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Receptor-Mediated and Protein Kinase-Dependent Growth Enhancement of Primary Human Fibroblasts by Platelet Activating Factor

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Chronic inflammation is a recognized risk factor for human cancer, but the causal mechanisms are poorly understood. We previously demonstrated that platelet activating factor (PAF) can induce alterations in the in vitro growth properties of primary rat fibroblasts. In the study reported here, exposure of primary human skin fibroblasts to PAF for 1 h in serum-free medium was shown to cause sustained proliferation over 50 d in medium containing low serum and anchorage-independent growth in soft agarose. Both properties could be inhibited by pretreatment with a PAF receptor antagonist, CV3988 (10 μM); a tyrosine-kinase inhibitor, genistein (1 μg/mL); or a protein kinase C (PKC) inhibitor, staurosporine (50 nM) but not with a cyclooxygenase inhibitor, indomethacin (200 nM–20 μM). PAF had no effect on doubling time, saturation density, or cell viability under normal monolayer growth conditions in complete medium. Treatment with *lyso*-PAF, an inactive metabolite of PAF, had no effect in either of the assays. Control and PAF-induced cell proliferation in low-serum medium was inhibited by PAF receptor antagonists present during the extended growth period. The presence of PAF receptor mRNA in human skin fibroblasts was demonstrated by reverse transcriptase–polymerase chain reaction. The presence of a functional receptor was indicated by an early (2 min) transient increase in PKC activity and an increase in *fos* mRNA after PAF treatment. PAF-induced PKC activity was blocked by pretreatment with either staurosporine (50 nM) or CV3988 (1 μM). These results suggest that PAF is a mitogenic factor that contributes to the known increase in risk of malignancy associated with chronic inflammatory conditions. *Mol. Carcinog. 20:366–375, 1997.*

Key words: inflammatory mediators; tumor progression; anchorage-independent growth; PAF receptor; mitogenicity

INTRODUCTION

Chronic inflammatory conditions have long been known to be associated with an increased predisposition to lymphoma and cancer in lung, bladder, bowel, breast, skin, and stomach [1–8]. The underlying mechanism is uncertain, but it has been postulated that factors such as oxyradicals, nitric oxide, lipooxygenase metabolites of arachidonic acid, and interleukins released by inflammatory cells may contribute to the malignant conversion of cells [3,9–18]. Another factor produced by inflammatory cells is platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine). PAF is a biologically active ether lipid that acts through specific receptors to produce diverse effects [19,20]. At sites of inflammation, it promotes cell aggregation, degranulation, adherence to endothelial cells, chemotaxis, and superoxide production [21–29]. There is an indication that it may also be involved in pulmonary and cardiovascular diseases [24,30,31] and premalignant inflammatory disorders of the bowel such as Crohn's disease and ulcerative colitis [32–36].

PAF acts through a G protein–coupled transmembrane receptor [37–40]. PAF receptor mRNA is expressed at low levels in human heart, spleen, lung, kidney, and brain and at higher levels in peripheral leukocytes and eosinophils [40,41]. Recently there was a report of PAF receptor mRNA in human lung fibroblasts [42]. Activation of the receptor has been reported to induce a number of early biochemical changes associated with signal transduction processes [20], including arachidonate and phosphoinositide turnover [38], protein kinase C (PKC) activation, and increases in protein-tyrosine phosphorylation [43–48]. PAF has been shown to increase mRNA expression of early response genes including c-*fos* [42,47,49,50].

We previously reported that brief exposure (1 h) to PAF can lead to a prolonged enhancement (over several weeks) in the proliferative capacity of primary rat fibroblasts and epithelial cells. This effect is modulated by protein kinase inhibitors [51]. In the study presented here, we extended our investigation of PAF to primary human skin fibroblasts (HF). Here we

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Abbreviations: PAF, platelet activating factor; PKC, protein kinase C; HF, human fibroblasts; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; RT, reverse transcriptase; PCR, polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BSA, bovine serum albumin; AI, anchorage independent; SEM, standard error of the mean.

present evidence that these cells expressed PAF receptor mRNA, that PAF enhanced proliferative capacity, and that PAF induced PKC activation. PAF-induced proliferation is shown to be both PKC- and tyrosine kinase–dependent. Our results provide the first indication that in human cells PAF may play a role in mediating the known increase in malignancy associated with inflammation.

MATERIALS AND METHODS

Cells and Culture Conditions

Normal diploid HF were prepared from neonatal foreskins as described previously [52]. HF cultures were between the third and seventh passages when treated. Human promyelocytic U-937 and HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). The cultures were grown in Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum (FCS; Life Sciences Technologies, Burlington, ON). Samples of U-937 cells, HL-60 cells, and HF were determined to be mycoplasma-free by staining with Hoechst 33258 and by reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis by using a mycoplasma PCR primer kit (Stratagene, La Jolla, CA).

Drugs and Chemicals

PAF and *lyso*-PAF were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), indomethacin from Sigma Chemical Co. (St. Louis, MO), CV3988 and 1,3-dioxolane from Calbiochem (La Jolla, CA), staurosporine from LC Services Corp. (Woburn, MA), genistein from Upstate Biotechnology, Inc. (Lake Placid, NY), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) from BIOMOL Res. Labs Inc. (Plymouth Meeting, PA).

