Uteroglobin reverts the transformed phenotype in the endometrial adenocarcinoma cell line HEC-1A by disrupting the metabolic

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UTEROGLOBIN REVERTS THE TRANSFORMED PHENOTYPE IN THE ENDOMETRIAL ADENOCARCINOMA CELL LINE HEC-1A BY DISRUPTING THE METABOLIC PATHWAYS GENERATING PLATELET-ACTIVATING FACTOR

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Uteroglobin, originally named blastokinin, is a protein synthesized and secreted by most epithelia, including the endometrium. Uteroglobin has strong anti-inflammatory properties that appear to be due, at least in part, to its inhibitory effect on the activity of the enzyme phospholipase A_2. In addition, recent experimental evidence indicates that uteroglobin exerts antiproliferative and antimetastatic effects in different cancer cells via a membrane receptor. The human endometrial adenocarcinoma cell line HEC-1A does not express uteroglobin. Thus, we transfected HEC-1A cells with human uteroglobin cDNA. The transfectants showed a markedly reduced proliferative potential as assessed by impaired plating efficiency as well as by reduced growth in soft agar. Cytofluorimetric analysis clearly indicated that in uteroglobin-transfected cells the time for completion of the cell cycle was increased. We previously demonstrated that HEC-1A cells actively synthesize platelet-activating factor, one of the products of phospholipase A_2 activity. In addition, we demonstrated that platelet-activating factor stimulates the proliferation of these cells through an autocrine loop. In uteroglobin transfectants, the activity of phospholipase A_2 and platelet-activating factor acetyl-transferase, which are involved in the synthesis of platelet-activating factor, was significantly reduced compared with wild-type and vector-transfected cells ($p < 0.05$). Our results indicate that enforced expression of uteroglobin in HEC-1A cells markedly reduced their growth potential and significantly impaired the synthesis of platelet-activating factor, an autocrine growth factor for these cells. These data suggest that one possible mechanism for the recently observed antineoplastic properties of uteroglobin may be the inhibition of the synthesis of platelet-activating factor. *Int. J. Cancer* 88:525–534, 2000.
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Uteroglobin (UG) (Beier, 1968) or blastokinin (Krishnan and Daniel, 1967) is a low molecular mass (15.8 kDa) secretory protein, first discovered in the rabbit uterine fluid during early pregnancy (see Miele *et al.,* 1994, for review) and subsequently found in other organs of the female/male genitourinary apparatus, in the respiratory and gastrointestinal tract and in the blood (Miele *et al.,* 1994). UG gene expression is under organ-specific hormonal control. For example, corticosteroids stimulate UG gene expression in the lung, whereas progesterone exerts an analogous stimulatory effect in the uterus (Miele *et al.,* 1994). Prolactin has been shown to augment progesterone-dependent UG gene expression (Kleis-San Francisco *et al.,* 1993). The human counterpart of UG was first found in nonciliated bronchiolar Clara cells (Plopper *et al.,* 1980) and was initially named CC10 kDa protein, based on its apparent molecular weight. Subsequently, it was demonstrated that the expression pattern (Peri *et al.,* 1993) and the biochemical properties (Mantile *et al.,* 1993) of CC10 kDa are very similar, if not identical, to those of rabbit UG, thus reinforcing the hypothesis that CC10 kDa can be regarded as the human UG. In particular, Peri *et al.* (1994) have previously demonstrated that UG mRNA and protein are readily detectable in the cycling human endometrium and that the highest levels of expression are observed in the lutheal phase of the menstrual cycle.

Several physical and biological properties of UG have been established. These include binding of progesterone and polychlorinated biphenyls, masking the antigenicity of pre-implantation embryos and epididymal spermatozoa *in vitro,* the inhibition of monocyte and neutrophil chemotaxis and phagocytosis and the inhibition of ADP- and thrombin-induced platelet aggregation (Miele *et al.,* 1994). The properties of UG can be due, at least in part, to its potent inhibitory effect on the activity of low molecular weight, secretory phospholipases A_2 ($sPLA_2$) (Miele *et al.,* 1994). Two major types of $sPLA_2$, structurally very similar, had been originally described (see Mukherjee *et al.,* 1992, for review): $sPLA_2$ type I, found primarily in cobra and sea snake venoms and in the mammalian pancreas, and type II, found in the venoms of *Crotalidae* family of snakes and in the human synovium, neutrophils and platelets. Various other mammalian $sPLA_2$s have been identified that are structurally related to types I and II. At least five different mammalian $sPLA_2$s have been described (Lambeau and Lazdunski, 1999). Various unrelated intracellular $cPLA_2$s have been described (Bingham and Austen, 1999; Mukherjee *et al.,* 1992). These are thought to participate in receptor-induced arachidonic acid (AA) release. Several lines of evidence suggest that both calcium-dependent and calcium-independent intracellular PLA_2s participate in receptor-induced AA release that is the first step in eicosanoid metabolism (Xing and Insel, 1996; Hiller and Sundler, 1999; Akiba *et al.,* 1999; Bingham and Austen, 1999). This metabolic network is receiving considerable attention as a potential target for chemopreventive intervention in human malignancies due to the growth-promoting effects of several eicosanoids (see Marks *et al.,* 1999, for review). In addition, $sPLA_2$s indirectly induce a variety of autocrine and paracrine effects, including cell proliferation, AA release and eicosanoid production, through cell membrane receptors (see Lambeau and Lazdunski, 1999, for review). This implies that $sPLA_2$ receptors can trigger the activation of intracellular PLA_2s (Xing *et al.,* 1995). Conversely, there is evidence indicating that UG is inversely correlated to neoplastic growth. UG mRNA is present in normal bronchiolar epithelium, whereas it becomes undetectable in human lung carcinoma by *in situ* hybridization (Broers *et al.,* 1992). Another report confirmed the markedly less frequent immunohistochemical positivity for UG in non-small-cell lung carcinoma compared with non-neoplastic lungs (Linnoila *et al.,* 1992). More recent reports showed that in a transgenic mouse model for lung carcinoma UG gene expression was lost with the progression of the tumor to the undifferentiated phenotype (Sandmoller *et al.,* 1995). Furthermore, UG-knockout mice have increased incidence of various malignancies (Zhang *et al.,* 1999). Enforced expression of UG or exogenous treatment with recombinant UG inhibit anchorage-independent growth and

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matrix invasion in various cancer cell lines (Leyton *et al.,* 1994, Kundu *et al.,* 1996, 1988; Szabo *et al.,* 1998; Zhang *et al.,* 1999).

