Embryonic Platelet-Activating Factor: Temporal Expression of the Ligand and Receptor

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Submitted July 23, 2001; accepted September 18, 2001

Purpose: Preimplantation embryos synthesize platelet-activating factor (PAF) and this embryo-derived PAF is required for development. PAF's signal transduction is receptor-mediated and PAF-receptor mRNA is present in early embryos. The study objective was to determine the relationship between PAF production and PAF-receptor mRNA expression levels in mouse preimplantation embryos.

Methods: Embryo-derived PAF levels were determined by radioimmunoassay. Embryonic PAF-receptor mRNA levels were determined by semiquantitated reverse transcription-polymerase chain reaction.

Results: Embryonic-PAF increased as time progressed at a relatively constant rate ($1.4\text{--}1.9\times$) except between the eight- and morula-cell stages where levels increased sevenfold. Embryonic PAF-receptor expression was highest at the two-cell stage and decreased steadily until the morula stage before increasing again. Regression analysis of embryo-derived PAF on PAF-receptor expression does not demonstrate a significant relationship.

Conclusions: PAF-receptor expression (mRNA) levels decrease, while embryo-derived PAF levels decrease, as the preimplantation embryo develops. Embryonic-PAF and PAF-receptor mRNA expression do not appear related. Therefore, embryonic-PAF does not appear to regulate expression of its own receptor in vitro. The data provide a clue to the complicated cell signaling system involving PAF production and receptor expression that may help our understanding of developmental events.

KEY WORDS: Mouse; embryo development; platelet-activating factor; receptor expression.

INTRODUCTION

Platelet-activating factor (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is a unique and novel signaling phospholipid that plays a significant role during fertilization and the preimplantation

period. Although the production of PAF by preimplantation embryos has been reported, physiologic roles of this potent mediator remain unclear. Embryonic PAF has been suggested to serve as a survival factor (1). Embryonic-PAF is synthesized and released by rabbit and mouse embryos during the preimplantation phase, with maximum levels at the expanded blastocyst stage (2,3). PAF production by human embryos has been correlated with pregnancy potential (4,5). Embryonic-PAF can act as an autocrine stimulator of embryo development (6,7), which can be blocked by PAF-antibodies or antagonists (8–10). Supplementation of culture medium with PAF improves two-cell development to the blastocyst stage in the mouse (11), apparently by stimulation of embryonic metabolism (6). The effect

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of exogenous PAF on in vitro mouse embryo development appears to be mouse-strain (12) and ligand-isoform (13) specific. PAF's mechanism of action in the embryo appears to be receptor-mediated since different PAF-antagonists competitively inhibit its action (9,10). Only just recently has the PAF-receptor been characterized in the mouse (14–16). Additionally, in other cell types, PAF has been found to influence expression of its own receptor (17). The study objective was to determine if a relationship exists between PAF production and PAF-receptor expression levels during preimplantation stage embryo development in the mouse model.

MATERIALS AND METHODS

Embryo Collection and Culture

Mice were housed in a specific-pathogen free facility with a 14 h light: 10 h dark photoperiod at 23°C and food and water provided ad libitum. Two-cell stage preimplantation embryos and white blood cells (WBC; PAF-receptor positive control for RT/PCR) were collected from PMSG and hCG (10 IU ea), primed Swiss-Webster (CFW; Charles River Breeding Labs, Wilmington, MA) female mice (4 weeks of age) mated with fertile CFW male (6 months of age) mice. Interval between PMSG and hCG was 46–48 h. Embryos ($n = 100$) were collected 16–18 h post-hCG and cultured (50 μ L microdrop) in triplicate in modified Minimum Essential Medium- α modification (α -MEM; Sigma, St. Louis, MO) under sterile-equilibrated mineral oil in an atmosphere of 5% CO₂ in air, 95% relative humidity at 37°C, as previously described (18). Embryos were cultured until a majority (>75%) of the embryos had reached the desired preimplantation embryonic-cell stage [i.e. two-cell stage (24 h), four-cell stage (36 h), eight-cell stage (48 h), morula-cell stage (72 h), and blastocyst-cell stage (96 h)]. Following the appropriate culture period the embryos were removed and the conditioned culture media recovered and treated with an equal volume of 20% glacial acetic acid [to prevent PAF degradation by PAF-acetyl hydrolases that may be present (19)] for phospholipid extraction.