Treatment with PAF and Related Compounds

Exponentially growing cultures were trypsinized and cells (either 100,000 for growth assays or 200 for cytotoxicity assays) were transferred to 6-cm tissue-culture dishes. After 18 h, 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 (BSA): vehicle (0.1% dimethyl sulfoxide), PAF (0.1 nM–10 μM), or *lyso*-PAF (0.1 nM–1 μM). The inhibitors used were CV3988 (1–10 μM), dioxolane (1 μM), indomethacin (0.2 μM–20 μM), staurosporine (50 nM), and genistein (1 μg/mL). Where treatment with inhibitor is indicated, the antagonist or inhibitor was added 15 min before PAF, and treatment was continued for 1 h after PAF administration. The cultures were then washed extensively to remove PAF and incubated for 24 h in DMEM with 10% FCS before being assayed for growth enhancement or cytotoxicity. To assess the role of PAF inhibitors on HF after PAF exposure, the cultures were treated with vehicle, PAF, or *lyso*-PAF for 1 h; washed in DMEM with 10% FCS; and assayed for growth in low-serum medium or for cytotoxicity as described below except that 1 μM CV3988 or 1 μM dioxolane was continuously present. In the latter assays, the PAF antagonists were added once daily to the cultures.

Assays of Cytotoxicity and Growth Enhancement

After treatment, the HF were assayed for changes in cell viability (cytotoxicity), doubling time, saturation density, growth in low serum, and anchorage-independent (AI) growth. The cytotoxicity measurements were made with a clonogenic assay that involved treating cultures (200 cells/plate) with PAF and allowing the cells to grow in DMEM with 10% FCS for 10 d with twice weekly feedings. The cultures were then washed with phosphate-buffered saline, fixed with methanol, and stained with 0.1% methylene blue in 50% ethanol. Colonies with more than 50 cells were scored. Cytotoxicity was also assessed by a trypan blue exclusion assay. Doubling time and saturation density were determined over 25 d by cell counts made with a Coulter Counter (Coulter Electronics, Hialeah, FL). Growth in low serum was assessed after treatment with PAF as described above by incubating cells in DMEM with 0.5% FCS for 25 d and estimating growth kinetics by Coulter counting. AI growth was determined by embedding cells 24 h after PAF treatment in 2 mL of 0.3% agarose over a 3-mm base layer of 0.6% agarose in DMEM with 10% FCS in a 6-cm dish. The soft-agarose layer was overlaid with 2 mL of liquid medium that was replaced every 5 d. AI colonies (those more than 80 μm in diameter) were scored microscopically between 10 and 60 d. Representative AI colonies were isolated at days 40 and 60 and cultured to assess plating efficiency.

RT-PCR Analysis of PAF Receptor mRNA Expression

Total cellular RNA was prepared from 2×10^6 HF, HL-60 cells, and U-937 cells essentially as described previously [53]. Ten picomoles of $(dT)_{20}$ was added to 500 ng of total RNA in 12 μL of H_2O and heated for 5 min at 70°C. The mixture was cooled, and 0.5 mM each dNTP (Pharmacia Inc., Piscataway, NJ) in Tris-HCl, pH 8.3 (50 mM), KCl (40 mM), $MgCl_2$ (6 mM), ribonuclease-free BSA (0.1 mg/mL), and dithiothreitol (10 mM) was added (final concentrations are shown). After heating for 1 min at 37°C, the RNA was transcribed by the addition of 5 U of Superscript reverse transcriptase (Life Science Technologies) in a total reaction volume of 20 μL. The reaction mixtures were incubated at 42°C for 1 h and then at 50°C for 30 min. Five microliters of the RT reaction mix was used for PCR amplification. The PAF receptor primer sequences used were 5′-GCATCCTACTTCCTCATCCT-3′ (forward) and 5′-ACTTCAGTGACCGTATCCGT-3′ (reverse) and defined a 537-bp fragment of the human leukocyte

mRNA [39]. PCR was performed in a volume of 50 μL containing dNTP mix (0.25 mM each dNTP); 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 50 μg/mL BSA; 4 mM $MgCl_2$; 1 U of Taq DNA polymerase; and 20 pmol of each primer. The reaction mixture was denatured at 94°C for 30 s, annealed at 57°C for 1 min, and extended at 72°C for 1 min for 30 cycles. The absence of genomic DNA contamination was demonstrated by performing identical reactions without RT. The PCR products and 100-bp markers (Pharmacia Inc.) were size-fractionated by electrophoresis on a 1.2% (w/v) agarose gel and visualized by ethidium bromide staining. Southern analysis of the RT-PCR products was performed. The probe was a ^{32}P-labeled 1-kb NotI/HindIII fragment of the human leukocyte PAF receptor cDNA pCDM8/PAFR [39] (kindly provided by Dr. N. Gerard, Beth Israel Hospital, Boston, MA). Hybridization was performed for 18 h and exposure to X-ray film for 48 h at –70°C.

