Partial characterization of the embryo-derived platelet-activating factor in mice

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Summary. The platelet-activating factor (PAF) produced by mouse embryos showed similar kinetics of action and dose–response curve, in a bioassay, as did 1-0-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (PAF-acether). The activity of the embryo-derived PAF was not affected by inhibitors of the ADP (pyruvate kinase with phosphoenol pyruvate) or cyclo-oxygenase (indomethacin) pathways of platelet activation. Chlorpromazine, an inhibitor of the PAF-acether pathway of platelet activation, caused a significant inhibition of the effects of embryo-derived PAF. Phospholipases A_2, C and D significantly inhibited the activity while lipase had no effect, suggesting a phospholipid structure. All the embryo-derived PAF was found in the chloroform fraction after chloroform:methanol (2:1 v/v) extraction, as was PAF-acether. Both factors migrated at a similar rate (R_f 0·10–0·12) on silica thin-layer chromatography (chloroform:methanol:water; 65:35:4 by vol.). The embryo-derived PAF therefore displays chemical, biochemical and physiological properties similar to those of PAF-acether.

Introduction

The initial maternal response to conception in mice was shown to be platelet activation, resulting in splenic contraction and mild thrombocytopenia (O'Neill, 1985a). This response to the presence of the zygote occurred within hours of fertilization. Early pregnancy-associated thrombocytopenia was not a consequence of the activation of the fluid phase of the blood coagulation cascade or of the maternal immunological recognition of the embryo: the preimplantation mouse embryo produced a soluble platelet-activating factor *in vitro* that had a temporal profile of induction of thrombocytopenia that was similar and a dose–response that was parallel to that caused by the platelet-activating factor, 1-0-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (PAF-acether) (O'Neill, 1985b). Further characterization of the embryo-derived platelet-activating factor was carried out and its biochemical and physiological characteristics were compared with those of PAF-acether.

Materials and Methods

Mice of the Quackenbush strain were exposed to light from 07:30 h to 21:30 h. Females, 6–8 weeks of age, were induced to superovulate by intraperitoneal (i.p.) injection of 5–10 i.u. PMSG (Folligon: Intervet, Boxmeer, The Netherlands) given at 15:00–17:00 h, followed 46–50 h later by

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an i.p. injection of 5–10 i.u. hCG (Chorulon: Intervet). After the second injection females were placed with fertile or vasectomized males and checked the following morning for copulation plugs (= Day 1 of pregnancy). On Day 2 embryos at the 8–16-cell stage, or unfertilized eggs, were collected from the oviducts of pregnant or pseudopregnant mice respectively. Unfertilized eggs and all morphologically normal embryos were pooled and groups of 30 were placed in 200 μl Medium W_1 containing 3 mg BSA/ml (O'Neill & Quinn, 1981) in disposable Petri dishes (Sterilin Ltd, Teddington, Middlesex, U.K.) for 24 h. After culture the embryos or eggs were removed and the culture medium was frozen immediately.

Bioassay for the embryo-derived platelet-activating factor

The assay was carried out as previously described (O'Neill, 1985b). Briefly, medium or test substances were given as a single i.p. injection (300 μl) into splenectomized mice. Platelet counts were performed immediately before and 30 min after injection. Animals were bled under light anaesthesia from the peri-orbital plexus and 10 μl blood were diluted into 2 ml ammonium oxalate (1% w/v). In each assay, samples of PAF-acether were run as positive controls.

PAF-acether (Calbiochem-Behring, Aust. Pty Ltd, Sydney, Australia) was resuspended in chloroform:methanol (2:1, v/v) under N_2 and was then diluted to 3·3 μg/ml in phosphate-buffered saline (pH 7·4) containing 0·25% BSA (Commonwealth Serum Laboratories, Melbourne, Australia).

Experiment 1

The aim of this experiment was to determine whether inhibitors of the three pathways of platelet activation could block the platelet-activating effects of the embryo culture medium.

