Mechanisms of Platelet-Activating Factor-induced Enhancement of VEGF Expression

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Key Words

PAF • p53 • Egr-1 • Sp-1 • VEGF • NF- κ B

Abstract

It has been previously reported that platelet-activating factor (PAF) induces the expression of vascular endothelial growth factor (VEGF) via the downregulation of p53 activity. In this study, we attempted to characterize the mechanism by which p53 activity negatively regulates PAF-induced VEGF expression. PAF increased luciferase activity as well as VEGF mRNA expression in human non-small cell lung cancer cell line H1299 transfected with VEGF luciferase reporter plasmid (VEGF-Luc). Cotransfection of the cells with wt p53, but not mutant p53, effected a blockage of PAF-induced VEGF mRNA expression. The ChIP assay revealed that p53 did not bind to the VEGF promoter. Transfection of Egr-1 or Sp-1 expression vector increased VEGF luciferase activity in VEGF-Luc-transfected cells, and this was inhibited by transfection with wt p53. The results of the Immunoprecipitation and immunoblot analysis showed that p53 binds to Egr-1 and Sp-1. Additionally, our electrophoretic mobility shift assay

demonstrated that PAF induced the mobilization of Egr-1 and Sp-1 to the nucleus, and this activity was inhibited by transfection with wt p53. These data indicate that PAF inhibits protein complexes between p53 and Egr-1/Sp-1 via the downregulation of p53 levels, thus increasing the free form levels of Egr-1 and Sp-1, ultimately resulting in the transcriptional activation of VEGF.

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Introduction

Platelet-activating factor (PAF), which is generated by a variety of inflammatory cells, is a potent lipid messenger known to be involved in cellular activation, fertilization, intracellular signaling, and a variety of inflammatory reactions [1-4]. With regard to the role of PAF in inflammation, PAF appears to function as the proximal inducer of nuclear factor (NF)- κ B, a critical transcription factor which regulates the expression of proinflammatory cytokines and a variety of immunoregulatory molecules [5] in response to inflammatory stimuli [6, 7] and microbial infection [8]. Further-

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more, we have demonstrated that PAF promotes angiogenesis via the production of vascular endothelial growth factor (VEGF) in an NF- κ B-dependent manner [9, 10].

VEGF is a potent peptide growth factor specific for vascular endothelial cells, which promotes neovascularization and increases vascular permeability *in vivo* [11]. As a mechanism by which NF- κ B enhances VEGF expression, we [12] and others [13–16] have reported that NF- κ B activation blocks the transactivation of the expression of the tumor suppressor gene, p53, which is one of the most frequently mutated genes in human cancers [17] and has also been reported to downregulate the activity of the VEGF promoter [18]. However, NF- κ B and p53 do not appear to regulate VEGF expression directly, as its promoter harbors no NF- κ B or p53 binding sites [19–21].

We have also determined that the NF- κ B-dependency of the PAF-induced increase in VEGF expression is attributable to reduced p53 activity, which is reciprocally regulated by increased NF- κ B activity [12]. In this study, we extended these findings by demonstrating that PAF inhibits the formation of protein complexes between p53 and Egr-1/Sp-1 via the downregulation of p53 levels, thereby increasing the levels of Egr-1 and Sp-1 in their free forms, ultimately resulting in the transcriptional activation of VEGF.

Materials and Methods

Reagents

PAF (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was purchased from Sigma (St. Louis, MO). The PAF receptor antagonist, WEB2170, was generously provided by Dr. Rhee, C.K. (College of Medicine, Dankook University, Korea). The passive lysis buffer and the luciferase assay kit were obtained from Promega (Madison, WI). Protein A/G PLUS agarose and antibodies for p53, GAPDH, Egr-1, and Sp-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Reporter and expression constructs

The AccI-NheI fragment encompassing -2018 to +50 bp of the VEGF promoter (pLuc 2068) was cloned upstream of the promoterless luciferase reporter vector in pAH 1409 (VEGF-Luc, a gift from Dr. Finkenzeller, G., Molecular Medicine Institute, Germany). Other constructs of the VEGF promoter (pLuc 465) were generated via the modification of pLuc 2068 [22]. Human wild-type (wt) p53 and mutant (mt) p53 were cloned into pcDNA3 (a gift from Dr. Xirodimas, D., Ninewells Hospital & Medical School, UK). The mt p53 is a mutant form with a point mutation (Arg→His) at codon 175. This mutant form is functionally inactive [23–25]. cDNAs encoding

for Egr-1 or Sp-1 were cloned into pcDNA3.