Our group has previously demonstrated that in the human endometrial adenocarcinoma cell line HEC-1A platelet-activating factor (PAF), one of the products of PLA_2 activity, is actively synthesized and stimulates cell proliferation via an autocrine loop (Maggi *et al.,* 1994). This cell line, in contrast to the normal endometrium, does not express UG. To investigate the role of UG as an inhibitor of cell proliferation, we transfected HEC-1A cells with UG cDNA. Clones expressing UG were selected and the proliferation rate was assessed using different experimental approaches. Wild-type HEC-1A cells and mock- (*i.e.,* vector alone) transfected cells were used as controls. Because of the growth-promoting effect of PAF in these cells (Maggi *et al.,* 1994), we investigated whether UG over-expression might affect the pathways generating PAF.

MATERIAL AND METHODS

Cell transfection and selection of the clones by RT-PCR

A full-length cDNA coding for human pre-UG was inserted between the HindIII and the XbaI site in the multiple cloning site of the expression vector pRc/RSV (Invitrogen, San Diego, CA). The vector was prepared by Dr. A. Peri and Dr. Z. Zhang in the Section on Developmental Genetics, Heritable Disorders Branch, National Institute of Child Health and Human Development, The National Institutes of Health, Bethesda, MD. This vector contains a neomycin resistance marker. The vector containing UG cDNA was linearized and HEC-1A (American Type Culture Collection, Gaithersburgh, MD) cells were transfected via electroporation and plated in McCoy's medium (Sigma, St. Louis, MO) supplemented with 10% FCS (GIBCO, Grand Island, NY), penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2.2 g/l sodium bicarbonate and G418 (Geneticin; Sigma). Forty-four clones were isolated and expanded. HEC-1A cells were also transfected with solely the vector (hereafter indicated as mock-transfected cells for convenience) as a control. Sixteen clones were isolated and amplified. Total RNA was extracted from different clones by using the RNAzol method (Cinna/Biotecx, Friendswood, TX). Extracted RNA was subjected to RT-PCR. UG-specific primers and experimental conditions were as described previously (Peri *et al.,* 1993). After RT-PCR, the amplified products were subjected to agarose gel electrophoresis, blotted onto nylon membranes and then hybridized to a UG-specific probe, as described previously (Peri *et al.,* 1993). Out of 44 isolated clones after UG cDNA transfection, 21 showed detectable levels of UG mRNA. Six of the UG-transfected clones expressing detectable levels of UG and 6 of the pRc/RSV-transfected clones were selected for subsequent experiments. The quality of the RNAs was assessed by RT-PCR using primers specific for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Peri *et al.,* 1993). The experimental conditions for GAPDH RT-PCR were as described previously (Peri *et al.,* 1993). The amplification process was stopped in the exponential phase of the reaction. Amplified products were electrophoresed on agarose gel, blotted onto nylon membranes and hybridized to a GAPDH-specific probe (Peri *et al.,* 1993).

Western blot analysis for UG detection

The selected clones in which UG mRNA was detectable were analyzed by Western blot to show the presence of the protein. Cells grown at confluence were scraped in cold PBS-1 mM EGTA, centrifuged and the pellets resuspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% Nonidet P40, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM EGTA). Total protein concentrations were determined, and aliquots containing 40 μg of proteins were diluted in equal volume of reducing 2× SB (Laemmli's sample buffer = 62.5 mM Tris, pH 6.8, containing 10% glycerol, 20% SDS, 2.5% pyronin and 10% β-mercaptoethanol) with 1 mM EGTA, incubated at 95°C for 5 min and loaded onto 20% polyacrylamide-bisacrylamide gel. As positive control, 0.5 μg of recombinant rabbit UG was used. After separation in SDS-PAGE, proteins were transferred onto nitrocellulose (Sigma) and stained with Ponceau to verify equal protein loading. The nitrocellulose was blocked in 5% BSA for 2 hr in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4), washed and then immunostained with a goat anti-rabbit UG antibody (1:100) followed by a peroxidase-conjugated secondary anti-goat IgG (1:3,000). The anti-rabbit UG antibody we used specifically recognizes human UG, as assessed in a previous study (Kikukawa *et al.,* 1988). Reacted bands were revealed by a chemiluminescent substrate (BM; Roche Diagnostics, Milan, Italy).

Monolayer cloning assay

The method described by Sobrero *et al.* (1985) was utilized. Briefly, a monocellular dispersion was obtained by passing the cells, upon trypsinization, through a 25-gauge needle. The cells were dispensed at different concentrations into sterile 60 mm Petri dishes (100-250-500-1,000 cells/well). The number of HEC-1A cells transfected with UG cDNA was increased (1,000-5,000-10,000-20,000 cells/well) after the first experiments, since no colony was observed at the original concentration. The cells were incubated at 37°C and 100% humidity with 7.5% CO_2. Clonal growth was determined after staining with orcein. Colonies containing more than 200 cells, as assessed by colony dimensions, were scored at 10× magnification using a dissecting microscope. Each experimental point was determined in triplicate.

Growth in soft agar

Two percent agar (Fluka, Buchs, Switzerland) was dissolved in boiling water. After cooling at 80°C, the agar was transferred to a 45°C bath. The agar (2%, 12 ml) was added to the culture medium (60 ml McCoy's, supplemented with G418 for the transfected cells) and was kept at 45°C. The cultured cells were trypsinized to obtain single-cell suspensions. The cell suspensions were counted and diluted to give 2,000, 667, 222, 74 cells/ml. Each experimental point was determined in triplicate. The cells were placed on ice, then 2.5 ml of the agar-containing medium was mixed with 0.5 ml cell suspension and poured into a dish immediately. After pouring, all dishes were placed at 4°C for 10 min, and then incubated in a humid CO_2 incubator. After 15 days, the cell colonies in each dish were counted.

Cell cycle analysis

Cell cycle studies were performed as previously described (Muratori *et al.,* 1997). Briefly, HEC-1A cells (wild-type, UG-transfected, mock-transfected) were cultured (approximately 0.7×10^6) in 100 mm tissue culture plates in 10 ml complete culture medium for 12 hr, then 5 μg/ml of aphidicolin was added. After 24 hr, partially synchronized cells (about 80% of total cells) were washed in PBS and fresh McCoy's medium was added. At the established times (every 3 hr for 36 hr), cells were washed twice with cold PBS without calcium and magnesium, and 1 ml fluorochrome solution (propidium iodide, 0.05 mg/ml, dissolved in 0.1% sodium citrate and 0.1% Triton X-100) was added. Each experimental point was determined in triplicate. Three experiments were performed. Dishes were kept for 60 min in the dark at 4°C and cells were then detached by scraping and pipetting and transferred to test tubes for cytofluorimetric analysis. Cell fluorescence was measured in a FACScan cytometer (Becton Dickinson, Mountain View, CA). The LYSYS II software was used to determine the distribution of cells in the various cell cycle stages as G_0/G_1, S and G_2.