Measurement of PKC Activity

Cultures were washed twice with serum-free DMEM, and approximately 6×10^6 cells were treated with vehicle (0.1% dimethylsulfoxide), PAF (1 pM–1 μM), or TPA (10 nM) in DMEM containing 0.2% BSA. In some cases, the cells were pretreated with either a PKC inhibitor, staurosporine (50 nM), or a specific PAF antagonist, CV3988 (10 μM). Staurosporine or CV3988 was added 15 min before the addition of TPA or PAF and maintained in the medium for the treatment duration. After stimulation, the cells were assayed for PKC activity essentially as described previously [54]. Briefly, the cells were washed with ice-cold phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 0.14 M NaCl, and 8 mM Na_2HPO_4) and suspended by scraping the cells from the plate with a rubber policeman into 1 mL of lysis buffer (0.1 M NaCl; 0.02 M Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 100 μM sodium vanadate; 100 μM sodium pyrophosphate; 1 mM sodium fluoride; and 100 μM phenylmethylsulfonylfluoride). The suspensions were sonicated for 5 s on ice with a Microson Ultrasonic Cell Disrupter (Mandel Scientific, Guelph, ON) at low power. Large cell fragments were removed by low-speed centrifugation at 500 × *g* for 5 min at 4°C, and the membranes present in the supernatant were isolated by centrifugation at 16 000 × *g* for 20 min at 4°C. The membrane pellet was suspended in 100 μL of assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, and 100 μM phenylmethylsulfonylfluoride, pH 7.5), and the protein content was measured according to the method of Bradford [55]. Membrane-associated PKC activity was assayed by quantifying the incorporation of ^{32}P from [γ-^{32}P]ATP into a PKC-selective substrate. This peptide substrate, FKKSFKL-NH_2, corresponds to the amino-acid sequence targeted by PKC in the MARCKS protein. The PKC assay reaction mixture consisted of 30 μL of membrane suspension (10 μg of protein), 10 μL of 750 μM peptide substrate, and 10 μL of 500 μM [γ-^{32}P]ATP (220 cpm/pmol, 0.5 μCi/tube) in assay buffer. After incubation at 25°C for 10 min, samples were immediately spotted on 1-cm^2 pieces of P81 Whatman paper (Fisher Scientific, Nepean, ON) and immersed in 5% acetic acid. The samples were washed twice in 5% acetic acid for 10 min. The amount of radioactivity bound to the filters was measured by using a liquid scintillation counter. Control reactions were performed either in the presence of membrane extract alone (no peptide) or in the presence of 10 μg of BSA alone (no peptide or membrane extract). These controls represent background attributable to phosphorylation of endogenous PKC substrates in the membrane preparation that may be capable of binding to P81 paper and nonspecific binding of [γ-^{32}P]ATP to P81 paper.

Measurement of *fos* mRNA

Cells were treated as described above for PKC activity. Fifteen min before exposure to PAF, cultures received 10 μg/mL cycloheximide. After stimulation with PAF for various time intervals, RNA was extracted and northern analysis performed [53,56]. In brief, total RNA (10 μg) was isolated, denatured with formaldehyde, electrophoretically separated on a 1.2% agarose gel containing 0.2 M formaldehyde, transferred to a Hybond-N nylon filter (Amersham Corp., Arlington Heights, IL) by using a vacuum system, and hybridized to ^{32}P-labeled DNA probes. The DNA probes used were a 1-kb PstI fragment of p-*fos*-1 (c-*fos* probe) [56] and a 1.5-kb PstI fragment of pGAPDH [57]. The probes were labeled with [^{32}P]dCTP by random primer labeling. Exposure to X-ray film was for 48 h.

Statistical Analysis

PKC kinetics data were analyzed by multiple analysis of variance. For each time point, data from drug-treated cells were compared with those from vehicle-treated cells. All other data were analyzed by using one-way factorial analysis of variance tests or Student's *t* tests, depending upon the number of groups (multiple groups or two groups, respectively). After analysis of variance and detection of a statistically significant F value with respect to the effect of a given series of treatments, post hoc Dunnett's *t* tests were used to identify which treated cells differed statistically from vehicle-treated cells. P values < 0.05 were considered statistically significant (shown as * in figures), and P values < 0.01 were considered highly statistically significant (shown as ** in figures).

RESULTS

PAF Enhancement of Growth of HF in Low-Serum Medium

We previously showed that PAF can promote the in vitro growth properties of normal rat embryo fi-

broblasts, increasing their saturation density and their ability to grow in low-serum medium as well as inducing growth of AI colonies in soft agarose [51]. Figure 1 illustrates that a 1-h treatment with PAF could affect the subsequent growth properties of HF. Although PAF had no observable effect on cell proliferation when cells were grown in complete medium (Figure 1A), a short treatment with 100 nM–1 μM PAF allowed cells to grow for an extended period of time under serum-limited conditions, as compared with untreated cells (Figure 1B). This effect was evident 10 d after PAF treatment and was statistically different by 25 d. When tested for another property, growth in soft agarose, AI colonies were seen in cultures treated with PAF at concentrations of 10 nM or higher but only very rarely in untreated cultures (Figure 1C). The growth rate of AI colonies was very slow, requiring 40 d for colonies to surpass 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. This apparent growth cessation was not due to extensive cell death because the cloning efficiencies of cells derived from colonies at day 40 and day 60 did not differ significantly. Twenty colonies were recovered from agarose at each time point (from 1 μM PAF-treated cultures). Of these, 13 of 14 colonies produced viable cultures. The plating efficiency (colonies per cell plated) was 9% (range 8–10%); fifth passage control HF had a plating efficiency of 12% (range 10–15%).