Indomethacin (Sigma Chemical Co., St Louis, MO, U.S.A.) was prepared as a 1 ml solution in ethanol and then diluted to 10 μM in 0·154 M-NaCl. Various volumes of this solution (10, 20 and 40 μl) were mixed with 300 μl of control medium, embryo culture medium or PAF-acether and the solutions were tested in the mouse bioassay.

Following the method of Haslam (1964), stock solutions of 28 mM-phosphoenol pyruvate (Calbiochem-Behring) and 300 U pyruvate kinase/ml (EC 2.7.1.40: Calbiochem-Behring) in Medium W_1 were prepared. Some (10 μl) of each stock was added to 200 μl of control medium, embryo culture medium or PAF-acether. The solutions were incubated at 37°C for 30 min before injection into splenectomized mice.

Chlorpromazine HCl (Chlorpromazine injection B.P., David Bull Laboratories Pty Ltd, Mulgrave, Victoria) was mixed with Medium W_1 to give a final concentration of 150, 300 and 450 μg/ml. Aliquants (200 μl) of other solutions were tested in splenectomized mice.

Experiment 2

Structural analysis of PAF-acether using phospholipases and lipase was described by Benveniste, Le Couedic, Polonsky & Tence (1977), and similar studies were performed on embryo-derived PAF. Control and embryo culture media were treated with the following enzymes at a final concentration of 0·5 i.u./ml: phospholipase A_2 (EC 3.1.1.4: from pig pancreas; Sigma); phospholipase C (EC 3.1.4.3: from *B. cereus*; Sigma); phospholipase D(EC 3.1.4.4: from cabbage; Sigma); and lipase (EC 3.1.1.3: pancreas; Calbiochem-Behring). Phospholipases A_2 and C were incubated for 30 min at 37°C with regular agitation. Phospholipase D was incubated for 1 h at room temperature and lipase was left for 2·5 h at 37°C with constant agitation. Immediately after treatment, the media were injected into splenectomized mice.

Experiment 3

Extraction of the lipid fraction of culture medium was carried out as described by Lotner, Lynch, Betz & Henson (1980). Culture media were extracted with a chloroform:methanol:water (6:6:5·4, by volume) system. Methanol (A.R. grade, Ajax Chemical Co., Australia) was added to the culture medium, or to the PBS–BSA containing PAF-acether, in a ratio of 6:4 (v/v). The contents were mixed at room temperature for 45 min. The protein was precipitated by the methanol and removed by centrifugation at 4000 g for 20 min at 4°C. Water and chloroform (Ajax Chemicals) were added to the supernatant with gentle mixing. The methanol–water layer was removed by a Pasteur pipette and the lipid-containing chloroform phase was retrieved.

The solvent was evaporated under a stream of N_2. The residue was resuspended in PBS–BSA for testing in the splenectomized mouse assay or in a minimal volume of chloroform:methanol (2:1 v/v) for characterization by thin-layer chromatography (t.l.c.). The samples were spotted onto silica-gel t.l.c. plates (silica gel, 0·2 mm thick: E. Merck AG, Darmstadt, West Germany) which had been previously washed with methanol. The plates were run in a solvent system of chloroform:methanol:water (65:35:4 by volume) (Lotner *et al.*, 1980). Sections (10 mm × 3 mm) of the chromatogram were scraped from the plate and suspended in chloroform:methanol (2:1 v/v) to elute active fractions, and the silica was removed by centrifugation at 700 g for 10 min. Each section was a progression of approximately 0·02 R_f units. The eluting substances were dried under N_2 and the residue was resuspended into 300 μl, PBS–BSA and tested in the splenectomized mouse system.

