Carcinogenesis vol.14 no.7 pp.1289–1296, 1993

Platelet activating factor, an endogenous mediator of inflammation, induces phenotypic transformation of rat embryo cells

S.A.L.Bennett, L.C.C.Leite[1] and H.C.Birnboim[2]

Department of Biochemistry, University of Ottawa and Ottawa Regional Cancer Centre, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6

[1]Present address: Instituto Butantan, Sao Paulo, Brazil

[2]To whom reprint requests should be sent

The ability of platelet activating factor (PAF), a potent endogenous inflammatory agent, to induce phenotypic transformation of primary rat embryo cells (RECs) was investigated. RECs are composed predominantly of fibroblasts, with some epithelial cells and a few neuronal and muscle cells. A 1 h period of treatment with PAF (1×10^{-8}–1×10^{-6} M) increased the ability of RECs to (i) form foci, (ii) reach a high saturation density in complete medium, (iii) grow in low serum-containing medium and (iv) exhibit anchorage-independent (AI) growth. Similar changes were achieved with C-PAF (1×10^{-10}–1×10^{-8} M), an active, non-metabolizable analog of PAF, but not by *lyso*-PAF (1×10^{-10}–1×10^{-6} M), a biologically inactive metabolite of PAF. All of the PAF-induced phenotypic changes could be inhibited by pretreatment with a PAF receptor antagonist, CV3988 (1×10^{-6} M). Pretreatment of RECs with genestein (1 μg/ml) also completely inhibited all four measures of PAF-induced REC transformation indicating that tyrosine kinase activity may be required for the observed changes in phenotype. Pretreatment with indomethacin (2×10^{-7} M) blocked the PAF-induced increases in focus formation and saturation density without affecting PAF-induced alterations in growth in low serum or AI growth. This indicates that PAF may exert some of its effects through a cyclooxygenase product. Pretreatment with staurosporine (5×10^{-8} M) failed to alter any of the PAF-induced effects, suggesting that protein kinase C activity is not involved in REC transformation by PAF. Our results provide the first evidence that PAF, released by activated phagocytes in and around areas of inflammation, may contribute to the process of malignant transformation.

Introduction

An association between chronic inflammation and increased predisposition to development of human cancer has been known for many years (1); specific examples have been found in cancer of the lung (2), bowel (3) and skin (4,5). This enhancement is presumed to be mediated by agents released by inflammatory cells at sites of inflammation. In support of this assumption, *in vitro* experiments have demonstrated that active oxygen species and arachidonic acid metabolites can induce malignant transformations of murine fibroblasts (6,7) and epidermal cells (8). However, numerous other factors are also likely present at sites of inflammation including nitric oxide, interleukins, tumour necrosis factor and platelet activating factor (PAF*). In this report, we present evidence concerning the tumourigenic potential of PAF, a mediator of inflammation synthesized and released by activated phagocytic cells.

PAF (1-*O*-alkyl-2-acetyl-glycero-3-phosphocholine) is a biologically active ether lipid with potent pro-inflammatory and hypertensive activities (for review see ref. 9). Its action is presumed to be through specific cell-surface receptors that have been identified in a wide variety of cell types and recently cloned from guinea pig lung (10), human lymphocytes (11), and human leukocytes (12). *In vitro*, PAF has been reported to induce cell aggregation (13–16), degranulation (16), adherence of phagocytic cells to endothelial cells (17–19), chemotaxis (20–22), superoxide production (23), a rapid, transient calcium influx (24), as well as arachidonate (25,26) and phosphoinositide (27) turnover and activation of a variety of protein kinases (28–30). *In vivo* exogenous administration or endogenous overproduction of this phospholipid has been shown to mediate the immediate hypersensitivity reactions characteristic of asthmatic (31,32) and anaphylactic attacks (33,34) as well as the delayed neuronal necrosis associated with ischemia-reperfusion injury (35–37). Depending upon the physiological conditions, endogenously produced PAF can act either as a normal signal transduction molecule or as a pathophysiological agent (9), but the possibility that the phospholipid may contribute to tumour progression has not previously been investigated. We now provide evidence incidating that a 1 h exposure to 0.01–1 μM PAF induces, in primary rodent cells, *in vitro* phenotypic changes commonly associated with malignant transformation. Some or all of these PAF-mediated transformations can be prevented by pretreatment with a PAF receptor antagonist, an inhibitor of protein tyrosine kinases or a cyclooxygenase inhibitor.

*Abbreviations: PAF, platelet activating factor; REC, rat embryo cell; DMEM, Dulbecco modified minimal essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; AI, anchorage-independent.

Materials and methods

Cells and culture conditions

Primary rat embryo cell (REC) cultures were prepared from day 14–16 Sprague Dawley rat embryos (Charles River, Quebec). Freshly dissected embryos were homogenized, incubated in 0.5% trypsin, 5.3 mM EDTA and dissociated into single cells. RECs were suspended in Dulbecco's minimal essential medium (DMEM) (Gibco, Ontario) plus 10% fetal calf serum (FCS) (Flow Laboratories, VA) (DMEM + 10% FCS), seeded at a density of $\sim 6 \times 10^6$/15 cm diameter tissue culture dish, and allowed to grow for 2 days at 37°C in a 5%-CO_2/95% air atmosphere. While still sub-confluent, cells were trypsinized and either passaged in fresh medium or stored at −100°C. Samples of third passage cells were determined to be mycoplasma-free, as judged by staining with a fluorescent dye, Hoechst 33258. The REC population was characterized immunohistochemically. RECs were plated at a density of 1×10^3 on gelatin-coated 22 mm^2 glass coverslips, washed in PBS (154 mM NaCl, 10 mM sodium phosphate, pH 7.2), fixed in cold 3.7% formaldehyde for 10 min, washed in PBS, exposed to 1% normal goat serum in PBS for 30 min at 25°C and reacted at 37°C for 2 h with monoclonal anti-vimentin (IgM: 1:100, Sigma, MO). Antibodies were diluted in Ab buffer [154 mM NaCl, 100 mM sodium phosphate, pH 7.2, 3% bovine serum albumin (BSA), 0.3% Triton X-100]. Samples were washed and immunopositive cells were visualized with a Texas Red-conjugated total Ig goat anti-mouse secondary antibody (Oncogene Science, NY). Sections were then double-labelled with one of the following primary antibodies: monoclonal anti-rat endothelium (1:100, Serotec, Ontario), monoclonal anti-cytokeratin 8.13 (1:100,

Sigma), rabbit anti-glial fibrillary acidic protein (1:100, Sigma), rabbit anti-neurofilament 200 kD (1:100, Sigma) or rabbit anti-desmin (1:50 Serotec). Samples were washed in PBS and immunopositive cells visualized with a fluorescein-conjugated IgG-specific goat anti-mouse or goat anti-rabbit (1:100, Oncogene Science) secondary antibody diluted in Ab buffer.