Lyso-PAF–treated cells (data not shown) were not distinguishably different in any of the assays from vehicle-treated cultures (Figure 1). *Lyso*-PAF treatment under the conditions used (1 h, serum-free medium, 1 μM) was not cytotoxic; the plating efficiency was 13% (range 10–15%).

Effect of Inhibitors on PAF-Mediated Enhancement of Growth Properties

To investigate the mechanism by which PAF might induce phenotypic changes in HF, a series of agents that affect signal transduction was tested (Figure 2). HF were pretreated for 15 min with CV3988 (a PAF receptor inhibitor), staurosporine (a PKC inhibitor), genistein (a protein tyrosine kinase inhibitor), and indomethacin (a cyclooxygenase inhibitor). PAF was then added and incubation was continued for 1 h. The effect of these compounds on PAF-induced enhancement of growth in low serum is depicted in Figure 2A. CV3988 (10 μM), staurosporine (50 nM), and genistein (1 μg/mL) all blocked the PAF-enhanced ability of HF to proliferate under serum-limited conditions but 0.2 μM indomethacin had no effect. Treatment with higher concentrations of indomethacin (1, 10, or 20 μM) also had no effect (data not shown). None of these inhibitors at the concentrations used affected cell growth of vehicle-treated or untreated HF (data not shown). The inhibitors were also tested for their effects on PAF-promoted AI growth (Figure 2B). As in the case of growth in low serum, AI growth was inhibited by CV3988, staurosporine, and genistein but not by indomethacin. None of the agents affected the AI phenotype of vehicle-treated cells (Figure 2B); nor did any treatment combination alter cell viability over the course of the experiments (data not shown).

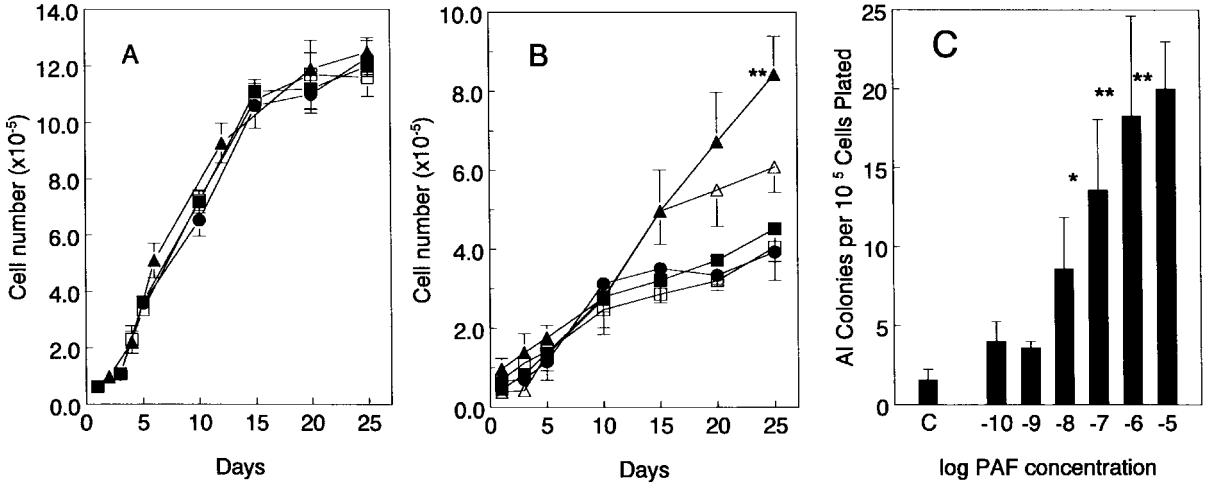

Figure 1. Growth enhancement of HF by PAF. Human fibroblasts were exposed for 1 h to different concentrations of PAF (0, □; 0.1 nM, ●; 10 nM, ■; 100 nM, △; 1 μM, ▲) and then grown either in complete medium (A), low-serum medium (B), or soft agarose AI growth (C). In panels A and B, each point represents the average cell count and standard error of the mean (SEM) of at least five plates. In panel C, the average number and SEM of AI colonies per 100 000 cells plated is shown. Colonies greater than 80 μm in diameter were scored at day 40 after treatment (n = 5 plates per condition). Other details are as described in Materials and Methods. Statistical tests (analysis of variance and post hoc Dunnett's *t* test) were performed on day 25 data points for panels A and B and on day 40 for panel C. No statistically significant differences were observed in panel A. For panel B, the control differed significantly from PAF (1 μM), $P < 0.01$. For panel C, the control differed significantly from 100 nM PAF ($P < 0.05$), 1 μM PAF ($P < 0.01$), and 10 μM PAF ($P < 0.01$).