Binding studies

Binding experiments were performed as previously described (Maggi *et al.,* 1994). HEC-1A cells (wild-type and UG-transfected) grown to confluence in 24-well plastic plates were incubated at 4°C for 24 hr in the presence of a fixed concentration (2 nM) of [^{3}H]PAF with increasing concentration (0.3–100,000 nM) of unlabeled PAF. All the measurements were obtained in triplicate. At the end of the incubation time, the supernatants were

removed, the cells were washed 6 times in ice-cold Dulbecco-modified PBS containing 3 mg/ml BSA and then solubilized in 200 μl of 1% SDS containing 0.25 N NaOH. In a beta counter, 190 μl of the total volume was counted.

Mitogenic assay ([^{3}H]thymidine incorporation)

Experimental conditions were as previously described (Maggi *et al.*, 1994). Briefly, cells were plated in 24-well plastic plates. After 2 days of culture, cells were maintained for 24 hr in serum-free medium, and PAF (3 or 10 μM) or vehicles were added. All treatments were performed in triplicate. The cells were then incubated for 24 hr, and [^{3}H]thymidine (1 μCi/well) was added during the last 4 hr. Cells were washed twice with ice-cold 5% trichloroacetic acid and solubilized with 0.25 N NaOH in 0.1% SDS at 37°C for 20 min. Aliquots were transferred to plastic vials and counted by liquid scintillation.

[^{3}H]AA release

These experiments were performed as previously described (Baldi *et al.*, 1993). Briefly, cells were grown to confluence on 6-well plastic plates, washed twice in serum-free McCoy's 5A medium containing 0.1% BSA and pre-incubated in the same buffer in the presence of [^{3}H]AA (2 μCi/ml) overnight. Each experimental point was determined in triplicate. The reaction was stopped by placing tubes on ice. Supernatants were collected and lipids extracted according to Bligh and Dyer (1959). The chloroform phase was dried under nitrogen, reconstituted in chloroform/methanol (9:1) and applied to silica gel t.l.c. plates. The solvent system used was hexane/ethyl ether/acetic acid (70:30:1). [^{3}H]AA was identified by co-chromatography with a known standard and visualized under a UV lamp after exposure to fluorescein. Areas corresponding to AA were scraped and counted by liquid scintillation.

Lyso-PAF:acetyl-CoA acetyl-transferase activity

PAF acetyl-transferase activity was determined in cell lysates by the incorporation of [^{3}H]acetyl from radiolabeled acetyl-CoA into [^{3}H]PAF, using lyso-PAF as substrate, as described previously (Villani *et al.*, 1991). Cells were sonicated in a buffer containing 0.1 M Tris-HCl (pH 7), 5 mM dithiothreitol, 0.5 mM EGTA. The homogenate was resuspended in the same buffer containing 1 mM $CaCl_2$ and centrifuged at 500 g for 5 min at 4°C. Aliquots of supernatant were incubated for 5 min at 37°C in a shaking water-bath in the presence of 30 μM lyso-PAF. [^{3}H]acetyl-CoA (5 μCi) was then added and incubation proceeded for 15 min. The reaction was stopped by addition of chloroform/methanol/acetic acid (1:2:0.04); lipids were extracted and PAF separated by t.l.c. as described above. All the measurements were obtained in triplicate.

Statistical analysis

Binding data were evaluated quantitatively with non-linear least-squares curve fitting using the computer program LIGAND (Munson and Rodbard, 1980), as previously described (Maggi *et al.*, 1994). The program provides objective measures of goodness-of-fit and objective criteria for distinguishing between models of different complexity. The selection of the best model was based on comparison of the weighted sum of squares and/or the root mean square error. An F-test based on the "extra sum of squares" principle was used. Results were expressed as mean ± SE or SD. Statistical comparisons were performed using unpaired Student's *t*-test.

RESULTS

Selection of transfected cell clones expressing UG

After transfection of HEC-1A cells with human UG cDNA, cell clones were tested for the presence of UG mRNA and protein. UG mRNA was assayed by RT-PCR. The expected 278 bp signal was detected by agarose gel electrophoresis of the RT-PCR products from 21 UG-transfected clones (not shown). The specificity of the signal was assessed by Southern blotting, using a UG-specific oligonucleotide as the probe. In Figure 1, the hybridization pattern of two (#4 and #7) of the clones exhibiting detectable levels of UG mRNA is shown. Neither HEC-1A wild-type cells nor mock-transfected cells (16 isolated clones) exhibited any positivity for UG mRNA (Fig. 1). Compared with the previously reported physiological expression of UG in the human endometrium (Peri *et al.*, 1994), UG mRNA levels in UG-transfected cells appeared similar in most cases and lower in some of the examined clones. Therefore, the observed effects of UG transfection on HEC-1A cells are unlikely to be due to over-expression of UG above the physiological range. The good quality of the RNAs used in these experiments was verified by RT-PCR, using a pair of primers specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH mRNA levels were virtually the same in all the samples examined (not shown). In Western blot experiments, UG was detected in HEC-1A cells transfected with UG cDNA and exhibiting detectable levels of UG mRNA, whereas neither mock-transfected nor wild-type cells showed any positivity (Fig. 2). Interestingly, no signal corresponding to UG monomer (around 5 kDa) was apparently detected, as expected under reducing conditions (see Material and Methods). However, the higher molecular weight signals observed in UG-transfected cells could be representative of enzymatic modifications of UG monomers. UG is a substrate for transglutaminase (Manjunath *et al.*, 1984), and this enzyme can crosslink UG monomers to amines altering its electrophoretic mobility (Porta *et al.*, 1990). Virtually identical UG migration patterns have been observed by our group in different cell lysates (*i.e.*, spermatozoa and lymphocytes; unpublished data).