Treatment of RECs with PAF and related compounds

Ampoules from frozen first passage cells were thawed and cultured for 5–7 days in 10 cm diameter tissue culture dishes using DMEM + 10% FCS. Exponentially growing cultures were trypsinized and 1×10^5 cells/6 cm tissue culture dish were allowed to attach overnight. The cultures were washed twice with serum-free DMEM and subjected to one of the following treatments for 1 h in DMEM containing 0.2% bovine serum albumin: vehicle [0.1% dimethyl sulfoxide (DMSO)], PAF (1×10^{-12}–1×10^{-6} M, Avanti Biochemicals, PA), C-PAF (1×10^{-12}–1×10^{-8} M, Biomol Research, PA), *lyso*-PAF (1×10^{-10}–1×10^{-6} M, Sigma) or PAF plus a specific PAF antagonist, CV3988 (1×10^{-6} M, Calbiochem, CA), a cyclooxygenase inhibitor, indomethacin (2×10^{-7} M, Sigma), a protein kinase C inhibitor, staurosporine (5×10^{-8} M, LC Services, MA) or a tyrosine kinase inhibitor, genestein (1 μg/ml, UBI, MA). Where treatment with both PAF and a second compound is indicated, the antagonist or inhibitor was added 15 min prior to PAF and incubation was continued for 1 h following PAF addition. Cultures were then washed and incubated thereafter in DMEM + 10% FCS.

Cytotoxicity

The cytotoxicity of each treatment was assessed by plating RECs at a density of 200 cells/6 cm dish, treating for 1 h as described above, and allowing cultures to grow in DMEM + 10% FCS for 10 days with twice weekly feedings. Cultures were washed with PBS, fixed with methanol and stained with 0.1% methylene blue in 50% ethanol. Colonies comprising >50 cells were scored.

Assays of cell transformation

Following treatment, cells were incubated undisturbed in DMEM + 10% FCS and assayed for changes in (i) doubling time, (ii) growth in low serum, (iii) saturation density, (iv) focus formation and (v) anchorage-independent (AI) growth. Doubling time was estimated over a 5 day period of exponential growth using both [^{3}H]thymidine (Dupont, ON) incorporation into DNA and cell counts. Cells were pulsed with [*methyl*-^{3}H]thymidine (0.1 μCi/ml) for 24 h. Cultures were then washed extensively with PBS. Cells were solubilized in 1 ml 0.5 N NaOH and DNA was precipitated by acidification with 150 μl 5 N HCl at 0°C for 30 min. Samples were centrifuged in a microfuge and the precipitates were washed twice with 0.5 ml of ice-cold 0.5 N HCl. Pellets were solubilized in 200 μl 0.2 N NaOH. Aquasol (Dupont, ON) scintillation fluid (1 ml) was added and radioactivity determined in a liquid scintillation counter. Cell counts were determined using a Coulter counter. Growth in low serum was assessed by incubating cells in DMEM + 0.5% FCS for up to 25 days following treatment and estimating growth kinetics by counting cells at intervals. Doubling time was estimated over the first 10 days of growth in low serum-containing medium, using both cell counts and [^{3}H]thymidine incorporation into DNA as described above. Saturation density was established by cell counts using a Coulter counter. Focus formation was determined by allowing cultures to grow undisturbed following treatment for 21 days, with gentle medium replacement every 5 days. Cultures were then washed with PBS, fixed with methanol and stained with 0.1% methylene blue in 50% ethanol. Foci of piled cells (>60 μm diameter) were scored using an Olympus inverted microscope. AI growth was estimated by suspending cells, 24 h after treatment, in 2 ml 0.3% molten agarose (BRL, MD) containing DMEM + 10% FCS at 37°C. This suspension was poured over a 3 mm layer of 0.6% agarose containing DMEM + 10% FCS. The soft agarose layer was overlaid with 2 ml DMEM + 10% FCS. The liquid layer was replaced every 5 days. AI colonies (>80 μm in diameter) were scored at 20, 40 and 60 days after plating, using an inverted microscope. Several individual foci and AI colonies were isolated and re-tested in the transformation assays without re-exposure to PAF. Some clones were analysed by immunohistochemistry as described above.

Statistical analysis

Data were analyzed using one-way factorial ANOVA tests or unpaired Student's *t*-tests, as applicable. Following detection of a statistically significant *F* value with respect to the effect of a given series of treatments, *post hoc* Dunnett's tests were used to identify which treatment condition differed statistically from control (vehicle-treated or untreated RECs, depending on the experiment). *P* values of <0.05 were considered statistically significant differences (shown as *); *P* values of <0.01 were considered highly statistically significant differences (shown as **).

Results

RECs are a population of cells; individual cell types were identified and quantitated immunohistochemically. Fibroblasts, endothelial cells and epithelial cells were identified using monoclonal antibodies directed against vimentin, rat endothelium and cytokeratin respectively. Muscle cells, glial cells and neurons were identified using polyclonal antibodies directed against desmin, glial fibrillary acid protein and neurofilament 200 kDa respectively. All cells in the REC explants were immunoreactive to anti-vimentin (data not shown), consistent with a predominance of fibroblasts in the population. Since cell types other than fibroblasts have also been shown to be vimentin-positive *in vitro*, cultures were double-labelled with additional antibodies. The majority (>85%) of cells were fibroblasts, i.e.they reacted only with anti-vimentin. Approximately 15% of cells reacted with anti-cytokeratin, indicating epithelial cells. Rarely (<0.1%), neuronal and muscle cells were detected by double-labelling with anti-vimentin and anti-neurofilament or anti-vimentin and anti-desmin respectively.

Possible cytotoxic effects of the various agents used in these experiments were examined, as described in Materials and methods. No indication of cytotoxicity was seen after treatment of RECs with any of the following: vehicle, (0.1% DMSO), PAF (1×10^{-12}–1×10^{-6} M), C-PAF (1×10^{-12}–1×10^{-8} M), *lyso*-PAF (1×10^{-10}–1×10^{-6} M), CV3988 (1×10^{-6} M), indomethacin (2×10^{-7} M) or genestein (1 μg/ml). Cloning efficiency was reduced by 30% when cells were treated with staurosporine (5×10^{-8} M).