Radioimmunoassay

Embryo-derived PAF levels were determined via a PAF specific radioimmunoassay, according to the manufacturer's instructions (NEN Research Products, DuPont, Boston, MA). Briefly, primary

antibodies were added to tubes containing the extracted embryo-derived PAF samples or working assay buffer. Tubes were mixed and incubated for 15 min at room temperature. Secondary antibodies and tracer were added to samples, standards, blanks and total count tubes, mixed and incubated for 24 h at room temperature. Following centrifugation (2000g; 30 min), the supernatant was decanted and the tubes blotted and counted in a Packard gamma counter (Packard Instrument, Downers, IL) for 1 min. The standard curve was calculated by regression analysis (logit value of normalized percent bound vs. log of ng PAF assayed). Content of extracted embryo-derived PAF is expressed as pmol per embryo.

Ribonucleic Acid Extraction

Ribonucleic acid (RNA) was extracted utilizing the Micro RNA Isolation Kit (Stratagene Cloning Systems, La Jolla, CA) per manufacturer's instructions. Embryos (1500 per cell stage) or WBC were added to individual 0.5 mL polypropylene tubes (sterile-RNase free) containing denaturing buffer (4 M guanidine thiocyanate, 1 M 2 β -mercaptoethanol, 0.02 M sodium citrate) on ice. Embryo and WBC lysis and homogenization were performed by liquid nitrogen flash-freezing. Embryonic and WBC RNA were precipitated by addition of 2 M sodium acetate (pH 4), water-saturated phenol and chloroform: isoamyl alcohol. Following a vigorous mixing, the nucleic acids were collected by centrifugation at 14,000g for 5 min at 4°C and the upper RNA phase precipitated with ice-cold isopropanol plus glycogen (2 mg/mL) followed by a second wash with 70% ethanol. The pellets were resuspended in DEPC-treated water and stored at –20°C. Total embryonic RNA recovered at each cell stage was determined by spectrophotometry.

Reverse Transcription (RT)

Embryonic and WBC RNA were placed into chilled first-strand buffer mixture: 1 $\times$ M-MLV RT reaction buffer, oligo dT, 5 mM 4 dNTP chase solution, 2 Units RNasin, embryonic-RNA (10 ng/ μ L), and 10 units M-MLV RT. One round of RT was carried out, 80°C, 3 min; 42°C, 60 min; 95°C, 5 min. Synthesized cDNA was frozen at –20°C until used for polymerase chain reaction amplification.

Qualitative Polymerase Chain Reaction (PCR)

The RT products were amplified in PCR reaction buffer, 50 pM PAF specific primer pair [upper

Fig. 1. Qualitative RT-PCR analysis of in vitro PAF-receptor expression at each developmental stage. Column 1: 123 bp DNA standard; column 2: white blood cell; positive (PAF-receptor) control; column 3: two-cell stage embryo; column 4: four-cell stage embryo; column 5: eight-cell stage embryo; column 6: morula embryo; column 7: blastocyst embryo. Row 1: PAF-receptor (600 bp); row 2: *Cyclophilin* positive (embryonic) control (200 bp).

primer: AGCGGCATCTCTTTGTCCC; lower primer: GGAGTCTGGTTGGC TGGCACTA (16)], 10× PCR buffer, and *Taq* DNA polymerase. The reaction mixture was amplified for 30 cycles at 94°C, 30 s; 55°C, 30 s; 72°C, 1 min. Following the final PCR round, the samples were heated for 5 min at 72°C then held at 4°C. Synthetic *Cyclophilin* (*Cyclophilin* Armored RNA Competicon, Ambion, Austin, TX) was used as an embryonic positive control primer (216 bp). *Cyclophilin* (peptidyl-prolyl, cis-trans isomerase) is an abundant, constitutively expressed, “housekeeping” gene that encodes a 162 amino acid protein in the mouse preimplantation embryo (20,21). The RT-PCR products and a 123 bp DNA standard ladder (Sigma) were analyzed by 1.5% agarose gel electrophoresis and transmitted ultraviolet light (Fig. 1). PCR without RT served as a negative control.