Effect of UG transfection on cell proliferation

Six of the UG-transfected clones, exhibiting detectable levels of UG, and 6 of the mock-transfected clones were used for the experimental work reported in this study. Two different approaches (monolayer cloning assay and growth in soft agar) were used to determine the effects of UG expression on cell proliferation. In the first approach, the cells were plated at different concentrations on solid supports (Petri dishes). The plating efficiency of UG-transfected, mock-transfected and wild-type HEC-1A cells is reported in Figure 3. The data show that transfection with UG cDNA severely impairs cell proliferation. The plating efficiencies of the different cell subtypes, calculated according to Sobrero *et al.* (1985) was 33 ± 4% (mean ± SD, n = 6, the mean value is inclusive of all the different cell concentrations plated) for HEC-1A wild-type cells, 25.66 ± 2.61% (n = 2) for mock-transfected cells and 1.17 ± 0.68% (n = 4) for UG-transfected cells. Using a different experimental design, HEC-1A wild-type and UG-transfected cells were plated at different concentrations (2,000/ml, 667/ml, 222/ml and 74/ml) and grown in soft agar. Anchorage-independent growth, as occurs in soft agar, is a feature

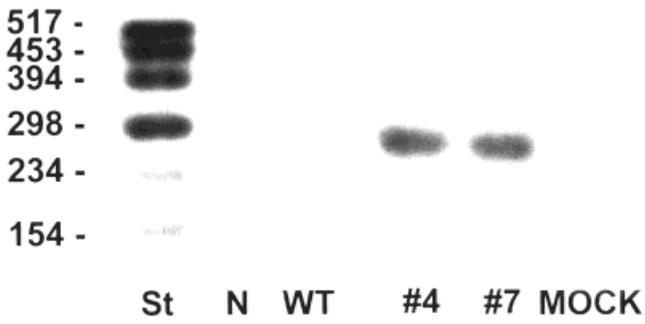

FIGURE 1 – Chemilumigram showing RT-PCR products from total RNAs using UG-specific primers, after hybridization with a UG-specific oligonucleotide probe. St, DNA molecular weight marker standard VI-digoxigenin labeled (Roche Diagnostics, Milan, Italy); N, no RNA control reaction; WT, wild-type HEC-1A cells; MOCK, one clone of HEC-1A cells transfected with pRc/RSV vector; #4 and #7, different HEC-1A UG-transfected clones, expressing UG.

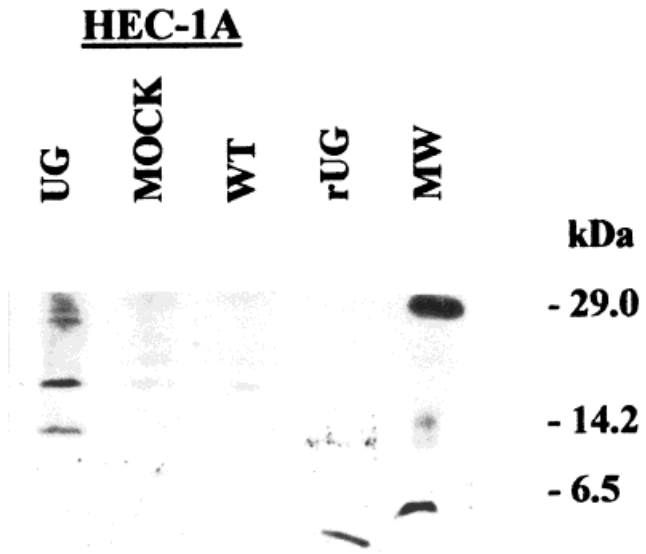

FIGURE 2 – Western blot analysis of UG content. The numbers on the right indicate the molecular weight (kDa) of the bands of the molecular weight marker. UG, HEC-1A clone transfected with UG cDNA; MOCK, HEC-1A clone transfected with pRc/RSV vector; WT, wild-type HEC-1A cells; rUG, recombinant rabbit uteroglobin; MW, molecular weight marker.

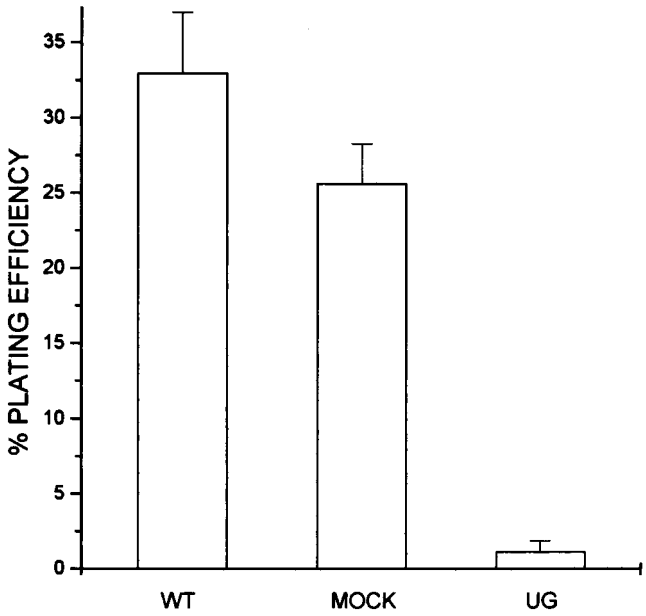

FIGURE 3 – Plating efficiency of HEC-1A wild-type (WT), mock-transfected (MOCK) and UG-transfected cells (UG) as determined by the method described by Sobrero *et al.* (1985). The numbers express the percentage of the cell colonies containing more than 200 cells.

of neoplastic cells. Wild-type HEC-1A cells formed large colonies in these conditions (Fig. 4*a*). The number of colonies ranged from 45 at the highest plating concentration to 3 at the lowest concentration. Conversely, UG-transfected cells appeared only as spread cells (Fig. 4*b*) and formed only a single colony at the highest plating concentration (not shown).

Cell cycle analysis

To determine the effect of UG transfection on cell cycle progression, flow cytometric analysis of DNA content in cells (HEC-1A wild-type, mock-transfected and UG-transfected) synchronized with aphidicolin was performed. Figure 5 shows the results of a typical experiment. After 24 hr exposure to 5 μg/ml aphidicolin, a potent nonspecific inhibitor of DNA polymerase α, about 80% of the cells in the 3 groups were in G_o/G_1-early S, as indicated by fluorescence emission of propidium iodide-stained nuclei of the cells (Fig. 5, top row). After removal of aphidicolin, wild-type and mock-transfected cells showed a faster progression through the cell cycle than UG-transfected cells, entering S and G_2/M at earlier times. Six hours after aphidicolin release, 73% of wild-type and 67% of mock-transfected cells were traversing S, whereas only 58% of UG-transfected cells were in S (Fig. 5, second row from the top). After a 12 hr release from aphidicolin block, 70% of wild-type and 54% of mock-transfected cells were in G_2/M (Fig. 5, third row). Forty-nine percent of UG-transfected cells were still traversing S and only 38% were in G_2/M. Six hours later, 41% of wild-type and 36% of mock-transfected cells were back in G_o/G_1 compared with only 14% of UG-transfected cells (Fig. 5, fourth row). Twenty-four hours after aphidicolin release, about 60% of the wild-type and mock-transfected cells were replenishing G_o/G_1 (45% of both cell subtypes) or were traversing S (15% and 18%, respectively) (Fig. 5, fifth row). Conversely, most of UG-transfected cells were still in G_2/M (68%) and only 23% were back in G_o/G_1. Only 12 hr later, UG-transfected cells partially accumulated in G_o/G_1 (46%; Fig. 5, lowest row), whereas a reduction in the amount of wild-type and mock-transfected cells in G_o/G_1 was observed compared with the previous experimental point shown in the figure (40% and 38%, respectively), concomitantly to an increased number of cells traversing S (19% and 31%, respectively).