Cell culture and establishment of stable transfectant cells

H1299 human non-small cell lung cancer (NSCLC) cells (p53-null cells) were obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco Invitrogen) at 37°C in a 5% CO₂ atmosphere. The H1299 cells were transfected with the wt p53 expression plasmid, using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. After transfection, the cells were selected in medium containing 500 µg/ml of G418 (Gibco Invitrogen) for 2 weeks, and individual G418-resistant clones were isolated. These clones were propagated and screened for p53 expression via Western blot analyses. Stable p53-expressing H1299 clones were maintained in medium containing G418.

Transient transfection and luciferase assay

Plasmid DNA was prepared using a QIAprep spin miniprep kit (Qiagen, Valencia, CA). H1299 cells (4×10⁴/well) were cultured to 40% confluence in 24-well plates. The cells were then transiently transfected with each plasmid DNA, using Lipofectamine™ 2000 reagent (Invitrogen) in accordance with the manufacturer's recommendations. After 16 h of incubation, the cells were then left untreated or were treated for 8 h with 2 µM PAF. The cells were harvested and the luciferase assays were conducted using a Lumat LB9501 luminometer (Berthold, Wildbad, Germany) in accordance with the manufacturer's recommended protocols (Promega, Madison, WI). Luciferase activity was normalized to β -galactosidase activity as an internal control. All experiments were repeated at least three times with two different batches of purified DNA.

Real-time RT-PCR

H1299 cells (4×10⁵/dish) were cultured to 40% confluence in 60 mm dishes. The cells were then transiently transfected with each plasmid DNA or siRNA. After 16 h of incubation, the cells were left untreated or were treated for 4 h with 2 µM PAF. Total RNA was extracted with TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions. Reverse transcription was conducted using 50 ng of total RNA in a 10 µl reaction mixture (Promega), containing oligo(dT) and avian myeloblastosis virus reverse transcriptase. PCR was conducted on a Roter-Gene 3000 System (Corbett Research, Morklake, Australia) using the SYBR Green PCR Master Mix Reagent Kit (Qiagen). The primers utilized were as follows: human VEGF; 5'-CTT GCC TTG CTG CTC TAC C-3' and 5'-CAC ACA GGA TGG CTT GAA G-3', and β -actin; 5'-GAT CTG GCA CCA CAC CTT CT-3' and 5'-GGG GTG TTG AAG GTC TCA AA-3'. The relative levels of VEGF mRNA were calculated using the standard curve generated from the cDNA dilutions. The mean cycle threshold (Ct) values from quadruplicate measurements were employed in the calculation of gene expression, with normalization to β -actin employed as an internal control. Calculations of the relative level of gene expression were conducted using complementary computer software (Corbett Research) with a standard curve.

Fig. 1. Inhibitory effect of p53 in PAF-induced VEGF expression. (A) H1299 cells (4×10^4) were transfected with 0.1 μ g of VEGF luciferase reporter plasmid (VEGF-Luc) and/or 0.1 μ g wt p53 or mt p53 expression vector ($n = 3$). The total concentration of DNA was adjusted to 0.2 μ g via transfection with an empty vector. The PAF antagonist, WEB2170 (10 μ g/ml), was added 30 min prior to treatment with 2 μ M PAF. VEGF promoter activity was assayed 8 h after PAF treatment, as described in the Materials and Methods section. Luciferase activity was normalized by β -galactosidase activity to determine transfection efficiency. All promoter activities are expressed as fold activations relative to the controls. (B and C) Cells (4×10^5) were incubated with WEB2170 30 min prior to PAF treatment or transfected with 0.5 μ g wt p53 or mt p53 expression vector, 16 h prior to PAF treatment ($n = 3$). (B) RNA was isolated 4 h after PAF treatment, and analyzed via Real-time RT-PCR, as described in the Materials and Methods section. (C) VEGF protein was analyzed via ELISA in the culture supernatants collected 8 h after PAF treatment. Results represent the mean $\pm$ S.E. of two or three independent experiments. *, $P < 0.001$ compared with the control group; #, $P < 0.001$ compared with the PAF-treated group.

Quantitation of cytokines by ELISA

H1299 cells (4×10^4 /well) were cultured to 40% confluence in 24-well plate. The cells were then transiently transfected with each plasmid DNA or siRNA. After 16 h of incubation, the cells were left untreated or were treated for 8 h with 2 μ M PAF. VEGF production from culture supernatants was quantitatively measured by using a human VEGF Quantikine ELISA kit (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from the cells, as previously described [6, 7]. 1 mM phenylmethylsulfonyl fluoride (PMSF) was added in order to inhibit endogenous protease activity. An oligonucleotide harboring the Igk-chain-binding site, Egr-1-binding site (Egr-1, 5'-CCG GTC CAG CGG GGG CGA GCG GGG GCG-3') or the Sp-1-binding site (Sp-1, 5'-CCG GAT TCG ATC GGG GCG GGG CGA GC-3') was synthesized as a probe for use in the gel retardation assay. The two complementary strands were then annealed and labeled with (α - ^{32} P)dCTP. Labeled oligonucleotides (10,000 cpm), 20 μ g of nuclear extracts, and binding buffer [10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly (dIdC) and 1 mM DTT] were incubated for 30 min at room temperature in a final volume of 20 μ l. The reaction mixture was analyzed via electrophoresis on a 5% polyacrylamide gel in 0.5 $\times$ Tris-borate buffer. Specific binding was controlled by competition with 50-fold excess of α - ^{32} P-unlabeled ('cold') Egr-1 or Sp-1 oligonucleotides as a competitor, or cAMP response element (CRE) oligonucleotides.