Semiquantitative Polymerase Chain Reaction (PCR)

Semiquantitated PCR was performed in duplicate utilizing the PCR MIMIC Construction Kit (Clontech Laboratories, Palo Alto, CA) per manufacturer’s instructions. This technique is based on a competitive PCR approach using nonhomologous internal standards referred to as PCR MIMICS. In competitive PCR, the second DNA fragment (PCR MIMIC) competes with the target DNA and acts as an internal standard. The internal standard is a nonhomologous PAF-receptor DNA fragment that has the same primer template as the target DNA but is designed to generate a PCR product of a different size (300 bp) than the target DNA (600 bp). By knowing the amount

of MIMIC added to the reaction, we are able to determine mRNA levels. The RT products were amplified in PCR reaction buffer: PAF specific primer pair (0.4 μ M each), PAF-receptor MIMIC specific primer pair (0.4 μ M each); 10× PCR buffer, dNTP mix (0.4 μ M each), *Taq* polymerase (0.04 μ M each), $MgCl_2$ (2 μ M each), MIMIC cDNA (serial dilutions; e.g. 1.56×10^{-4} to 5.0×10^{-3} μ M). The reaction mixture was amplified for 30 cycles at 94°C, 30 s; 60°C, 1 min; 72°C, 1 min. Following the final PCR round, the samples were heated for 5 min at 72°C, then held at 4°C. The RT-PCR products were visualized following 1.5% agarose gel electrophoresis with the fluorescent dye ethidium bromide and transmitted ultraviolet light (e.g. Fig. 2). By visual evaluation of electrophoretic bands, we compare band intensities, generated by the PAF-receptor MIMIC against those generated by the target PAF-receptor. A 123 kb DNA ladder was used to approximate size the cDNA fragments. Content of PAF-receptor mRNA in early embryos is expressed as amol per embryo.

Statistical Analysis

Embryo-derived PAF production data were analyzed by one-way analysis of variance and all pairwise comparisons analyzed by Tukey’s test. The relationship between embryo-derived PAF and PAF-receptor mRNA expression levels was analyzed by regression analysis. Statistical calculations were performed with SigmaStat for Windows, version 2.03 (Jandel Scientific, San Rafael, CA).

RESULTS

Embryo-derived PAF was detected in all conditioned embryo culture media and the data [mean

Fig. 2. Illustration of the MIMIC-PCR analysis of PAF-receptor expression. Column 1: 123 bp DNA standard; column 2: empty; column 3: positive control mimic primer; column 4: negative control; column 5: 5.0×10^{-3} amol; column 6: 2.5×10^{-3} amol; column 7: 1.25×10^{-3} amol; column 8: 6.25×10^{-4} amol; column 9: 3.125×10^{-4} amol; column 10: 1.56×10^{-4} amol. Row 1: PAF-receptor (600 bp); row 2: MIMIC PAF-receptor (300 bp).

Table I. In Vitro Embryonic-PAF Production in CFW Mice^{a,b}

	Hours				
	24	36	48	72	96
pmol per embryo ($\pm$ SEM)	6.03 (1.69)	8.32 (1.65)	13.63 (2.61)	96.29 (13.35)	183.56 (13.83)
Difference from previous stage (relative increase)	na	2.29 (1.4 $\times$)	1.64 (1.64 $\times$)	82.66 (7.1 $\times$)	87.27 (1.91)

^a ANOVA analysis revealed the data are significantly different ($P < 0.001$).

^b All pairwise comparisons (Tukey's test) were significantly different ($P < 0.01$).

($\pm$ SE) pmol per embryo] are presented in Table I and illustrated in Fig. 3. The lowest amount of embryonic-PAF measured in conditioned embryo culture media was in the 24-h [6.03 picomoles/embryo] culture group and the highest in the 96-h (183.56 pmol/embryo) culture group. Embryonic-PAF levels increased as time progressed at a relatively constant rate (1.4–1.9 $\times$) except for between the 48th and 72nd hours of culture (eight-cell to morula stage) where in vitro embryonic-PAF production increased by sevenfold. Analysis of variance revealed a significant difference ($P < 0.001$) among all groups in embryonic-PAF production. All pairwise multiple comparisons were also found to be significantly different ($P < 0.01$).

The presence of in vitro cultured embryonic mRNA at each preimplantation embryonic-cell stage encoding the PAF-receptor was demonstrated using RT-PCR. Complimentary DNA from mouse CFW preimplantation embryos was amplified as described, and each preimplantation embryonic-cell stage produced a single band of the predicted 600 bp size (Fig. 1). As expected, *Cyclophilin* expression remained relatively constant throughout development. Sequence analysis and GenBank query of the

RT-PCR products confirmed the identity for the PAF-receptor. Embryonic PAF-receptor expression was highest at the two-cell stage (3.13 amol/embryo) and decreased steadily until the morula stage (0.14 amol/embryo) before beginning to rise again (Fig. 4). The coefficient for regression (Fig. 5) of embryo-derived PAF production on PAF-receptor expression was not significant. Thus, PAF and PAF-receptor expression do not appear to be related in preimplantation stage mouse embryos. Embryonic-PAF does not appear to regulate its own receptor.