PAF-mediated loop

Because UG-transfected cells exhibited a lower proliferative activity than mock-transfected and nontransfected controls, we speculated that UG expression may affect the expression or the downstream signaling of molecules acting as growth factors for these cells. We therefore investigated whether or not UG expression interferes with the autocrine proliferative loop mediated by PAF, a phospholipid growth factor for HEC-1A cells. First, we studied the effect of UG transfection on the density and affinity of PAF receptors. Binding studies were performed as described (Maggi *et al.*, 1994). Figure 6 shows the results of a typical PAF binding experiment performed simultaneously in wild-type and UG-transfected HEC-1A cells. Mathematical analysis of binding data using the program LIGAND (Munson and Rodbard, 1980) indicated that the two displacement curves were virtually identical. The curves share the same binding parameters (high-affinity site $B_{max} = 31.41 \pm 11.62$ fmol/10^6 cells and $K_d = 0.3 \pm 0.16$ nM; low-affinity site $B_{max} = 16.46 \pm 14.59$ nmol/10^6 cells and $K_d = 0.66 \pm 0.40$ μM). These data indicate that UG transfection does not affect the expression of PAF receptors. To verify whether or not UG transfection interferes with PAF responsiveness, we stimulated wild-type, mock-transfected and UG-transfected HEC-1A cells with PAF and studied the effect of PAF on DNA synthesis. The results are shown in Table I, expressed as percentage [^{3}H]thymidine uptake vs. control, *i.e.*, untreated cells. In each of the 3 subgroups, a 24 hr incubation with PAF, added to the cultured cells at 2 different concentrations (3 and 10 μM), induced a statistically significant increase of [^{3}H]thymidine incorporation ($p < 0.05$) compared with controls. The mean of the control values in each subgroup was given an arbitrary value of 100. However, we found no differences in the ability of the 3 distinct subgroups to respond to PAF. This confirms that UG transfection does not affect the capability of HEC-1A cells to respond to PAF.

We therefore sought to determine whether the activity of 2 key enzymes in the synthesis of PAF, namely PLA_2 and PAF acetyltransferase, was reduced in UG-transfected cells. We determined PLA_2 activity in cultured cells by measuring AA release. The results, reported in Figure 7, indicated that the release of AA in HEC-1A cells and in mock-transfected cells is not statistically

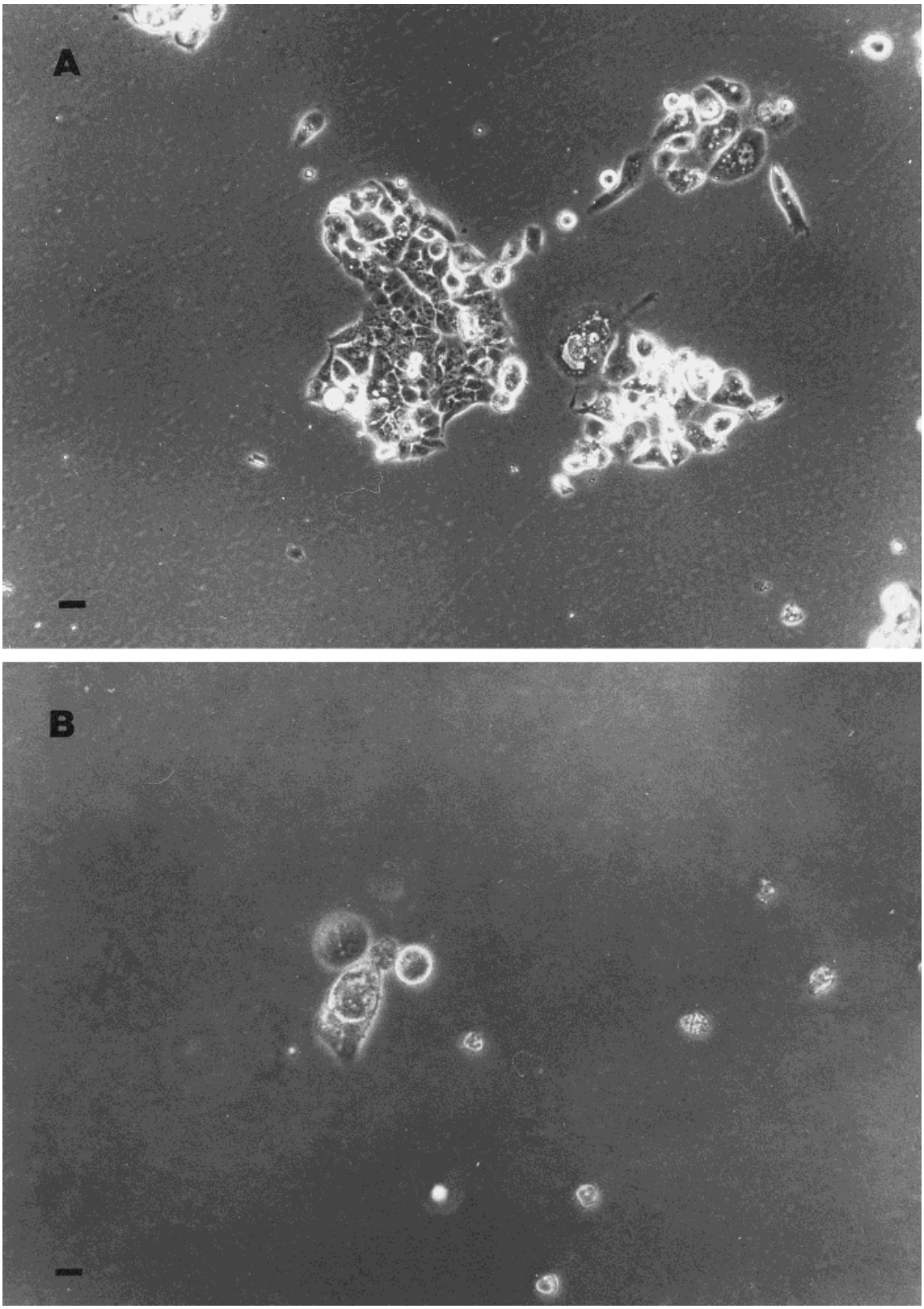

FIGURE 4 – Cell growth in soft agar. (*a*) HEC-1A wild-type cells were able to form colonies in these conditions; (*b*) HEC-1A cells transfected with UG cDNA did not retain this characteristic and only single spread cells were observed. Scale bar = 10 μm.