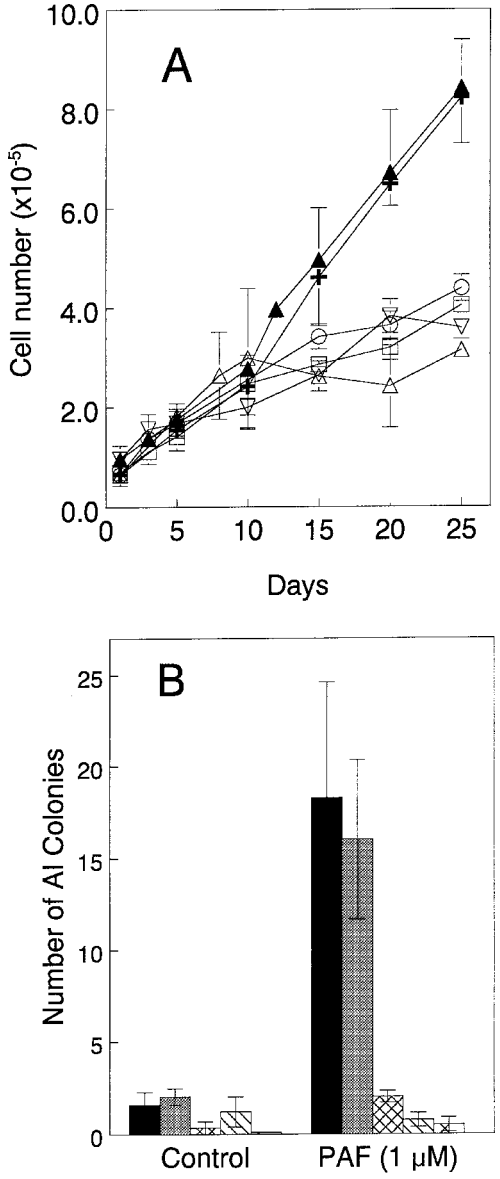

Figure 2. (A) Effect of inhibitors on PAF-induced growth of HF in low serum. HF were treated for 1 h with vehicle (□) or with 1 μM PAF (▲) in the presence of 200 nM indomethacin (+), 10 μM CV3988 (▽), 50 nM staurosporine (△), or 1 μg/mL genistein (○) and then grown in low-serum medium. The data represent cell counts from five or more plates per data point. The statistical analysis shown is for day 25. Statistically significant differences were found between PAF and PAF plus CV3988 ($P < 0.01$), PAF plus staurosporine ($P < 0.01$), PAF plus genistein ($P < 0.01$), and control ($P < 0.01$). Higher concentrations of indomethacin (1, 10, and 20 μM) had no effect (data not shown). (B) Effect of inhibitors on PAF-induced AI growth. HF were treated with PAF and inhibitors as in panel A and then plated in soft agarose to determine their capacity for AI growth. The data represent the number of AI colonies (greater than 80 μm diameter) per 100 000 cells plated as scored on day 40 after treatment (n = 5 plates/condition). The inhibitors used were (from left to right) none, indomethacin, staurosporine, genistein, CV3988. The concentrations used are as in panel A. Statistically significant differences were found between PAF and PAF plus staurosporine ($P < 0.01$), PAF plus genistein ($P < 0.01$), and PAF plus CV3988 ($P < 0.01$). Higher doses of indomethacin (1, 10, and 20 μM) had no effect (data not shown). Other details are as described in Materials and Methods.

PAF receptor inhibitors were also tested for their effects on growth enhancement in low serum when added after the initial PAF treatment (Figure 3). As in previous experiments, a short treatment with PAF produced extended growth in low serum compared with control cultures. Inclusion of either CV3988 or dioxolane (PAF receptor antagonists) in the culture medium 24 h after a 1-h treatment with PAF completely blocked growth in low serum to a level below that of control cultures. These agents were, however, not toxic because the cells responded to the addition of complete medium with the expected rapid increase in growth (Figure 3) and were not toxic in cytotoxicity tests (data not shown).

PAF-Induced Growth Enhancement in Low Serum Medium Lost After 50 d

The above experiments demonstrated that enhanced growth of PAF-treated HF continued for at least 25 d. To determine whether this property of the treated cell population is permanent or reversible, HF cultures were maintained in low serum for up to 75 d (Figure 4). The medium was replaced twice weekly, and the cells were passaged at 20-d intervals. Growth rate enhancement was evident for up to 50 d, but thereafter the slope of the growth curve appeared parallel to that of the untreated cultures. After 75 d, addition of complete medium produced

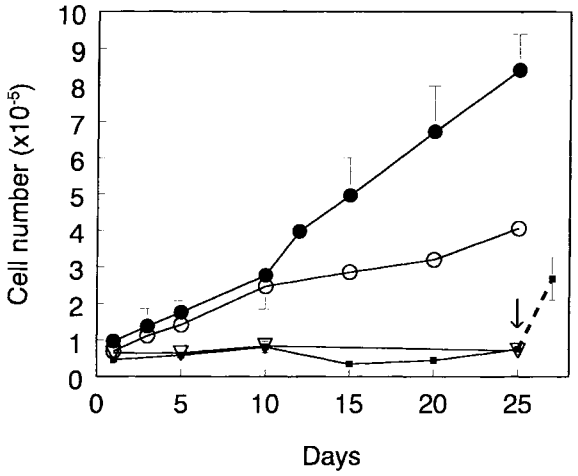

Figure 3. Effect of inhibitors added during growth in low serum. HF were treated with PAF (●) or with vehicle (○) as in Figure 2A. Twenty-four hours after removal of PAF and addition of low-serum medium, PAF receptor antagonists CV3988 (1 μM) (▽) and dioxolane (1 μM) (■) were added and replenished at each medium change. To assess viability after 25 d of treatment, complete medium (without inhibitor) was added to the dioxolane cultures (arrow). The data represent cell counts from at least five plates per point. Means ± SEM are shown; where no error bars are shown, the SEM is within the data point. Statistical tests (analysis of variance and post hoc Dunnett's *t* test) were performed for the day 25 data points. Statistically significant differences were seen for PAF versus CV3988 ($P < 0.01$) and PAF versus dioxolane ($P < 0.01$) and for control (vehicle) versus CV3988 ($P < 0.01$) and control versus dioxolane ($P < 0.01$).