The effects of PAF, C-PAF and *lyso*-PAF on doubling time, focus formation, saturation density, growth in low serum and AI growth are presented in Table I. PAF had no effect on REC doubling time in complete medium. Both PAF and vehicle-treated REC cultures exhibited foci of transformed cells, that is, tightly packed three-dimensional growths of piled cells, >60 μm in diameter, surrounded by a monolayer of contact-inhibited cells. No attempt was made to categorize foci according to degrees of transformation, although the morphology of foci derived from PAF-treated cultures appeared to differ from that of foci arising spontaneously in vehicle-treated cultures. PAF-induced foci were more irregular in shape, denser (i.e. cells stained more intensely) and exhibited a more disorderly pattern of growth than foci seen in vehicle-treated cultures. In addition to altered morphology, PAF induced a dose-dependent increase in the number of foci observed (Table I). This enhancement was also seen following treatment with C-PAF (at lower concentrations), but not by *lyso*-PAF. The maximal increase in focus formation (~3-fold) was observed in RECs following treatment with 1×10^{-8} M PAF. At higher concentrations, scoring of individual foci was not possible because a relatively uniform lawn of darkly staining cells was seen. Since PAF induced a dose-dependent increase in saturation density up to 3-fold increase compared to controls at 1×10^{-6} M PAF, it appears that this overgrowth interfered with accurate quantification of discrete foci. Similarly, C-PAF treatment significantly increased focus formation, with a maximal increase observed at 1×10^{-10} M. As with PAF, individual foci could not be distinguished at higher concentrations of C-PAF, although the highest concentration of C-PAF tested for saturation density was 1×10^{-10} M. *Lyso*-PAF had no statistically significant effect on either the number of foci observed or saturation density.

Growth in low serum containing medium is one measure of autonomous cell growth, i.e. the ability of cells to proliferate with limiting amounts of exogenous growth factors. As shown in Table I, PAF significantly reduced the doubling time of RECs grown in DMEM + 0.5% FCS and increased the length of time that RECs were able to sustain growth in low serum, as compared to untreated RECs. Similar changes in low serum doubling time

Table I. Transformation-associated properties in PAF, C-PAF and *lyso*-PAF treated RECs

Properties	Vehicle	Treatment[a]				
		PAF (M)				
		10^{-12}	10^{-10}	10^{-8}	10^{-7}	10^{-6}
Doubling time[b]						
Cell count	33.5	37.2	31.6	32.4	ND	36.2
Thymidine incorp.	33.5	30.1	35.4	35.5	ND	36.3
Focus formation[c] (colonies/plate)	50 ± 18.1 $n = 5$	63.4 ± 19.3 $n = 5$	78.3 ± 25.9 $n = 6$	135.9 ± 32.4* $n = 7$	OG	OG
Saturation densities[d] (cell/plate)	$1.1 \times 10^6 \pm 3 \times 10^5$	$8.8 \times 10^5 \pm 5 \times 10^4$	$1.1 \times 10^6 \pm 9 \times 10^4$	$2.1 \times 10^6 \pm 1 \times 10^{5,*}$	ND	$3.8 \times 10^6 \pm 5 \times 10^{5,**}$
Growth in low serum[e]						
Doubling time (h)	127.6 ± 4.9	ND	90.6 ± 1.3**	83.8 ± 5.5**	ND	86.0 ± 0.3**
Days of growth	6		21	21		21
AI colonies[f]	0	0	0	24	43*	110**
	C-PAF (M)			*Lyso*-PAF (M)		
	10^{-12}	10^{-10}	10^{-8}	10^{-10}	10^{-8}	10^{-6}
Doubling time[b]						
Cell count	ND	29.8	ND	ND	30.7	ND
Thymidine incorp.	ND	49.1	ND	ND	34.0	ND
Focus formation[c] (colonies/plate)	66.4 ± 10.9 $n = 3$	95.5 ± 7.3* $n = 3$	OG	43 ± 4.0 $n = 3$	31 ± 2.0 $n = 3$	23.8 ± 6.5 $n = 5$
Saturation densities[d] (cells/plate)	ND	$9.3 \times 10^5 \pm 2 \times 10^4$	ND	ND	$8.4 \times 10^5 \pm 3 \times 10^4$	ND
Growth in low serum[e]						
Doubling time (h)	ND	85.0 ± 0.3**	ND	ND	135.6 ± 5.2	ND
Days of growth		21			6	
AI colonies[f]	4	112**	ND	0	0	0

[a]Data represent the mean (± SEM) of $n = 5$ plates, unless otherwise indicated. *Indicates a result statistically significantly different from vehicle-treated control data (ANOVA., *post hoc* Dunnett's *t*-test $P < 0.05$); **indicates a highly statistically significant difference (ANOVA, *post hoc* Dunnett's *t*-test $P < 0.01$), as described in Materials and methods. ND, not determined.

[b]Doubling time (h) was calculated during the first 5 days in culture in DMEM + 10% FCS, using cell counts ($n = 3$ plates) and [^{3}H]thymidine incorporation ($n = 2$ plates), as described in Materials and methods. Mean values are indicated.

[c]Foci (>60 μm in diameter) were counted 21 days after treatment, as described in Materials and methods. *n*, the number of plates is indicated. OG, overgrowth of cells to a degree that individual foci could not be scored. PAF ANOVA: $F = 2.7$, $df = 3,43$, $P < 0.05$; PAF (10^{-8} M) versus Vehicle: Dunnett $t = 2.4$; C-PAF ANOVA: $F = 3.0$, $df = 2,15$, $P < 0.05$; C-PAF (10^{-10}M) versus Vehicle: Dunnett $t = 2.2$.

[d]Cells/6 cm diameter dish were counted 10 days after saturation had been reached in vehicle-treated cultures as described in Materials and methods. PAF ANOVA: $F = 8.7$, $df = 4,19$, $P < 0.01$; PAF (10^{-8} M) versus Vehicle: Dunnett $t = 2.4$; PAF (10^{-6} M) versus Vehicle: Dunnett $t = 4.4$.

[e]Following each treatment, medium was replaced with DMEM + 0.5% FCS. Doubling time was estimated during the first 10 days of culture as described above and in Materials and methods. The number of days that cells were able to sustain growth in this medium is indicated; observations were carried out for 21 days. PAF ANOVA: $F = 30.8$, $df = 3,16$, $P < 0.01$; PAF (10^{-10} M) versus Vehicle: Dunnett $t = 7.0$; PAF (10^{-8} M) versus Vehicle: Dunnett $t = 8.3$; PAF (10^{-6} M) versus Vehicle: Dunnett $t = 7.9$; C-PAF, Student's $t = 8.7$, $df = 8$, $P < 0.01$.

