any PAF-inhibitory activity, platelets were treated with 5 μl sample and, 1–2 min later, were stimulated with 50 pg PAF in BSA/PBS, while monitoring the optical transmission.

Materials. The 1-0-hexadecyl PAF (C₁₆-PAF) and 1-0-octadecyl PAF (C₁₈-PAF) were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Poly-ethylene glycol MW 6000 and gelatin were purchased from BDH (Kilsyth, Victoria, Australia) and Bio-Rad (Richmond, CA, USA) respectively. Organic solvents were obtained from Ajax Chemicals (Sydney, Australia). Sheep anti-PAF antiserum, normal sheep serum and donkey anti-sheep immunoglobulin were generous gifts from Silenus Laboratories (Melbourne, Australia) (these are now available from New England Nuclear (Boston, MA, USA) as a PAF RIA kit product number NEK-062). [¹²⁵I]PAF (sp. act. 2200 Ci/mmol) was obtained from New England Nuclear. All other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Phosphate-buffered saline (PBS) was 0.05 M-potassium phosphate pH 7.4 with 0.9% (w/v) NaCl, and the RIA assay buffer was 0.05 M-sodium acetate pH 6.0 containing 0.05% Tween 20.

Results

Measurement of PAF by RIA

Under the assay conditions described, the binding of [¹²⁵I]PAF to anti-PAF antiserum at the concentrations used was 55–60%, while non-specific binding was below 1.6%. The standard inhibition curve was generally linear in the range 25 to 1000 pg PAF per tube, with 50% inhibition occurring with approximately 200 pg. The assay was sensitive down to 10 pg PAF per tube.

Initial attempts to assay directly for PAF in embryo culture medium showed that the presence of BSA in the RIA buffer interfered with the performance of the assay. Samples of culture medium were therefore extracted with chloroform/methanol (Bligh & Dyer, 1959), achieving recovery rates greater than 85%.

Analysis of 24-h cultures of 2-cell embryos, compacted morulae or blastocysts, showed that PAF was not detected in the culture media from any of the embryo stages examined (Table 1). Likewise, immunoreactive PAF could not be detected in direct extracts from the embryos (Table 1). Calculations show that the total amount of PAF in each culture tube was below the sensitivity of the assay, being less than 26 pg per 25–45 embryos.

Table 1. PAF levels in mouse embryos and culture media, as determined by RIA

Development stage of embryos*	No. of embryos	Total cell-associated PAF (pg)	Total PAF in culture medium (pg)
Medium only	—	—	< 26
2-Cell	30	< 25	< 26
Compacted morula	45	< 25	< 26
Blastocyst	25	< 25	< 26

*Embryos were cultured in duplicate in 0.25 ml M16 culture medium, and each sample was assayed in duplicate.

Platelet aggregation studies

Samples of embryo culture medium were also tested for PAF biological activity using the washed rabbit-platelet aggregation bioassay. In each case, levels of PAF were below the detectable limit of 10 pg. To determine whether inhibitors of PAF activity were present in the samples which could inhibit the response of endogenous PAF, platelets were incubated with the extract before stimulation with synthetic PAF. In all cases, the response of platelets to added PAF was similar to that of controls which contained no embryo culture medium or embryo extracts. A typical result is shown in Fig. 1. This indicates that the inability to detect PAF biological activity in these embryo samples is not due to embryo factors which inhibit PAF activity.

Fig. 1. Profiles for the aggregation of rabbit washed platelets stimulated with extracts (5 µl) followed by PAF (50 pg). C, control (ethanol); M, 2-cell embryo culture medium (10 µl) extract; E, extract of 2-cell embryos (equivalent to 1.25 embryos).