different, whereas a statistically significant lower value was obtained in UG-transfected cells compared with the 2 other groups of cells ($p < 0.05$). Furthermore, the activity of PAF acetyl-transferase was determined in wild-type as well as in UG- and mock-transfected HEC-1A cells. The experimental results are shown in Figure 8. As expected, PAF acetyl-transferase activity did not

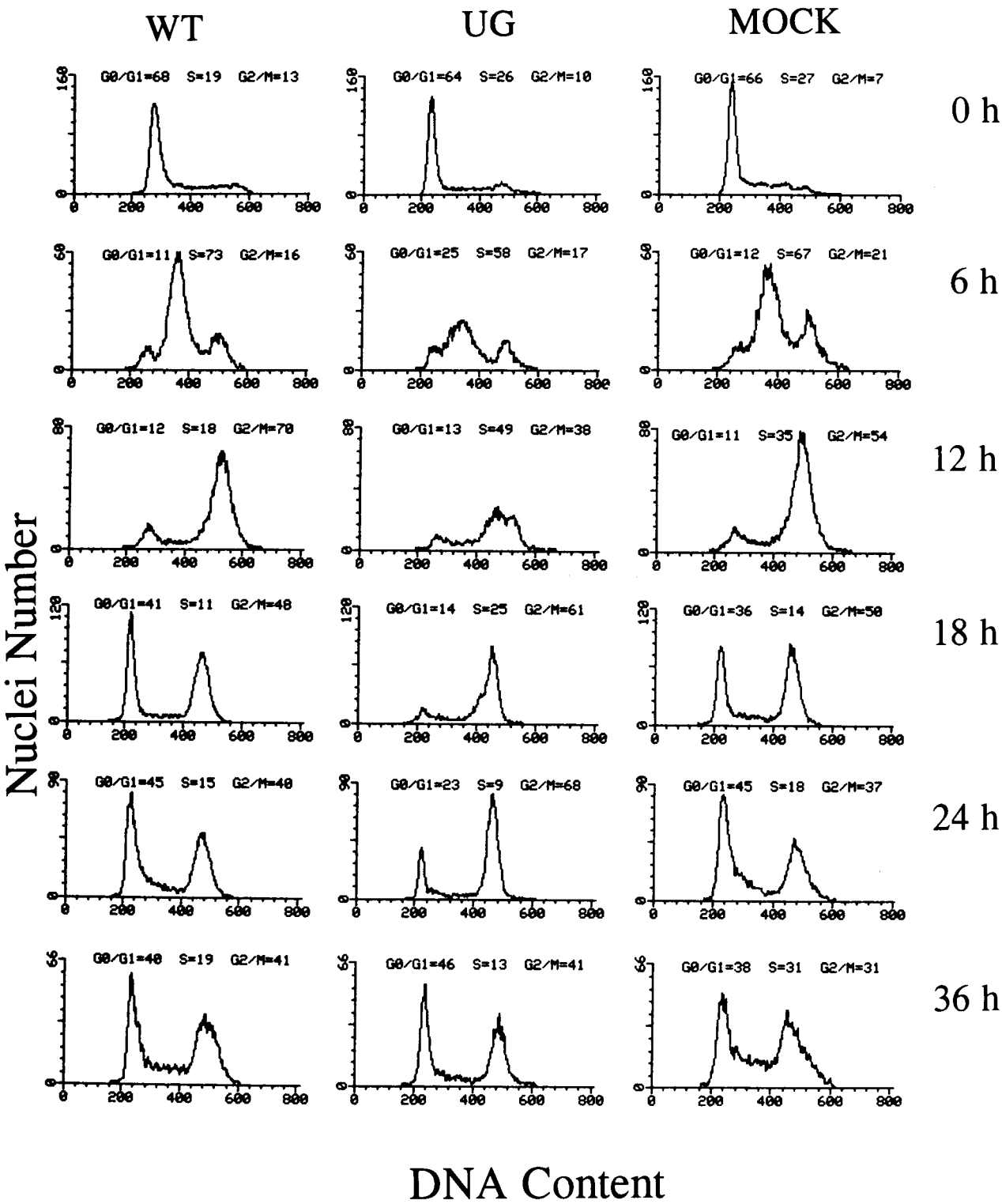

FIGURE 5 – Flow cytometric analysis of cell cycle distribution of synchronized HEC-1A wild-type cells (WT), UG-transfected (UG) and mock-transfected (MOCK) cells 0, 6, 12, 18, 24 and 36 hr after release from aphidicolin block.

significantly differ ($p = 0.13$) in wild-type and mock-transfected cells. Conversely, a significant reduction in enzymatic activity was observed in UG-transfected cells compared with wild-type and mock-transfected cells ($p < 0.05$).

DISCUSSION

In this study, we demonstrated that UG inhibits the proliferation and cell cycle progression of neoplastic cells from the human endometrium and that this effect appears to be mediated by a

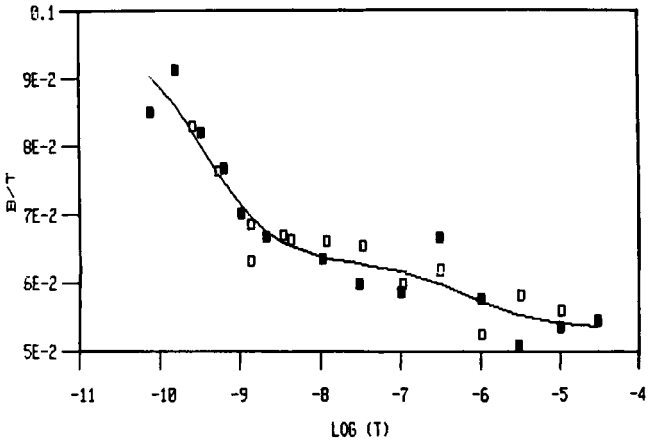

FIGURE 6 – Displacement curve for PAF binding to wild type (■) and UG-transfected (□) HEC-1A cells. Ordinate, B/T; abscissa, total ligand concentration (labeled and unlabeled) (T) on log scale.