[f]AI colonies (>80 μm in diameter) were counted 40 days following treatment. Values shown are the total number of AI colonies observed per 5×10^5 cells plates (7.5×10^4 viable cells, based upon a cloning efficiency of 15%). PAF ANOVA; $F = 12.4$, $df = 5,24$, $P < 0.01$; PAF (10^{-7} M) versus Vehicle: Dunnett $t = 2.5$; PAF (10^{-6} M) versus Vehicle: Dunnett $t = 6.3$; C-PAF ANOVA: $F = 28.3$, $df = 2,12$, $P < 0.01$; C-PAF (10^{-12} M) versus Vehicle: Dunnett $t = 6.4$; PAF (10^{-10} M) versus Vehicle: Dunnett $t = 6.6$.

and growth in DMEM + 0.5% fetal calf serum were observed following treatment with C-PAF, but not with *lyso*-PAF.

AI growth represents the ability of cells to form colonies in a semi-solid medium such as soft agarose. In our experiments, colonies >80 μm in diameter were scored at 20, 40 and 60 days after treatment. Only data from day 40 are presented in Table I. Both PAF (1×10^{-8}–1×10^{-6} M) and C-PAF (1×10^{-10} M) induced a dose-dependent increase in AI growth. *Lyso*-PAF had no effect. The growth rate of colonies appearing in PAF-treated cultures was very slow. Small colonies (~50 μm) could be seen at day 20 after treatment. By day 40, the colonies were >80 μm in diameter. By day 60, colony size was not significantly larger than at day 40, suggesting that the growth of the AI cells had slowed or stopped. However, growth cessation was not due to cell death since cloning efficiency of cells derived from days 40 and 60 colonies did not differ.

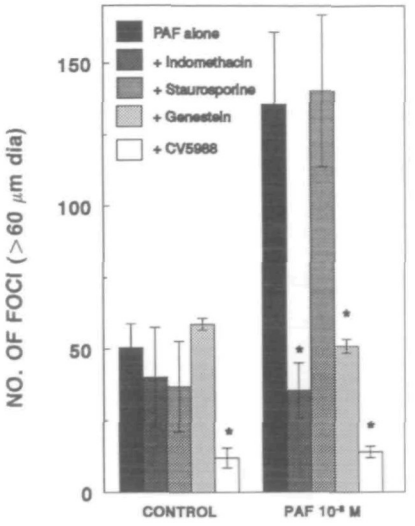

Fig. 1. Effect of indomethacin (2×10^{-7} M), staurosporine (5×10^{-8} M), genestein (1 μg/ml) and CV3988 (1×10^{-6} M) on PAF-induced increase in focus formation. Asterisks indicate a statistically significant decrease ($P < 0.05$) in the number of foci compared to PAF treatment alone. ANOVA: $F = 5.2$, $df = 4,29$, $P < 0.05$.

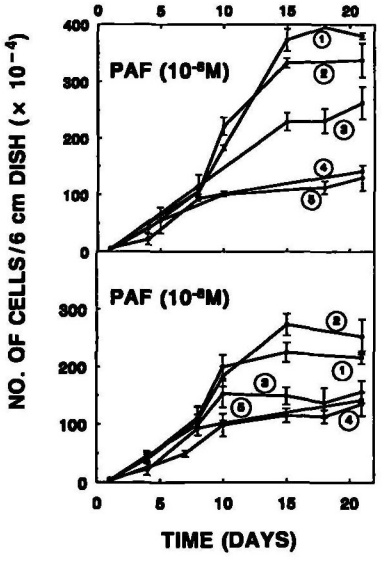

Fig. 2. Effect of indomethacin (2×10^{-7} M), staurosporine (5×10^{-8} M) and genestein (1 μg/ml) on PAF-induced increase in saturation density. 1, PAF treatment; 2, PAF + staurosporine; 3, PAF + indomethacin; 4, PAF + genestein; 5, vehicle. Indomethacin and genestein treatment significantly decreased the PAF-induced enhancement in final saturation density. PAF (1×10^{-6} M) ANOVA: $F = 21.4$, $df = 3,16$, $P < 0.01$; PAF (1×10^{-6} M) ANOVA: $F = 7.1$, $df = 3,16$, $P < 0.05$.

In an attempt to determine the mechanism(s) underlying PAF-mediated transformation of RECs, cells were treated with a variety of pharmacological agents, in each case administered 15 min prior to PAF. The effects of these agents are depicted in Figures 1–4. PAF-induced increases in focus formation are shown in Figure 1, saturation density in Figure 2, growth in low serum in Figure 3 and AI growth in Figure 4. Administration of 1×10^{-6} M CV3988, a PAF receptor antagonist, prevented PAF-induced increase in each of the four parameters of transformation, suggesting that the PAF likely works through its specific receptor. Indomethacin (2×10^{-7} M), an inhibitor of the cyclooxygenase pathway of arachidonic acid metabolism,

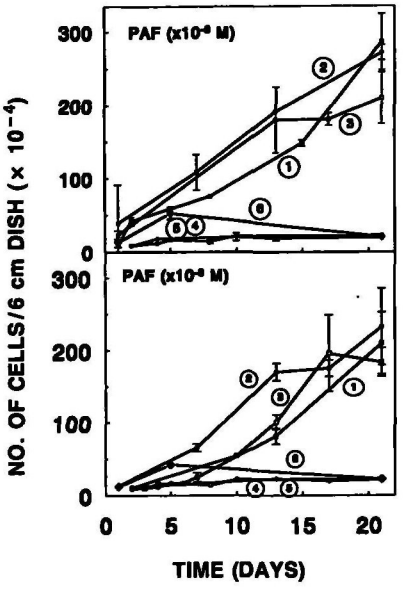

Fig. 3. Effect of indomethacin (2×10^{-7} M), staurosporine (5×10^{-8} M), genestein (1 μg/ml) and CV3988 (1×10^{-6} M) on PAF-induced increase in growth in DMEM + 0.5% FCS. 1, PAF treatment; 2, PAF + staurosporine; 3, PAF + indomethacin; 4, PAF + genestein; 5, vehicle; 6, PAF + CV3988. Genestein and CV3988 treatment significantly decreased the PAF-induced enhancement in growth in low serum containing medium. PAF (1×10^{-6} M) ANOVA: $F = 31.5$, $df = 4,20$, $P < 0.01$; PAF (1×10^{-6} M) ANOVA: $F = 23.6$, $df = 4,20$, $P < 0.01$.