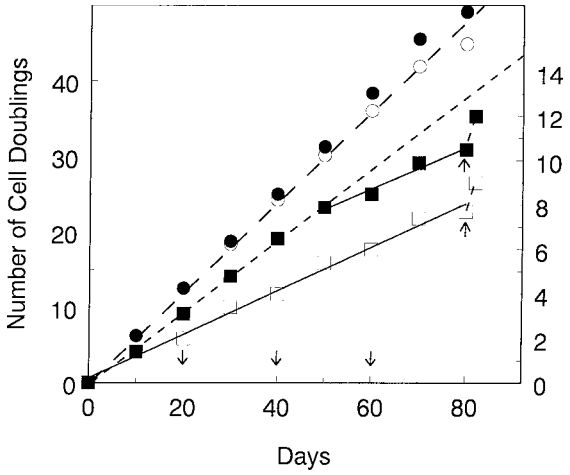

Figure 4. Stability of PAF-induced growth in low serum. HF were treated with vehicle (control) (○, □) or with PAF (●, ■) as in Figure 2. Cells (1×10^5) were then cultured in 6-cm dishes either in complete medium (left ordinate) (○, ●) or in low serum (right ordinate) (□, ■), and the increase in cell number was estimated with a Coulter counter. The cultures were fed twice weekly and replated at 20-d intervals (indicated by the downward arrows). Each data point is based on counts from five to 12 replicate dishes. At 80 d (indicated by the upward arrows), the low-serum medium was replaced with complete medium. Multiple regression analysis was performed on the growth curves in low serum; PAF-treated cells grew faster for 50 d ($P < 0.05$), and thereafter the slope was similar to control cells. There was no effect of PAF on growth in complete medium.

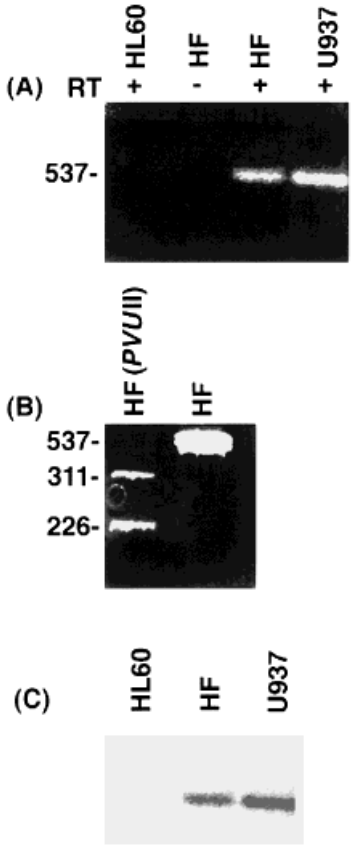

Figure 5. PAF receptor mRNA in HF. cDNA was prepared by reverse transcription of 500 ng of total cellular RNA extracted from HF, HL-60, and U-937 cells as described in Materials and Methods. The RT products were amplified by PCR with primers specific for a 537-bp fragment of the human PAF receptor gene. (A) First lane, undifferentiated HL-60 cells (PAF receptor negative); second lane, HF cells processed in the absence of RT (control for genomic contamination); third lane, HF cells; fourth lane, U-937 cells (PAF receptor positive). (B) PvuII digestion (first lane) of the HF PCR product (second lane) produced the expected 226- and 311-bp fragments. (C) The identities of the fragments were confirmed by Southern analysis of the PCR products with a 1-kb fragment of the PAF receptor human leukocyte gene [39]. First lane, HL-60 cells; second lane, HF; third lane, U-937 cells.

a growth spurt in both treated and untreated cultures, indicating that both populations were not yet senescent (Figure 4).

PAF Receptor mRNA Detected in HF by RT-PCR

HF were tested for the presence of mRNA for PAF receptor by RT-PCR (Figure 5A). We observed a 537-bp RT-PCR fragment (lane 3) identical in size to that obtained from U-937 cells (lane 4), which are known to express PAF receptor [39]. Digestion of the PCR fragment with PvuII generated fragments of the expected size (311 and 226 bp) (Figure 5B). Probing with full-length PAF receptor cDNA further confirmed the identity of the amplified product (Figure 5C). The negative control reactions in Figure 5A were HL-60 RNA (no PAF receptor mRNA [58]) (first lane) and PCR (i.e., no RT) (second lane) and controlled for false priming and genomic DNA contamination, respectively.

PAF Activation of PKC and Stimulation of *fos* mRNA Expression in HF

The inhibitor studies involving staurosporine suggested that PKC may be involved in PAF-induced growth enhancement. To test whether PAF induces PKC in HF, membrane-bound PKC activity was measured. PAF elicited a transient, dose-dependent increase in PKC activity, with maximal activation occurring 2 min after stimulation with 10 nM and 1 μM PAF (Figure 6). The increase was blocked by pretreating cells with CV3988 (10 μM) or staurosporine (50 nM) (data not shown).