Stability of PAF during embryo culture

To determine whether embryos produce enzymes which may degrade PAF during the culture period, culture media with or without embryos were supplemented with known concentrations of C_{18} -PAF at the start of embryo culture. The amount of PAF remaining after 24 h culture at 37°C was determined by RIA (Table 2). The amount of PAF recovered in embryo-free culture medium after 24 h was identical to that added (0 h culture). The presence of embryos in the culture medium resulted in no significant decrease in PAF activity at any concentration of PAF added (1.78, 7.12 and 17.8 ng/ml).

Table 2. Stability of PAF during embryo culture

	Added C_{18} -PAF (ng/ml)*	Incubation time (h)	Observed PAF (ng/ml)†
Medium only	1.78	0	2.14 ± 0.03
Medium only	1.78	24	2.35 ± 0.29
Blastocysts	1.78	24	1.88 ± 0.07
Medium only	7.12	0	7.95 ± 0.49
Medium only	7.12	24	7.65 ± 0.19
Blastocysts	7.12	24	7.20 ± 0.32
Medium only	17.8	0	20.8 ± 0.5
Medium only	17.8	24	20.6 ± 0.8
Blastocysts	17.8	24	18.3 ± 0.8

*Stock concentration was 35.6 ± 1.6 µg/ml, determined by RIA.

†Values are the mean ± s.e.m. of 4 determinations by RIA.

Discussion

The results presented in this study demonstrate that significant quantities of PAF are not produced by cultured mouse preimplantation embryos. Using a highly specific and sensitive RIA with a detection limit of 1 pg per embryo (Smal *et al.*, 1990b), PAF was not observed in the embryo

culture medium or in cell-associated material extracted from 2-cell embryos, compacted morulae, or blastocysts. The possibility that PAF was synthesized and secreted by preimplantation embryos, but was degraded during the culture period was tested by the ability to recover PAF which was added exogenously to the embryo cultures. No significant degradation of exogenous PAF was demonstrated by 2-cell embryos or blastocysts over a 24-h period of incubation. The possibility of intracellular metabolism of endogenous PAF could not be excluded by these experiments.

The PAF RIA described here and elsewhere (Smal *et al.*, 1990b) is based on an antiserum raised to a chemically defined PAF antigen (Smal *et al.*, 1989). Recent combining-site studies have shown that the antiserum is highly specific for PAF (Smal *et al.*, 1990a). The immunoreactivities of PAF analogues that are likely to occur naturally are summarized in Table 3. In brief, the antiserum is specific for (1) an ether-linked alkyl chain at carbon 1 of the glyceryl backbone of PAF, (2) a short-chain acyl group (2 or 3 carbons) at carbon 2, and (3) to a lesser extent for a phosphorylcholine group at carbon 3. Thus the PAF radioimmunoassay will detect, in addition to PAF itself, only the various 1-0-alkyl homologues of PAF, the 2-0-propionyl analogue, and the various N-demethylated analogues. All of these analogues have some degree of biological activity (Braquet & Godfroid, 1987) and should therefore be detected in any measurement of 'PAF'. The analogues of PAF which are not detected by the assay are the 1-0-acyl and 2-0-long-chain acyl analogues. While these are likely to occur naturally, they have little or no biological activity (Braquet & Godfroid, 1987). The commonly occurring lipids show no cross-reactivity at concentrations up to 20 µg/ml (Smal *et al.*, 1990a,b). We conclude, therefore, that mouse embryos do not synthesize either PAF itself, or any of the PAF analogues that have biological activity similar to PAF.