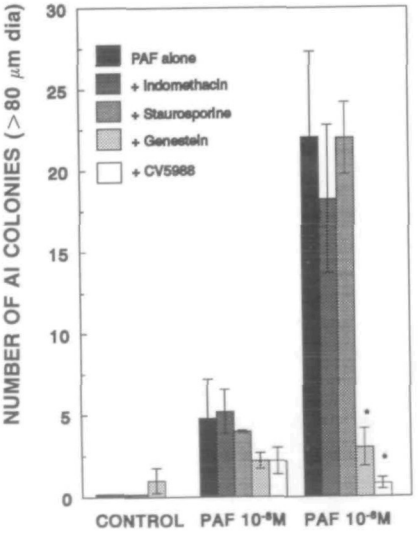

Fig. 4. Effect of indomethacin (2×10^{-7} M), staurosporine (5×10^{-8} M), genestein (1 μg/ml) and CV3988 (1×10^{-6} M) on PAF-induced increase in AI growth. Data represent number of AI colonies per 6 cm plate scored 40 days after treatment. Asterisks indicate a highly statistically significant decrease ($P < 0.01$) in the number of foci compared to PAF treatment alone. PAF (1×10^{-8} M) ANOVA: $F < 1$, $df = 4,20$, $P > 0.05$. PAF (1×10^{-6} M) ANOVA: $F = 10.0$, $df = 4,20$, $P < 0.01$;

blocked PAF-induced changes in saturation density and focus formation, but failed to alter PAF-induced growth in low serum and AI growth. Staurosporine (5×10^{-8} M) failed to affect any of the PAF-induced transformation measures. Interestingly, genestein (1 μg/ml), a tyrosine kinase inhibitor, prevented all of the observed PAF-induced changes.

The stability of the PAF-induced phenotypes was determined by re-testing (without re-exposure to PAF) clones isolated from

Table II. Transformation-associated properties of untreated RECS, PAF (10^{-8} M)-induced focus clones, PAF (10^{-6} M)-induced AI clones, and C-PAF (10^{-10} M)-induced AI clones

Parameters	Untreated RECs	Clones[a]			
		PAF (10^{-8} M)-induced focus clones			
		PF-81	PF-82	PF-83	PF-84
Cell type[b]	F/E	F	E	E	F
Doubling time (h)					
Cell count	31.4	33.1	52.8	58.3	40.5
Thymidine incorp.	31.4	32.7	50.2	60.1	41.2
Focus formation[c] (colonies/plate)	50.6 ± 8.3	58.0 ± 9.8	108 ± 6.3**	53.7 ± 2.6	69.4 ± 11.4
Saturation densities[d] (cell/plate)	1.2×10^6 ± 3×10^5	8.8×10^5 ± 5×10^4	8.0×10^5 ± 2×10^5	7.2×10^5 ± 3×10^4	6.7×10^5 ± 4×10^4
Growth in low serum[e]					
Doubling time (h)	142.0 ± 10.8	71.2 ± 0.5**	120.8 ± 4.1*	69.4 ± 2.6**	158.4 ± 0.6
Days of growth	6	8	8	30	30
AI colonies[f]	0	0	0	8*	0

	PAF (10^{-6} M)-induced AI clones					C-PAF (10^{-10} M)-induced AI clones	
	PA-61	PA-62	PA-63	PA-64	PA-65	CP-101	CP-102
Cell type[b]	F	F	F	F	F	F	F
Doubling time (h)							
Cell count	33.1	41.7	35.4	37.7	37.1	44.7	29.2
Thymidine incorp.	30.1	28.1	37.9	38.6	38.1	46.5	25.1
Focus formation[c] (colonies/plate)	>500	>500	88.4 ± 0.98	515.4 ± 30.9**	465.2 ± 48.2**	ND	ND
Saturation densities[d] (cells/plate)	3.9×10^6 ± 5×10^5	2.2×10^6 ± 6×10^5	1.0×10^6 ± 1×10^5	1.3×10^6 ± 1×10^4	1.0×10^6 ±2×10^4	1.6×10^6 ± 9×10^4	1.4×10^6 ± 7×10^4
Growth in low serum[e]							
Doubling time (h)	115.6 ± 0.3	66.1 ± 0.9**	147.4 ± 5.8	92.6 ± 2.8**	48.5 ± 0.0**	83.9 ± 2.5**	71.6 ± 1.7**
Days of growth	20	30	6	30	20	30	30
AI colonies[f]	17*	10*	0	10*	11*	13*	7*

[a]Details as described in the legend to Table I.
[b]Cells were characterized by immunohistochemical staining as described in Materials and methods. F, fibroblasts; E, epithelial cells. F/E, mixed population (85% fibroblasts/15% epithelial cells/rare <0.1% muscle and neuronal cells).
[c]Foci (>60 μm in diameter) were counted 21 days after plating, as described in Materials and methods. PAF focus clones ANOVA: $F = 8.1$, $df = 4,20$, $P < 0.01$; PF82 versus Control: Dunnett's $t = 4.9$; PAF AI clones ANOVA: $F = 71.3$, $df = 3,16$, $P < 0.01$; PA64 versus Control: Dunnett's $t = 11.4$; PA65 versus Control: Dunnett's $t = 10.1$.
[d]PAF AI clones ANOVA: $F = 10.0$, $df = 5,24$, $P < 0.01$; PA61 versus Control: Dunnett's $t = 5.4$; PA62 versus Control: Dunnett's $t = 2.1$.
[e]Growth was observed over a 30 day period. PAF focus clones ANOVA: $F = 58.5$, $df = 4,20$, $P < 0.01$; PF81 versus Control: Dunnett's $t = 9.4$; PF82 versus Control: Dunnett's $t = 2.8$; PF83 versus Control. Dunnett's $t = 9.7$; PF84 versus control: Dunnett's $t = 2.2$; PAF AI clones: $F = 77.2$, $df = 5,24$. $P < 0.01$; PA61 versus Control: Dunnett's $t = 4.0$; PA62 versus Control: Dunnett's $t = 11.7$; PA64 versus Control: Dunnett's $t = 7.6$; PA65 versus Control: Dunnett's $t = 14.4$. C-PAF AI clones ANOVA: $F = 33.7$, $df = 2,12$, $P < 0.01$; CP101 versus Control: Dunnett's $t = 6.3$; CP102 versus Control: Dunnett's $t = 7.7$.
[f]PAF focus clones ANOVA: $F = 51.2$, $df = 4,19$, $P < 0.01$; PF83 versus Control: Dunnett's $t = 11.7$; PAF AI clones: $F = 5.0$, $df = 5,24$, $P < 0.01$; PA61 versus Control: Dunnett's $t = 4.9$; PA62 versus Control: Dunnett's $t = 2.9$; PA64 versus Control: Dunnett's $t = 3.1$; PA65 versus Control: Dunnett's $t = 2.9$; C-PAF AI clones ANOVA: $F = 16.9$, $df = 2,12$, $P < 0.01$; CP101 versus Control: Dunnett's $t = 5.8$; CP102 versus Control: Dunnett's $t = 3.0$.