Activation of PKC in quiescent fibroblasts is known to produce upregulation of transcription of the *fos* proto-oncogene. To provide additional evidence that PAF treatment can activate PKC, the cells rendered quiescent by growth to confluence were treated with 10 nM PAF in serum-free medium 15 min after the addition of cycloheximide. Total cell RNA was prepared and *fos* mRNA levels were measured by northern analysis. As shown in Figure 7, an increase in *fos* mRNA was detected within 5 min of exposure to PAF.

DISCUSSION

Although the effect of PAF has been widely studied in a variety of cell types [21,26,59–62], there have

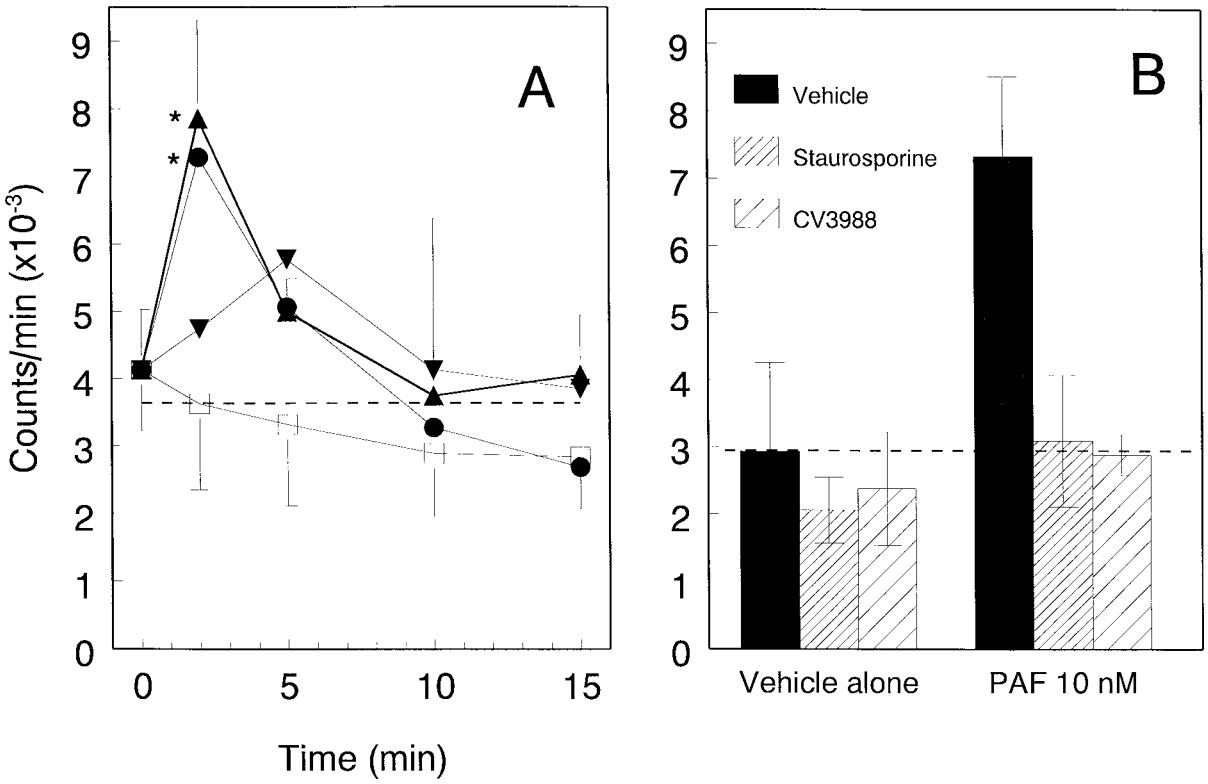

Figure 6. (A) PAF increase of membrane-associated PKC activity in HF. Cells were treated with PAF, and at the indicated times extracts were prepared and PKC assays were performed as described in Materials and Methods. The data are expressed as the average cpm ± SEM for three independent experiments, each conducted in triplicate. The range of the triplicates was less than 10%. The specific activity of the [γ-^{32}P]ATP in the reaction mixture was 220 cpm/pmol. □, Vehicle (0.1% dimethyl sulfoxide); ▼, 0.1 nM PAF; ●, 10 nM PAF; ▲, 1000 nM PAF. Dotted line, nonspecific binding of [γ-^{32}P]ATP to P81 paper. Statistically significant differences in PKC activity across time and treatments are as follows: 10 nM PAF versus vehicle multiple analysis of variance: 2-min treatment, $P < 0.05$. 1 μM PAF versus vehicle multiple analysis of variance: 2-min treatment, $P < 0.05$. (B) Inhibition of PAF-induced PKC activity by staurosporine (50 nM) and by CV3988 (10 μM). The experimental details are as in Materials and Methods and panel A.

been relatively few investigations into its effect on fibroblasts [51,63]. In other cell types, the effect of PAF is mediated through a specific receptor [37–40,42]. The report presented here documents the presence of PAF receptor mRNA in human skin fibroblasts, as recently shown in human lung fibroblasts [42]. We also showed that HF responded to PAF treatment by an increase in PKC activity and c-*fos* mRNA levels, as also reported for other cell types [42,46,47,50,64,65]. This evidence indicates that a functional PAF receptor is present in human fibroblasts and that its activation triggers PKC and other signal transduction events.