Results

Embryo culture medium induced thrombocytopenia ($P < 0.01$) in splenectomized mice while medium in which unfertilized eggs were cultured had no significant effect (Table 1). The platelet-activating effects of embryo culture medium were not altered by treatment with indomethacin or

Table 1. The relative change to the platelet count from splenectomized mice caused by: (1) culture medium in which unfertilized eggs were incubated; (2) culture medium in which 8–16-cell embryos were cultured for 24 h and (3) PBS containing 3·3 μg PAF-acether/ml and the effect of treatment of these fluids with various inhibitors of platelet activation

Treatment	Platelet count relative to prior count (%)		
	Control medium	Embryo medium	PAF-acether
Control			
(untreated media)	103·1 ± 4·7	72·1 ± 3·1	65·3 ± 8·1
Phosphoenol pyruvate			
and pyruvate kinase	106·6 ± 4·9	70·6 ± 5·7	63·9 ± 3·8
Indomethacin	101·3 ± 6·1	69·0 ± 2·1	61·8 ± 3·8
Phosphoenol pyruvate/			
pyruvate kinase and			
indomethacin	104·9 ± 3·6	70·2 ± 2·1	67·1 ± 4·3
Control	104·7 ± 3·0	72·7 ± 2·9	67·1 ± 3·8
Chlorpromazine	98·7 ± 4·5	90·6 ± 2·3*	83·3 ± 3·7*
Control	101·2 ± 1·9	73·5 ± 3·7	68·1 ± 1·2
Phospholipase A_2	92·1 ± 6·7	91·8 ± 2·3*	79·9 ± 2·1*
Phospholipase C	106·1 ± 3·2	86·5 ± 3·6*	78·3 ± 2·7*
Phospholipase D	94·7 ± 4·5	93·6 ± 6·4*	80·1 ± 3·5*

The results are the mean ± s.e.m. of 3 replicates.
*$P < 0.05$ compared with control value (analysis of variance).

Table 2. The platelet count of splenectomized mice after injection of the lipid fraction, before and after thin-layer chromatography (t.l.c.) of control and embryo culture medium and platelet-activation factor (in PBS containing 0·25% BSA)

Treatment	Platelet count relative to prior count (%)		
	Control	Embryo	PAF
Untreated medium	98·8	74·2	70·8
Chloroform fraction	105·1	104·2	99·1
t.l.c. fractions			
(R_f units)			
0·02–0·04	105·2	104·4	99·8
0·04–0·06	103·4	100·0	106·2
0·06–0·08	96·8	99·7	102·7
0·08–0·10	102·4	98·1	94·3
0·10–0·12	100·7	80·5	76·4
0·12–0·14	97·3	86·3	79·4
0·14–0·16	104·8	106·3	104·3
0·16–0·18	101·7	101·7	97·6
0·18–0·20	109·3	98·4	101·2

The results are the mean of 2 replicates.

the removal of ADP by phosphoenol pyruvate and pyruvate kinase (Table 1). Chlorpromazine caused a significant inhibition of the effects of embryo-derived PAF and PAF-acether (Table 1). Both substances showed a significant reduction in activity after treatment with phospholipase A_2, phospholipase C and phospholipase D (Table 1): lipase had no inhibitory effect on the activity of the embryo culture medium or PAF-acether.

The lipid fraction of the embryo culture medium contained all of the embryo-derived PAF. The identity of the factor was further characterized by t.l.c. The bulk of the activity migrated at R_f 0·10–0·12 (Table 2). Synthetic PAF-acether displayed an identical profile (Table 2).

Discussion

Promoters of platelet activation are many and varied, but can be divided into a number of categories: (1) macromolecules, e.g. collagen; (2) enzymes, e.g. thrombin; (3) small molecules, e.g. adrenaline, ADP, PGs and PAF-acether; and (4) particles, e.g. antigen–antibody complexes and latex (reviewed by Holmsen, Salganicoff & Fukami, 1977). Despite this array of known activators, they are all thought to act via one of three pathways (Haslam, 1964) which converge to give a single response. Such 'stimulus–response coupling' by platelets simplifies, to some extent, the study of the causes of platelet activation.