TABLE I – EFFECTS OF 2 DIFFERENT CONCENTRATIONS OF PAF ON [^{3}H]THYMIDINE UPTAKE IN WILD-TYPE (WT), MOCK-TRANSFECTED (MOCK) AND UG-TRANSFECTED (UG) HEC-1A CELLS

PAF [M]	HEC-1A WT	HEC-1A MOCK	HEC-1A UG
3E-6	148.33 ± 6.74	153.33 ± 8	154 ± 12.5
1E-5	167 ± 5.68	147.66 ± 9.33	160.33 ± 12.66

Values (mean ± SE, n = 3) are expressed as percentage of control (100%). The differences among all pairs at the same concentration of PAF were not statistically significant ($p \geq 0.05$).

disruption in the metabolic pathways generating PAF. In a previous study investigating for the first time UG expression in human organs, Peri *et al.* (1993) found that UG is expressed in a number of human tissues and that epithelial cells are the primary site of UG synthesis. Subsequently, we demonstrated that UG expression in the endometrium is dependent on the hormonal environment of sex steroids. In particular, the levels of UG mRNA and protein were shown to peak in the secretory phase of the ovarian menstrual cycle (Peri *et al.,* 1994). However, when we investigated UG expression in a human endometrial cancer cell line, HEC-1A, no detectable amounts of either mRNA or protein were found.

In view of this discrepancy between normal and neoplastic endometrium, we investigated a possible relationship between UG and endometrial cancer. The first step was to stably transfect HEC-1A cells with an expression vector containing UG cDNA. The obtained cell clones were selected on the basis of the presence of UG transcript and protein. Subsequently, 6 of the clones expressing UG were subjected to studies assessing their neoplastic phenotype compared with wild-type cells. Mock-transfected cells (transfected with the vector in the absence of UG cDNA) were generated and used as controls in our experiments to rule out nonspecific effects due to the expression vector or the manipulations associated with cell transfection and selection. UG-transfected cells unequivocally exhibited markedly reduced proliferation rate and impaired anchorage-independent growth compared with wild-type and mock-transfected cells. High plating efficiency (clonogenicity) and anchorage-independent growth are important phenotypic parameters that characterize transformed cells. Flow cytometric analysis of aphidicolin-synchronized cells indicated that in UG transfectants cell cycle progression is markedly slowed compared with wild-type and mock-transfected HEC-1A cells. This effect appeared to be equally distributed among the different phases of the cell cycle rather than being the result of arrest at a single cell cycle checkpoint.

In principle, a tumor suppressor-like effect of UG is not surprising. In fact, the UG gene maps to chromosome 11q12.2–13.1

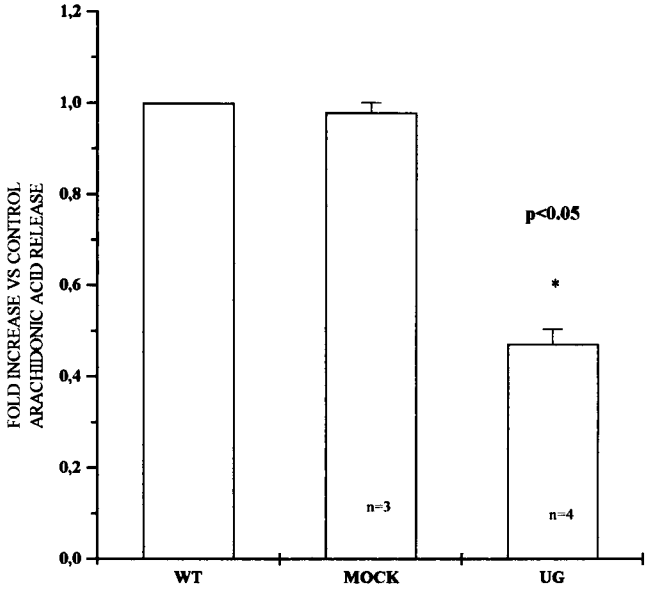

FIGURE 7 – Arachidonic acid release in wild-type (WT), mock-transfected (MOCK) and UG-transfected (UG) HEC-1A cells. n, number of experiments. The value representing the mean among the experimental determinations (in triplicate in 3 different experiments) for WT cells was considered as 1. The determinations (mean ± SE) for MOCK and UG cells are expressed as fold increase vs. WT. *, statistically significant at the 0.05 level.

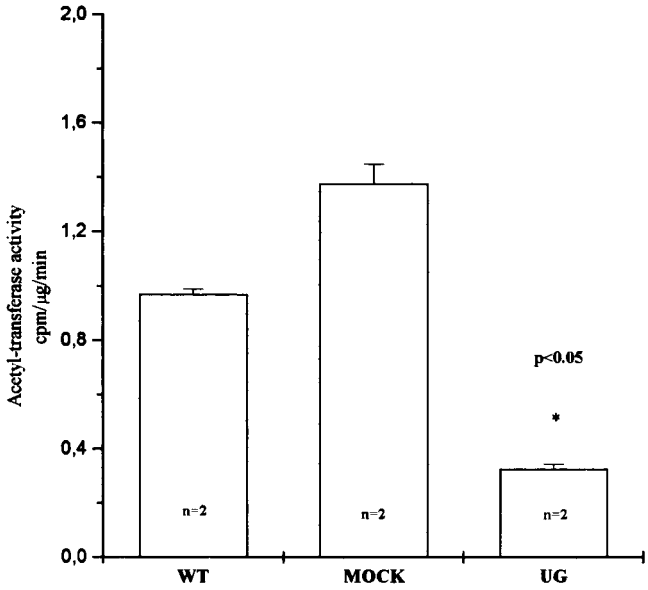

FIGURE 8 – Acetyl-transferase activity in wild-type (WT), mock-transfected (MOCK) and UG-transfected (UG) HEC-1A cells. n, number of experiments. The values (mean ± SE) are expressed in cpm/μg/min, where μg indicates μg of protein. *, statistically significant at the 0.05 level.