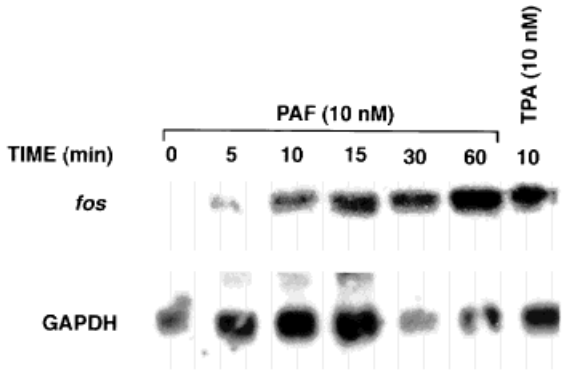

Figure 7. Induction of c-*fos* mRNA expression in HF by PAF. HF were pretreated with 10 μg/mL cycloheximide for 15 min before exposure to 10 nM PAF or to 10 nM TPA (positive control). Total RNA was extracted and northern analysis performed as described in Materials and Methods. The blots were stripped and reprobed with pGAPDH to demonstrate the presence of equivalent amounts of RNA in each lane.

Activation of the PAF receptor may affect different target cell types differently. Previously, we showed that PAF can enhance growth of primary rat embryo fibroblasts under low-serum conditions and induce the ability to form colonies in soft-agarose medium [51]. The study presented here extends these findings to human fibroblasts. Inhibitors of PKC (staurosporine) and tyrosine kinase activity (genistein) administered during PAF treatment effectively blocked HF growth potentiation, suggesting that signal transduction pathways mediate these phenotypic changes. Coadministration of PAF and the PAF receptor antagonist CV3988 repressed the autacoid-mediated growth alterations, but *lyso*-PAF, the

immediate PAF metabolite, was inactive, indicating the ligand-specific nature of these effects. Thus, the altered growth properties of HF in response to PAF appeared to be receptor mediated.

We observed that the altered growth pattern of HF induced by a brief exposure to PAF lasted 50 d, or approximately 40 cell doublings. Despite the persistence of this altered growth pattern over a fairly long time, transformation of PAF-treated cultures was not stable, and eventually the cells reverted to a normal phenotype. The mechanism underlying this "reversible transformation" is uncertain. There is no evidence that PAF treatment is genotoxic. Although PAF has been reported to have biological activity in alveolar macrophages at concentrations as low as 1 fM [66], it is highly improbable that the growth potentiation observed under both low-serum and AI conditions was due to the carry over of residual trace amounts of the initially added PAF because of the extensive washing and multiple medium changes performed. It is also known that PAF has a short half-life in serum due to the presence of PAF acetylhydrolases [67,68]. Rather, we postulate that brief treatment with PAF stimulates a self-sustaining PAF-driven autocrine loop. Such an autocrine loop has been demonstrated for certain tumor cells and mesangial cells [44,65,69]. Supporting evidence for the existence of such a loop in HF was provided by our finding that PAF antagonists administered after PAF treatment effectively suppressed PAF-induced growth potentiation as well as blocked the growth of untreated cells in serum-limited medium. The same antagonists produced no detectable cytotoxicity. However, it is possible that the two structurally different antagonists had an inhibitory effect unrelated to the PAF receptor. PAF would therefore appear to be a mitogen for HF, as shown earlier for human endometrial adenocarcinoma cells and pulmonary fibroblasts in defined medium [42,65]. However, in the case of HF, the mitogenic activity and establishment of an autocrine loop appears to be evident only under serum-deprived growth conditions. This loop may be broken with time, and the rate of cell proliferation may eventually return to normal.

While these data are consistent with and extend our earlier findings using rodent fibroblasts [51], they also reveal differences in PAF-induced stimulation between cell types and species. As with HF, PAF-induced growth enhancement of rat fibroblasts is receptor mediated, PAF specific, and tyrosine kinase dependent. However, rodent and human fibroblasts respond differently to cyclooxygenase and PKC inhibitors. PAF-induced transformation of rodent cells is partially inhibited by indomethacin and insensitive to staurosporine, whereas PAF-mediated HF transformation is unaffected by indomethacin and inhibited by staurosporine. These results suggest that there are some species or cell-type differences either in PAF signaling pathways or in sensitivity to the inhibitors used. In addition, PAF treatment of rodent cells results in a growth advantage under both optimal and suboptimal culture conditions; brief exposure increases cell saturation density and focus formation in complete medium and enhances growth in low-serum and AI growth. In HF, PAF-induced growth potentiation was seen only in the latter two conditions. These data highlight the differences in PAF mitogenic signals between rodent and human fibroblasts.

Inflammatory diseases and malignancy are associated with elevated levels of PAF [32,70,71]. We postulate that these elevated levels may enhance proliferation of cells at sites where they may also be exposed to genotoxic oxyradicals or nitric oxide, thus increasing the probability of malignant conversion. Our findings lend support to the hypothesis that endogenous inflammatory agents such as PAF can affect the growth of cells at sites of chronic inflammation and, in this manner, contribute to tumorigenesis or tumor progression.

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