The three pathways of platelet activation are initiated by: (1) adenosine diphosphate (ADP) (Born, 1962); (2) the products of cyclo-oxygenase action on arachidonic acid (Vargaftig & Zirinis, 1973); and (3) platelet-activating factor (PAF-acether) (Chignard, Le Couedic, Tence, Vargaftig & Benveniste, 1979). In any particular circumstance it is possible to test which pathway is responsible by their sequential and concurrent inhibition. The ADP pathway is inhibited by the addition of phosphoenol pyruvate and pyruvate kinase which converts ADP to ATP (Born, 1962). The enzyme cyclo-oxygenase converts arachidonic acid to endoperoxide derivatives (Samuelsson, 1969) which cause platelet activation. This enzyme is inhibited by aspirin or indomethacin (Ferreira & Vane, 1974) and so indomethacin blocks the second pathway of activation. The PAF-acether pathway is characterized by its property of causing platelet activation in the presence of inhibitors of the adenosine diphosphate- and cyclo-oxygenase-dependent pathways (Chignard *et al.*, 1979). The

involvement of this pathway is confirmed by examination of the biochemical properties of the activator, i.e. its sensitivity to phospholipases (Benveniste *et al.*, 1977), its characteristic mobility during t.l.c. (Tence, Polonsky, Le Couedic & Benveniste, 1980), and its inhibition by membrane-active drugs such as chlorpromazine (Cazenave, Benveniste & Mustard, 1979).

The present results confirm earlier reports (O'Neill *et al.*, 1985; O'Neill, 1985b) that the pre-implantation embryo of the mouse produces a platelet-activating factor. It was previously reported that neither ADP nor arachidonic acid could mimic the effects of the embryo-derived PAF in the mouse bioassay (O'Neill, 1985b). In support of those findings, the platelet-activating effect of embryo culture medium was not altered by inhibition of the ADP and cyclo-oxygenase pathways of platelet activation (Table 1) and therefore did not appear to be acting along either of the first two pathways of platelet activation.

Embryo-derived PAF had a temporal profile of induction of thrombocytopenia in splenec-tomized mice that was similar to and a dose–response curve that was parallel to that caused by PAF-acether (O'Neill, 1985b). Both factors were partly deactivated by phospholipases A_2, C and D but not by lipase (Table 1). The degree of deactivation was greater for embryo-derived PAF than for PAF-acether. This may suggest different chemical characteristics or it may be due to slight difference in concentration of the two chemicals. The dose–responses of both are relatively flat at these doses (O'Neill, 1985b) so that apparently similar doses may in fact be significantly different. Chlorpromazine caused a significant inhibition of the platelet-activating effects of embryo-derived PAF and PAF-acether (Table 1).

After chloroform:methanol extraction embryo-derived PAF and PAF-acether activity were found in the chloroform fraction and both factors displayed the same rate of migration (R_f 0·10–0·12) on silica t.l.c. (Table 2). Using the same methods, Lotner *et al.* (1980) showed that PAF-acether had an R_f value of 0·10–0·11.

Insufficient material from the embryo culture medium has been collected to provide definitive chemical characterization of the platelet-activating factor produced by the zygote. Tence *et al.* (1980) proposed that if platelet activation occurs independently of the ADP and cyclo-oxygenase pathways and if the factor is partly purified and shown to migrate with characteristic mobility on t.l.c. or HPLC, that is sufficient evidence to assign the activation to the PAF-acether pathway. Embryo-derived PAF fulfils these criteria and it displays other chemical, biochemical and physio-logical similarities to PAF-acether.

PAF-acether is synthesized by the release of 1-0-alkyl-glycero-3-phosphocholine from phos-pholipids by phospholipase A_2 followed by the addition of the acetyl moiety of acetyl-coenzyme A by acetyl transferase (Ninio, Mencia-Huerta, Heymans & Benveniste, 1982). The preimplantation mouse embryo was capable of incorporating methyl-[^{3}H]choline into phosphocholine, phos-phatidylcholine and lysolecithins, via the Kennedy pathway (Pratt, 1980). Embryo extracts showed considerable phospholipase A activity, resulting in the deacylation of exogenous phospholipids (Pratt, 1980). Whether preimplantation embryos also contain an acetyl transferase capable of con-verting the lyso-phosphocholines into active PAF-acether is still to be determined. Preimplantation embryos, prelabelled with [^{3}H]choline, lost 10–40% of labelled phospholipids during a 24-h culture period and it was suggested that this was due to turnover of membrane phospholipids (Pratt, 1980). In view of the present findings, it will be necessary to perform pulse–chase studies to determine whether some of this label is released as embryo-derived PAF.