RNA Interference

Small interfering RNA (siRNA) strands for Egr-1 (sc-29303), Sp-1 (sc-29487), and the negative control [siRNA-A, non-targeting control siRNA (sc-37007)] were purchased from Santa Cruz Biotechnology. The cells were transfected with Lipofectamine^{TM} 2000 Reagent (Invitrogen) in the presence of siRNAs, in accordance with the manufacturer's recommendations.

Immunoprecipitation and immunoblot analysis

Nuclear extracts were prepared from the cells, as previously described [6, 7]. The extracts were then incubated overnight with 2 μ g of anti-p53 antibody at 4°C and precipitated with Protein A/G PLUS-Agarose beads. The immune complexes were washed 4 times and electrophoretically analyzed on 8-10% SDS-polyacrylamide gels, then transferred onto Protran nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Membranes were blocked via 1 h of incubation at room temperature in 5% skim milk in TBS, followed by overnight incubation with primary antibody at 4°C. The blots were then washed, and then incubated for 2 h at room temperature with the HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). The blots were subsequently washed again and finally developed using LumiGLO Reagent (Cell Signaling Technology, Beverly, MA).

Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was conducted using a ChIP assay kit (Upstate Biotechnology, Waltham, MA) in accordance with the manufacturer's recommendations. In brief, H1299 cells sta-

bly expressing wt p53 (2×10^6 /dish) were cultured to 80% confluence in 100 mm dishes and then treated with PAF for 1 h. Cells were cross-linked with 1% (v/v) formaldehyde for 10 min at 37°C. Cells were washed twice with ice-cold PBS with protease inhibitor (1 mM PMSF), scraped, and pelleted by centrifugation for 4 min at 2000 rpm at 4°C. Pellets were resuspended in a lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, and 10 mM EDTA) with protease inhibitor, incubated for 10 min on ice, and sonicated to shear DNA. Remaining insoluble material was removed by centrifugation for 10 min at 13,000 rpm at 4°C. The supernatant was diluted in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1% Triton X-100, 2 mM EDTA, and 167 mM NaCl) with protease inhibitors, and then pre-cleared with 60 μ l of Salmon Sperm DNA/protein A agarose beads for 1 h at 4°C. The DNA/protein complex was immunoprecipitated overnight using 5 μ g of anti-p53 or anti-Egr-1 antibody. 60 μ l of Salmon Sperm DNA/protein A agarose beads were added in order to purify the immune complexes, and the DNA was purified via phenol/chloroform extraction. Primers designed for the VEGF and p21 promoter were utilized for PCR amplification. The VEGF promoter primer set (-222/-3 containing the p53 response region) was 5'-TCA GGC TGT GAA CCT TGG TGG G-3' and 5'-CTC CCC GCT ACC AGC CGA CTT TTA A-3'. The p21 promoter primer set (p53-binding sites) was 5'-GTG GCT CTG ATT GGC TTT CTG-3' and 5'-CTG AAA ACA GGC AGC CCA AG-3'. The expected products were subsequently resolved on 1.5% agarose gels.

Statistical analysis

Data were expressed as the means $\pm$ SE. Statistical significance was evaluated via one-way ANOVA (StatView; Abacus Concepts, Berkeley, CA). A *P* value of < 0.05 was considered statistically significant. All experiments were conducted at least twice. Reproducible results were obtained, and thus representative data are shown in the figures.