(Zhang *et al.,* 1997*a*). Rearrangements or deletions of this region of chromosome 11 have long been known to be associated with human malignancies (Misra and Srivastan, 1989; Lammie *et al.,* 1991; Jesudasan *et al.,* 1995). In addition, it has been reported that the introduction of chromosome 11 into cervical cancer cells (HeLa) suppresses the neoplastic phenotype of these cells (Saxon

et al., 1986). Recombinant human UG inhibits the *in vitro* invasiveness of human metastatic prostate cancer cells (Leyton *et al.,* 1994). Furthermore, Szabo *et al.* (1998) over-expressed UG in the non-small-cell lung cancer (NSCLC) cell line A549; this cell line, like most NSCLC lines, does not express UG. A549 cells, upon transfection with UG, showed a marked reduction in invasiveness that was paralleled by diminished metalloproteinase activity and by decreased anchorage-independent growth. In addition, the UG transfectants exhibited decreased adhesion to fibronectin. The authors hypothesize that since the binding of fibronectin to its integrin receptors initiates a cascade leading to stimulation of growth or suppression of apoptosis, the interruption of such a signal cascade by UG may be responsible, at least in part, for its antineoplastic activity. A severe renal glomerular disease associated with massive glomerular deposition of fibronectin has been observed in mice in which the UG gene had been disrupted (Zhang *et al.,* 1997*b*). The molecular mechanism that normally prevents fibronectin deposition appears to involve high-affinity binding of UG with fibronectin to form heterodimers that counteract fibronectin self-aggregation. Most interestingly, preliminary results of aging studies on UG-knockout mice revealed a high incidence of malignancies (UG-knockout mice, 16/16 vs. wild-type littermates, 0/25) (Zhang *et al.,* 1999).

The mechanism(s) of the apparent tumor suppressor-like effects of UG require further investigation. Our data suggest that one of these mechanisms may involve the inhibition of AA release and of the synthesis of lipid growth factors such as PAF. It is unclear which PLA_2(s) participate in AA release in HEC-1A cells. The inhibition of AA release by UG in this system may be due to direct inhibition of one or more mammalian $sPLA_2$(s) and/or to effects mediated by the UG receptor (Kundu *et al.,* 1996, 1998), to inhibition of integrin-fibronectin binding (see above) or to hitherto unrecognized mechanisms. UG has been demonstrated to be a very strong inhibitor of $sPLA_2$ activity, and this effect appears to be due to direct interaction between UG and $sPLA_2$ with interference with the process of interfacial activation (Miele *et al.,* 1994). A direct inhibition of $sPLA_2$ in a system in which such enzymes release AA from intact membranes could in principle result in decreased production of growth-promoting eicosanoids and PAF. On the other hand, $sPLA_2$s also stimulate AA release (Xing *et al.,* 1995), prostaglandin synthesis (Kishino *et al.,* 1995), cell growth (Arita *et al.,* 1991) and extracellular matrix invasion (Kundu *et al.,* 1997) through receptor-mediated mechanisms that do not require their catalytic activity (see Lambeau and Lazdunski, 1999, for review). UG is structurally similar to $sPLA_2$s (Mukherjee *et al.,* 1992), and it is an intriguing possibility that UG may function as an antagonist to receptor-mediated $sPLA_2$ effects, either by directly binding to $sPLA_2$ or to the $sPLA_2$ receptor or indirectly through its own receptor. Finally, inhibition of integrin-fibronectin binding could in principle lead to reduced AA release by preventing the activation of intracellular PLA_2(s) via protein kinase C or MAP kinases.

PAF is an acetylated alkyl ether glycerophospholipid produced by a variety of cell types and tissues, including inflammatory cells, epithelial cells, neurons, heart, lung, liver, kidney and exocrine glands (Bussolino and Camussi, 1995). After synthesis, PAF may either be released in extracellular fluids or remain exposed on the outer plasma membrane (Bussolino and Camussi, 1995). The synthesis of PAF from its precursor lyso-PAF depends on an acetyl-transferase reaction. The pathway by which PAF is generated through a tightly coupled reaction of PLA_2 and an acetyltransferase is indicated as the *remodeling pathway* (Bussolino and Camussi, 1995). Alternatively, PAF synthesis occurs also by a different route, named the *de novo pathway,* through the activity of the enzyme cholinephosphotransferase on the substrate 1-alkyl-2-acetyl-*sn*-glycerol (Bussolino and Camussi, 1995). Both enzymatic activities occur at relatively high levels in most cells.

We previously reported that HEC-1A cells synthesize PAF and respond to it via specific cell membrane receptors (Maggi *et al.,* 1994). PAF binding to HEC-1A cells induced DNA synthesis, expression of the nuclear proto-oncogene *c-fos* and calcium influx (Maggi *et al.,* 1994). In addition, other reports indicated that PAF increases tyrosine kinase activity both in platelets (Danhauser-Riedl *et al.,* 1991) and in proliferating cells (Chao *et al.,* 1992; Tripathi *et al.,* 1992). Recently, our group demonstrated that the mitogenic effect of PAF in HEC-1A cells is mediated by the activation of multiple signaling pathways, involving protein tyrosine kinase, mitogen-activated protein kinase and protein kinase C (Bonaccorsi *et al.,* 1997).

It should be noted that Camussi *et al.* (1990) have shown that synthetic oligopeptides derived from the putative active site of UG, named antiflammins (see Miele, 2000, for review), have essentially the same effects on human neutrophils as we have seen in HEC-1A cells with UG expression. These authors have shown that antiflammins inhibit AA release, the activation but not the catalytic activity of human neutrophil PLA_2 and the activity of lyso-PAF acetyltransferase. Our results indicated that AA release and acetyltransferase activity were reduced in UG transfectants compared with wild-type and mock-transfected cells. These findings suggest the possibility that the antiproliferative effects of UG in HEC-1A cells may be in part correlated to suppression of the enzymatic activities ultimately leading to the synthesis of PAF. The molecular mechanism(s) responsible for these effects of UG and antiflammins will require further investigation. However, our results suggest that the inhibition of PAF synthesis may be a common mechanism that contributes to the anti-inflammatory and antineoplastic properties of UG. The possible role of the $sPLA_2$ receptors and/or the UG receptors deserves careful consideration in light of the receptor-mediated effects of $sPLA_2$s on growth and invasion (see above) and of the fact that the antichemoinvasive effects of UG appear to require the presence of UG receptors (Zhang *et al.,* 1999).

In conclusion, in this study we have addressed the potential role of UG as a secretory tumor-suppressor protein in human endometrial carcinoma cells. We have demonstrated that UG expression significantly affects cell cycle progression and anchorage-independent growth and inhibits AA release and PAF synthesis. The latter effect may be responsible for its antiproliferative effect in this experimental model.

Together with reports from other groups (Broers *et al.,* 1992; Linnoila *et al.,* 1992; Leyton *et al.,* 1994; Sandmoller *et al.,* 1995; Nord *et al.,* 1998; Szabo *et al.,* 1998; Zhang *et al.,* 1999), this study suggests that loss of UG expression may potentially be a diagnostic/prognostic marker in some human malignancies and that UG itself, administered as a recombinant protein or through gene therapy strategies, may have significant antineoplastic activity.

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