References

Benveniste, J., Le Couedic, J.P., Polonsky, J. & Tence, M. (1977) Structural analysis of purified platelet-activating factor by lipase. *Nature, Lond.* **259**, 170–171.

Born, G.V.R. (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature, Lond.* **194**, 927–929.

Cazenave, J.P., Benveniste, J. & Mustard, J.F. (1979)

Aggregation of rabbit platelet by platelet-activating factor is independent of the release reaction and the arachidonate pathway and inhibited by membrane active drugs. *Lab. Invest.* **41**, 275–285.

Chignard, M., Le Couedic, J.P., Tence, M., Vargaftig, B.B. & Benveniste, J. (1979) The role of platelet activating factor in platelet aggregation. *Nature, Lond.* **279**, 799–800.

Ferreira, S.H. & Vane, J.R. (1974) Aspirin and prostaglandins. In *The Prostaglandins*, Vol. 2, pp. 1–34. Ed. P. Ramwell. Plenum Press, New York.

Haslam, R.J. (1964) Role of adenosine diphosphate in the aggregation of human blood-platelets by thrombin and by fatty acids. *Nature, Lond.* **202**, 765–768.

Holmsen, H., Salganicoff, L. & Fukami, M.H. (1977) Platelet behaviour and biochemistry. In *Haemostasis: Biochemistry, Physiology and Pathology*, pp. 239–319. Eds D. Ogston & B. Bennett. John Wiley and Sons. London.

Lotner, G.Z., Lynch, J.M., Betz, S.J. & Henson, P.H. (1980) Human neutrophil derived platelet activating factor. *J. Immunol.* **124**, 666–684.

Ninio, E., Mencia-Huerta, J.M., Heymans, F. & Benveniste, J. (1982) Biosynthesis of platelet activating factor. I. Evidence for an acetyl-transferase activity in murine macrophages. *Biochim. Biophys. Acta* **710**, 23–31.

O'Neill, C. (1985a) Thrombocytopenia is an initial maternal response to fertilization in mice. *J. Reprod. Fert.* **73**, 559–566.

O'Neill, C. (1985b) Examination of the cause of early pregnancy-associated thrombocytopenia in mice. *J. Reprod. Fert.* **73**, 567–577.

O'Neill, C. & Quinn, P. (1981) Interaction of the uterine flushings with mouse blastocysts *in vitro* as assessed by the incorporation of [^{3}H]uridine. *J. Reprod. Fert.* **62**, 257–262.

O'Neill, C., Pike, I., Porter, R.N., Gidley-Baird, A.A., Sinosich, M.J. & Saunders, D.M. (1985) Maternal recognition of pregnancy prior to implantation—methods for monitoring embryonic viability in-vitro and in-vivo. *Ann. N. Y. Acad. Sci.* **680**, (in press).

Pratt, H.D.M. (1980) Phospholipid synthesis in the preimplantation mouse embryo. *J. Reprod. Fert.* **58**, 237–248.

Samuelsson, B. (1969) On the incorporation of oxygen in the conversion of 8,11,14–eicosatrienoic acid to prostaglandin E$_1$. *J. Am. Chem. Soc.* **87**, 3011–3013.

Tence, M., Polonsky, J., Le Couedic, J.P. & Benveniste, J. (1980) Release, purification and characterization of platelet-activating factor. *Biochimie* **62**, 251–259.

Vargaftig, B.B. & Zirinis, P. (1973) Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from PGE$_2$ and PGF$_2$. *Nature, New Biol.* **244**, 114–116.