DISCUSSION

This is the first report on the temporal expression of PAF and the PAF-receptor throughout the preimplantation embryonic period. Embryo-derived PAF production levels peaked around the time interval when the mouse preimplantation embryo normally enters the uterus. The highest production levels for embryonic-PAF were at the blastocyst stage, confirming earlier reports (3,22). Preimplantation stage embryos in a variety of species (human, mouse, sheep,

Fig. 3. Bar graph illustration of embryonic-PAF production levels in vitro (with standard curve). ANOVA analysis revealed the data are significantly different ($P < 0.001$); all pairwise comparisons (Tukey's test) were also significantly different ($P < 0.05$).

Fig. 4. Bar graph illustration of quantitative RT-PCR analysis of PAF-receptor expression in preimplantation mouse embryos in vitro.

and rabbit) produce and release PAF (23–25). Furthermore, mouse preimplantation embryos cultured in the presence of PAF have an enhanced developmental (26) and higher implantation rates upon transfer to synchronized recipients that may be due, in part, to stimulation of embryonic metabolism by embryo-derived PAF (27). PAF directly influences the oxidative metabolism of glucose and lactate in the mouse preimplantation embryo (28). Cholinephosphotransferase and acetyltransferase (the enzymes that catalyze the final step in the biosynthetic

pathways for PAF production) are present in mouse preimplantation stage embryos (19). PAF antibodies inhibit embryo development (9) and antagonists inhibit implantation (29,30) providing further evidence, albeit indirect, for the presence and requirement of embryo-derived PAF during the pre- and periimplantation periods. Additionally, PAF production by human embryos is related to their subsequent pregnancy potential (4).

Molecular evidence for the PAF-receptor was found at each preimplantation developmental stage.

Fig. 5. Bar graph illustration of in vitro PAF ligand and receptor levels at each developmental stage.

PAF-receptor expression was highest at the two-cell stage and lowest at the morula stage. The results further demonstrate that PAF-receptor expression decreases as the embryo develops from the two-cell stage through the morula stage before increasing again at the blastocyst stage. Although Sharkey *et al.* (31) were unable to detect the transcript encoding the cloned PAF-receptor in a single human preimplantation stage embryo, several reports have demonstrated the presence of PAF-receptor transcripts throughout preimplantation embryo development in the mouse (15,16). Embryo-derived PAF does not appear to regulate expression of its own receptor. However, additional studies are required to further elucidate this finding. We are currently investigating the effect of exogenous PAF, as well as the effect of PAF-antagonists, or embryonic PAF-receptor expression throughout the preimplantation period.

The cDNA for the PAF receptor cloned from a mouse macrophage cDNA library is a G-protein-linked receptor with seven transmembrane loops and has an open reading frame encoding 341 amino acid residues and was mapped to a region of the D2.2 band of Chromosome 4 (32). PAF receptors, expressed by the transformed endometrial cell line HEC-IB, are functionally linked to inositol lipid hydrolysis, the mobilization of intracellular-free calcium and activation of a tyrosine kinase pathway (33). Although not reported in preimplantation stage embryos, PAF binds to cell surface receptors causing the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (34). Exogenous PAF has been demonstrated to affect intracellular calcium levels in mouse preimplantation two-cell stage embryos (15). PAF may bind to cell surface receptors on preimplantation embryos, initiating the formation of IP_3 and DAG, so increasing intracellular calcium.

In summary, preimplantation stage embryos produce PAF, with the highest levels produced just after the embryo enters the uterus; PAF binds to its receptor, which can be blocked by PAF-antibodies (9) or antagonists (29,30), resulting in increased intracellular calcium levels (15), increased mitotic rates (6), enhanced growth and development (8,11), and implantation (10,26). The collective data provides further evidence that PAF's effect on preimplantation embryo development is receptor-mediated and may involve the IP_3 system. PAF may effect preimplantation embryonic development by regulation of intracellular calcium levels via a G-protein- IP_3 signaling system.

The data provide a clue as to the complicated signal-transduction system following PAF synthesis

and PAF-receptor expression and may help in our understanding of the communication between the preimplantation stage embryo and the maternal endometrium required for implantation and pregnancy.

ACKNOWLEDGMENTS

This work was supported, in part, by NICHD-1RO3HD3523301 to W. E. Roudebush.

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