Table 3. Cross-reactivity of PAF analogues in the PAF RIA

Compound	Substituent*	Position†	IC ₅₀ /IC ₅₀ C ₁₆ -PAF
C ₁₆ -PAF	—O—(CH ₂) ₁₅ CH ₃	C1	1.0
C ₁₈ -PAF	—O—(CH ₂) ₁₇ CH ₃	C1	1.85
C _{18:1} -PAF	—O—(CH ₂) ₈ CH = CH(CH ₂) ₇ CH ₃	C1	1.23
Palmitoyl-AGPC	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—C—(CH}_2\text{)}_{16}\text{CH}_3 \end{array}$	C1	> 256
Lyso-C ₁₆ -PAF	—OH	C2	40 000
PGEPC	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—C—CH}_2\text{CH}_3 \end{array}$	C2	4.4
BuGEPC	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—C—(CH}_2\text{)}_2\text{CH}_3 \end{array}$	C2	> 256
HGEPC	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—C—(CH}_2\text{)}_4\text{CH}_3 \end{array}$	C2	> 256
AGEPDME	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—P—O—(CH}_2\text{)}_2\text{N}^+\text{H(CH}_3\text{)}_2 \\ \\ \text{O}^- \end{array}$	C3	5.9
AGEPE	$\begin{array}{c} \text{O} \\ \parallel \\ \text{—O—P—O—(CH}_2\text{)}_2\text{NH}_3^+ \\ \\ \text{O}^- \end{array}$	C3	53.8

*Replacement of the substituent groups in the structure of PAF (1-0-alkyl-2-0-acetyl-*sn*-glycero-3-phosphocholine).

†Carbons 1, 2 and 3 of the glyceryl backbone of PAF.

In addition to negative results in the RIA, we were unable to detect PAF activity in embryo culture media by the washed rabbit-platelet bioassay. Endogenous inhibitors of PAF activity in crude cell extracts which can mask the presence of endogenous PAF have been reported (Miwa *et al.*, 1987). However, embryo extracts used in our study were found to contain no factors which inhibited platelet aggregation in the bioassay.

Our results show that the amount of PAF produced by mouse embryos after 24-h culture is less than 1 pg/embryo, using either the RIA or the bioassay. Very low levels of mouse-embryo PAF (≤ 40 pg/embryo) have been detected using the platelet serotonin-release bioassay (Angle *et al.*, 1988a). Maximal production of PAF appeared to coincide with the morula stage of development, and was influenced by the concentration and source of human serum in the culture medium. The embryo culture methods used in these studies differ from those used by Angle *et al.* (1988b) but nevertheless routinely produce blastocysts which have 80–90% implantation rates after transfer to pseudopregnant foster mice (unpublished observations). The values in the present study differ significantly from the previously published levels of 800–16 700 pg PAF/embryo/24 h, when measured by a rabbit whole-blood bioassay (Collier *et al.*, 1988). The splenectomized mouse bioassay has also been used to quantitate embryo-derived PAF, with similar results (O'Neill *et al.*, 1987). The specificity of these bioassays for PAF detection needs to be questioned, since stimulation of the many cellular components present in these assays may lead to activation of platelets. Indeed, mouse platelets appear to be unresponsive to synthetic PAF (Lanara *et al.*, 1982), suggesting that the decreased platelet count in the splenectomized mouse bioassay might be mediated by factors other than PAF. Culture media of human embryos have been tested by both the splenectomized mouse bioassay and, concurrently, rabbit washed-platelet aggregation assay. PAF-activity was detected by the former but not by the latter assay (Amiel *et al.*, 1989). However, there may be species differences in the production of PAF by preimplantation embryos, and the specific RIA used in this study will be useful for such an analysis.

Although our studies indicate that viable mouse embryos do not produce significant quantities of PAF or one of the biologically active analogues of PAF, the disparity between our results and those previously published may be due to a different molecular nature of embryo-derived PAF. Since no mass spectral data are available for embryo-derived PAF, the exact chemical structure is not known. While the chromatographic and pharmacological behaviour of embryo-derived PAF indicate a PAF-like molecule (Collier *et al.*, 1988), it remains possible that even a small variation from the established structure of PAF (Hanahan *et al.*, 1980) may be sufficient to eliminate immunoreactivity in the RIA, and abolish biological activity as measured by the rabbit washed-platelet bioassay. Further chemical analyses are required before it can be established whether preimplantation embryos produce molecules which are structurally similar to PAF and can hence be called PAF-like, or structurally distinct molecules which nevertheless elicit responses similar to PAF in certain biological assays.

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