4 foci, 5 AI colonies and 2 C-PAF-induced AI colonies. Table II summarizes the results obtained in the four transformation assays. Cell types in these clones were identified immunohistochemically; two were epithelial in origin, all others were fibroblast. The doubling times in DMEM + 10% FCS of the majority of clones was comparable to untreated RECs, except for PF-82, PF-83, PF-84 and CP-101. Most of the PAF- and C-PAF-treated AI clones exhibited significantly altered growth in low serum and were able to maintain growth in low serum throughout the 30 days of testing. Saturation density of induced clones was comparable to untreated cultures in all but two cases (PA-61 and PA-62), which exhibited a significantly increased saturation density. Only PAF-treated AI clones demonstrated an increased ability to form foci compared to untreated RECs. PA-61

and PA-62 exhibited a very high capacity for focus formation such that accurate quantification was not possible, although discrete foci could still be identified. Finally, all PAF- and C-PAF-induced AI colonies and 1 PAF-induced focus clone (PF-83) demonstrated a weak but statistically significant AI phenotype, compared to untreated RECs.

Discussion

Although a correlation between chronic inflammation and predisposition to cancer in humans has been recognized for many years, mechanisms underlying this correlation have yet to be elucidated. The inflammatory milieu is considered to be very complex. In this report, we have studied the potential of PAF, a known endogenous inflammatory agent, to affect carcinogenic processes. Our results provide the first evidence, using concentrations of PAF that may well be found *in vivo* at sites of inflammation, that this agent may contribute to the multi-step process of malignant transformation. Characteristic phenotypic traits of transformed cells cultured *in vitro* include altered morphology, growth factor independence, increased saturation density, focus formation and AI growth (for a review, see ref. 38). These *in vitro* phenotypic traits, most notably AI growth, are believed to correlate with *in vivo* tumorigenicity. Treatment of RECs with PAF for 1 h in medium lacking serum induced an increase in their ability to (i) form foci, (ii) reach a high saturation density, (iii) sustain growth under low serum conditions and (iv) acquire the property of AI growth. In all assays, maximal effects were observed at 1×10^{-6} M PAF with the exception of focus formation, which could only be accurately quantified at a treatment dose of 1×10^{-8} M PAF. At 1×10^{-6} M PAF, individual foci could not be distinguished from the dense background of cells. It is noteworthy that 1×10^{-6} M PAF increased saturation density by ~3-fold compared to vehicle-treated cells. In addition to increasing focus formation and saturation density, PAF also reduced doubling time of REC in low serum-containing medium by up to 1.5-fold (PAF 1×10^{-6} M) and increased the length of time cells were able to sustain growth in low serum from 6 to >30 days. The most striking phenotypic change induced by PAF was a dose-dependent increase in AI growth, since no AI colonies were ever observed in untreated or vehicle-treated RECs. Our results clearly indicate that PAF can induce, in early passage rat embryo fibroblast and epithelial cells, phenotypic alterations characteristic of malignancy.

The PAF effects we have observed are likely to be receptor-mediated since the effects follow the known potency of closely related compounds, C-PAF and *lyso*-PAF, which were more potent or inactive respectively. C-PAF increased three of the four phenotypic properties tested at doses two orders of magnitude below that seen with PAF. This 'shift to the left' in the dose–response curve is consistent with the fact that C-PAF is non-metabolizable and hence capable of stimulating cells for a longer period of time than the natural ligand (39). However, it has a lower affinity for the PAF receptor than PAF itself and does not produce the same magnitude of response as the natural ligand in other assays (39); this may explain the reduced response in our assays, compared to PAF. Conversely, no phenotypic changes were observed after treatment with *lyso*-PAF, which is also inactive in other assays. Furthermore, a specific PAF receptor antagonist, CV3988, attenuated all of the PAF-induced effects. Taken together, these observations provide strong evidence that PAF-mediated phenotypic alterations of RECs are likely to be receptor-mediated.

The biological effects of PAF have been studied in inflammatory and other cell types. PAF can activate phospholipase A2, releasing arachidonic acid from the plasma membrane, which is then metabolized by the lipoxygenase pathway in granulocytes (40). PAF has also been shown to activate protein kinase C (28,29) and to induce tyrosine phosphorylation of a number of proteins in rat Kupffer cells and human granulocytes (29,30). Although these effects have yet to be demonstrated in fibroblasts and epithelial cells, murine fibroblasts are known to respond to PAF by exhibiting calcium transients (24) and increasing interleukin-6 production (41). Murine epithelial cells respond to PAF by permitting blastocyst implantation (42) while a human epidermoid carcinoma cell line has been shown to increase c-*fos* and TPA-Inducible Sequence (TIS-1) mRNA expression (43). The possibility that PAF might cause the phenotypic alterations we have observed via the cyclooxygenase pathway of archidonic acid metabolism, protein kinase C activation, or alterations in tyrosine kinase activity was investigated by treating RECs with indomethacin, staurosporine or genestein respectively. Indomethacin blocks the synthesis of cyclooxygenase metabolites (e.g. prostaglandins) of arachidonic acid. A lipoxygenase pathway metabolite, 5-hydroxyeicosatetranoic acid, has been demonstrated to cause clastogenic effects in murine fibroblasts and epithelial cells, contributing to malignant transformation (44). No highly specific lipoxygenase inhibitors are available and therefore none were tested. In the present study, indomethacin inhibited PAF-induced increases in saturation density and focus formation but failed to alter PAF-induced growth in low serum or AI growth, suggesting that PAF acts through multiple pathways and that the anchorage-dependent phenotypes (focus formation and saturation density) may depend upon the PAF-induced synthesis of cyclooxygenase metabolites. Staurosporine, a protein kinase C inhibitor, was ineffective in altering any PAF-induced effect, providing evidence that a protein kinase C pathway is not involved. Most notably, a marked decrease in all of the PAF-induced phenotypic alterations was seen following pretreatment of RECs with genestein, a putative tyrosine kinase inhibitor. This observation suggests that stimulation of PAF receptor by its ligand leads to a protein tyrosine phosphorylation event that is important in PAF-mediated transformation of RECs.

The altered phenotypic properties induced in RECs by the 1 h treatment with PAF were not genetically stable. When PAF-transformed foci were isolated and re-assayed (in the absence of further PAF treatment), the clones demonstrated neither increased focus forming ability nor increased saturation density compared to control cells, and exhibited only a modest increase in growth in low serum and occasional AI growth. PAF-transformed AI colonies, however, retained more transformed properties upon re-testing, such as markedly enhanced focus formation and growth in low serum; to a lesser degree, they also exhibited AI growth. It appears that some relatively stable epigenetic event has occurred as the result of PAF treatment. When second generation AI colonies (derived from the original PAF-induced AI clones) were re-tested for phenotypic transformation, they exhibited a substantial increase in all the phenotypic properties tested, including AI growth (data not shown). Our results demonstrate that exposure to PAF is able to induce malignant transformation-like changes in RECs and thus is a potentially important endogenous factor in the process of tumourigenesis. The reason(s) for the apparent lack of stability of these changes remains uncertain.

In summary, the present study provides the first evidence that administration of the endogenous inflammatory agent, PAF,

causes malignant transformation of rodent cells in culture. Inhibition of these phenotypic changes by genestein is evidence that protein tyrosine phosphorylation may be important in this process. Differential inhibition of two of the four measures of transformation by indomethacin is evidence that cyclooxygenase products play a role in the development of the 'anchorage-dependent' phenotypes. Our findings suggest that PAF, released by activated phagocytic cells in and around areas of chronic inflammation, may be an important contributor to tumour progression.

Acknowledgements

The authors thank E.Bartlett and Cai-Ying Zhou for their skilful technical assistance. This research was supported by a grant from the Medical Research Council for Canada to HCB. SALB is a recipient of a Medical Research Council studentship. LCCL was supported by a fellowship from CNPq (Brazil). HCB is a career scientist of the Ontario Cancer Treatment and Research Foundation.

References

1. Templeton,A.C. (1975) Acquired diseases. In Fraumeni,J.F. (ed.), *Persons at High Risk of Cancer: An Approach to Cancer Etiology and Control*. Academic Press, New York, pp. 69–84.
2. Creasia,D. and Netteshim,P. (1975) Particulates and chronic inflammation increase rates of carcinogenesis. In Karbe,E. and Parke,J. (eds), *Experimental Lung Cancer*. Springer Verlag, New York, pp. 234–235.
3. Alexander-Williams,J. (1976) Inflammatory disease of the bowel. *Dis. Colon Rectum*, **19**, 579–581.
4. Argyris,T. and Slaga,T. (1981) Promotion of carcinomas by repeated abrasion in initiated skin of mouse. *Cancer Res.*, **41**, 5193–5195.
5. Camisa,C. (1984) Squamous cell carcinoma in acne conglobata. *CUTIS*, **33**, 185–190.
6. Floyd,R. (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB*, **4**, 2587–2597.
7. Zimmerman,R. and Cerutti,P. (1984) Active oxygen acts as a promoter of transformation in mouse embryo C3H/10T1/2/C18 fibroblasts. *Proc. Natl. Acad. Sci. USA*, **81**, 2085–2087.
8. Muehlematter,D., Larson,R. and Cerutti,P. (1981) Active oxygen induced DNA strand breakage and polyADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells. *Carcinogenesis*, **9**, 239–245.
9. Braquet,P., Touqui,L., Shen,T. and Vargaftig,B. (1987) Perspectives in platelet-activating research. *Pharmacol. Rev.*, **39**, 99–145.
10. Honda,Z., Nakamura,M., Miki,I., Minami,M., Watanabe,T., Seyma,Y., Okado,H., Toh,H., Ito,K., Miyamoto,M. and Shimizu,T. (1991) Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature*, **349**, 342–345.
11. Ye,R., Prossnitz,E., Zou,A. and Cochrane,C. (1991) Characterization of a human cDNA that encodes a functional receptor for platelet-activating factor. *Biochem. Biophys. Res. Commun.*, **180**, 105–111.
12. Kunz,D., Gerard,N.P. and Gerard,C. (1992) The human leukocyte platelet-activating factor receptor. cDNA cloning, cell surface expression and construction of a novel epitope-bearing analog. *J. Biol. Chem.*, **267**, 9101–9106.
13. Tran,A., Vanhée,D., Capron,A., Vorng,H., Braquet,P. and Joseph,M. (1992) Separate induction of human blood platelet aggregation or cytotoxicity by different concentrations of PAF-acether and thrombin. *Agents Actions*, **36**, 39–43.
14. Phenekos,C., Siafaka-Kapadai,A., Trapali,M., Botitsi,E. and Mavris,M. (1992) Effect of gliclazide on platelet-activating factor-induced platelet aggregation in patients with non-insulin-dependent diabetes mellitus. *Metabolism*, **41**, 30–32.
15. Bin,Z. and Kun,L. (1992) Inhibition by glaucocalyxin A of aggregation of rabbit platelets induced by ADP, arachidonic acid and platelet-activating factor, and inhibition of [^{3}H]-PAF binding. *Thromb. Haemost.*, **67**, 458–460.
16. Doeffier,E., Borrel,M., Lefort,J., Chignard,M., Brozuet,C., Heyman,F., Godfroid,K. and Vargaftig,B. (1986) Effects of PAF-acether and structural analogues on platelet activation and bronchoconstriction in guinea pigs. *Eur. J. Pharmacol.*, **131**, 179–188.
17. Milhoan,K.A., Lane,T.A. and Bloor,C.M. (1992) Hypoxia induces endothelial cells to increase their adherence for neutrophils: Role of PAF. *Am. J. Physiol. Heart Circ. Physiol.*, **263**, H956–H962.
18. Dilon,P., Fitzpatrick,M., Ritre,A. and Durna,W. (1988) Effect of platelet-activating factor on leukocyte adherence to microvascular endothelium. *Inflammation*, **12**, 563–573.
19. Hirafuji,M. and Shinoda,H. (1991) Platelet–leukocyte interaction in adhesion to endothelial cells induced by platelet activating factor. *Br. J. Pharmacol.*, **103**, 1333–1338.
20. Okada,C., Hopp,R.J., Miyagawa,H., Sugiyama,H., Nair,N.M., Bewtra,A.K. and Townley,R.G. (1992) Effect of terfenadine on neutrophil and eosinophil chemotactic activities after inhalation of platelet-activating factor *in vivo* and on neutrophil chemotaxis *in vitro*. *Int. Arch. Allergy Appl. Immunol.*, **97**, 181–186.
21. Townley,R.G. (1992) Antiallergic properties of the second-generation H_1 antihistamines during the early and late reactions to antigen. *J. Allergy Clin. Immunol.*, **90**, 720–725.
22. Miyagawa,H., Nabe,M., Hopp,R.J., Okada,C., Bewtra,A.K. and Townley,G. (1992) The effect of WEB 2086 on PAF-induced eosinophil chemotaxis and LTC_4 production from eosinophils. *Agents Actions*, **37**, 39–43.
23. Mabuchi,K., Sugiura,T., Ojima-Uchiyama,S., Masuzawa,Y.and Waku,K. (1992) Differential effects of platelet-activating factor on superoxide anion production in human eosinophils and neutrophils. *Biochem. Int.*, **26**, 1105–1113.
24. Chau,L.-Y., Hu,C.-Y., Chang,W.-T. and Hsu,Y.-S. (1992) Possible existence of two subsets of platelet-activating factor receptor to mediate polyphosphoinositide breakdown and calcium influx in neuroblastoma × glioma hybrid NG 108-15 cells. *J. Neurochem.*, **59**, 1090–1098.
25. Trochtenberg,D.S., Lefferts,P.L., King,G.A., Hwang,Y.S., Christman,B.W. and Snapper,J.R. (1992) Effects of thromboxane synthase and cyclooxygenase inhibition on PAF-induced changes in lung function and arachidonic acid metabolism. *Prostaglandins*, **44**, 555–577.
26. Kunievsky,B. and Yavin,E. (1992) Platelet-activating factor stimulates arachidonic acid release and enhances thromboxane B_2 production in intact fetal rat brain *ex vivo*. *J. Pharmacol. Exp. Ther.*, **263**, 562–568.
27. Yue,T.-L., Gu,J.-L. and Feuerstein,G. (1992) Protein kinase C activator phorbol 12, 13-dibutyrate inhibits platelet activating factor-stimulated Ca^{2+} mobilization and phosphoinositide turnover in neurohybrid NG108-15 cells. *Neurochem. Res.*, **17**, 997–1004.
28. O'Flaherty,J., Redman,J., Jacobson,D. and Rossi,A. (1990) Stimulation and priming of protein kinase C translocation by a Ca^{2+} transient-independent mechanism. *J. Biochem.*, **265**, 21619–21623.
29. Chao,W., Liu,H., Hanahan,D.J. and Olson,M.S. (1992) Platelet-activating factor-stimulated protein tyrosine phosphorylation and eicosanoid synthesis in rat Kupffer cells. Evidence for calcium-dependent and protein kinase C-dependent and -independent pathways. *J. Biol. Chem.*, **267**, 6725–6735.
30. Gomez-Cambronero,J., Wang,E., Johnson,G., Huang,C. and Sha'afi,R. (1991) Platelet-activating factor induces tyrosine phosphorylation in human neutrophils. *J. Biol. Chem.*, **266**, 6240–6245.
31. Dorsch,W., Bittinger,M., Kaas,A., Müller,A., Kreher,B. and Wagner,H. (1992) Antiasthmatic effects of *Galphimia glauca*, gallic acid and related compounds prevent allergen- and platelet-activating factor-induced bronchial obstruction as well as bronchial hyperreactivity in guinea pigs. *Int. Arch. Allergy Appl. Immunol.*, **97**, 1–7.
32. Chung,K.F. (1992) Platelet-activating factor in inflammation and pulmonary disorders. *Clin. Sci.*, **83**, 127–138.
33. Rabinovici,R., Yue,T.L., Vernick,J. and Feuerstein,G. (1991) PAF and TNFα interactions in the pathophysiology of septic shock. *Adv. Exp. Med. Biol.*, **314**, 193–203.
34. Heuer,H. and Casals-Stenzel,J. (1989) The pathophysiological role of PAF in anaphlactic lung reaction in the guinea pig and in endotoxin shock evidenced by the specific PAF-antagonist WEB 2086. In *Second Vienna Shock Forum*. Alan Liss, New York, pp. 925–930.
35. Mozes,T., Braquet,P. and Filep,J. (1989) Platelet-activating factor: an endogenous mediator of mesenteric ischemia-reperfusion-induced shock. *Am. J. Physiol.*, **257**, R872–R877.
36. Feurstein,G., Yue,G. and Lysko,R. (1990) Platelet activating factor: a putative mediator in central nervous system injury? *Stroke*, **21**, III–90–94.
37. Braquet,P., Paubert-Braquet,M., Koltai,M., Bourgain,R., Bussolino,F. and Hosford,D. (1989) Is there a case for PAF antagonists in the treatment of ischemic states? *TIPS*, **10**, 23–30.
38. Heidelberger,C. (1977) Chemical oncogenesis in culture. *Mutat. Res.*, **114**, 283–291.
39. O'Flaherty,J., Redman,J., Schmitt,J., Ellis,J., Surles,J., Marx,M., Piantadosi,C. and Wykle,R. (1987) 1-*O*-alkyl-2-*N*-methylcarbamyl-glycerophosphocholine: a biologically potent, non-metabolizable analog of platelet-activating factor. *Biochem. Biophys. Res. Commun.*, **147**, 18–24.
40. Nakashima,S., Suganama,A., Sato,M., Tohamatsu,T. and Nowawa,Y. (1989) Mechanism of arachidonic acid liberation in platelet-activating factor stimulated human polymorphonuclear neutrophils. *J. Immunol.*, **143**, 1295–1302.
41. Braquet,P., Pignol,B., Maisonnet,T. and Mencia-Heurta,J. (1991) Platelet-activating factor modulates interleukin-2 production by mouse fibroblasts. *Int. Arch. Appl. Immunol.*, **94**, 165–166.
42. Harper,M., Kudolo,G., Alecozay,A. and Jones,M. (1989) Platelet-activating factor (PAF) and blastocyst-endometrial interactions. In *Development of*

Preimplantation Embryos and their Environment. Alan Liss, New York, pp. 305–315.
43. Tripathi,Y., Kandala,J., Lim,R. and Shulka,S. (1991) Platelet activating factor induces expression of early response genes c-fos and TIS-1 in human epidermoid carcinoma A-431 cells. *Life Sci.*, **49**, 1767–1769.
44. Ochi,T. and Cerutti,P. (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblast C3H/10T1/2. *Proc. Natl. Acad. Sci. USA*, **85**, 990–994.

Received on February 5, 1993; revised on April 6, 1993; accepted on April 15, 1993