Results

p53 inhibits PAF-induced VEGF expression in H1299 cells

In this study, we used the p53-null cells, human non-small cell lung cancer cells (H1299 NSCLC). We transfected the cells with VEGF-Luc. The treatment of the cells with PAF resulted in increases in luciferase activity (Fig. 1A) as well as VEGF mRNA expression (Fig. 1B) and protein synthesis (Fig. 1C), all of which were blocked by treatment with the PAF antagonist, WEB2170. Additionally, PAF-induced luciferase activity (Fig. 1A) as well as VEGF mRNA expression (Fig. 1B) and protein synthesis (Fig. 1C) were significantly diminished by wt p53 as compared to the mt p53-transfected cells. We conducted a ChIP assay to evaluate the possibility that p53 regulates VEGF expression via direct binding to the VEGF promoter. Protein-DNA complexes were

Fig. 2. p53 does not bind directly to the VEGF promoter. H1299 cells stably expressing wt p53 (8×10^5) were treated for 1 h with 2 μ M PAF. The nuclear extracts were immunoprecipitated with normal IgG, p53, or Egr-1 antibody and a ChIP assay was performed. The PCR primers were amplified in the -222 to -3 region of the VEGF promoter and in the p53 binding site of the p21 promoter. Input refers to the same dose of nuclear extract administered prior to immunoprecipitation. Egr-1 binding to the VEGF promoter and p53 binding to the p21 promoter were used as a positive control for the ChIP assay. One representative data from three independent experiments is shown.

immunoprecipitated with antibodies against p53, Egr-1 (as a positive control), or normal IgG (negative control) and the DNA was isolated and purified. An aliquot of non-immunoprecipitated chromatin was employed as the input control was included to show specificity. Densitometry demonstrated no increase in p53 binding to the VEGF promoter after incubation with PAF (Fig. 2).

p53 inhibits Egr-1- and Sp-1-induced VEGF expression

Considering that the nucleotides between -414 and +50 within the VEGF promoter harbor Egr-1 [20] and Sp-1 [21] binding sites, we subsequently explored the possibility that p53 indirectly suppresses VEGF expression via interaction with Egr-1 and/or Sp-1. We initially attempted to characterize the manner in which Egr-1 and Sp-1 regulate VEGF expression. Transfection with Egr-1 or Sp-1 expression vectors increased VEGF luciferase activity in a dose-dependent manner in VEGF-Luc-transfected H1299 cells (Fig. 3A and B). Furthermore, either Egr-1 or Sp-1 siRNA significantly inhibited the increases in VEGF luciferase activity (Fig. 3C) as well as mRNA expression (Fig. 3D) and protein synthesis (Fig. 3E) that occur in response to PAF. These data demonstrate that both Egr-1 and Sp-1 are key transcription factors in PAF-induced VEGF expression.

We next attempted to characterize the manner in which p53 regulates Egr-1- or Sp-1-induced VEGF expression. H1299 cells were cotransfected with VEGF luciferase reporter plasmid (pLuc 2068), Egr-1, Sp-1, wt p53, or mt p53 expression vector. Transfection with Egr-1 or Sp-1 expression vector resulted in enhanced

Fig. 3. Effect of Egr-1 or Sp-1 on PAF-induced VEGF expression. (A and B) H1299 cells (4×10^4) were cotransfected with 0.1 μg VEGF-Luc and Egr-1 or Sp-1 expression vector at the indicated concentrations ($n = 3$). (C) Cells (4×10^4) were cotransfected with 0.1 μg VEGF-Luc and 100 nM siRNAs 16 h prior to treatment with 2 μM PAF. VEGF promoter activity was assayed 8 h after PAF treatment ($n = 3$). RNA (D) and VEGF protein (E) were analyzed via real-time RT-PCR and ELISA ($n = 3$), as described in the legend for Fig. 1, respectively. Results represent the mean $\pm$ S.E. of two or three independent experiments. *, $P < 0.001$; **, $P < 0.05$ compared with the control group; #, $P < 0.001$ compared with the PAF plus negative control siRNA-treated group.

Fig. 4. H1299 cells (4×10^4) were cotransfected with Egr-1, Sp-1, wt p53 or mt p53 expression vectors and modified VEGF luciferase reporter plasmids. (A) The nucleotide sequence -2018 to +50 bp of the human VEGF gene promoter was modified by 5'-deletion. Potential consensus binding sites for AP-1, Sp-1 and Egr-1 response regions are indicated. (B and C) VEGF promoter activity was assayed ($n = 3$), as described in the legend for Fig. 1. Results represent the mean $\pm$ S.E. of two or three independent experiments. *, $P < 0.001$ compared with the control group. #, $P < 0.001$ compared with the Egr-1 or Sp-1 expression vector transfected group.

luciferase activity, and this enhanced activity was completely inhibited by transfection with wt p53 (Fig 4B and C). The binding sites of Egr-1 and Sp-1 congregate in the -414/+50 VEGF promoter region. Therefore, we generated the modified VEGF luciferase reporter pLuc 465 (Fig. 4A). When this reporter construct was cotransfected with other expression vectors, the luciferase activity was determined to be similar to that of pLuc 2068 (Figs. 4B and C).

p53 binds to Egr-1 and Sp-1

In an effort to determine the molecular mechanisms underlying the p53-mediated inhibition of Egr-1- or Sp-1-induced VEGF expression, we evaluated the possible physical interaction occurring between p53 and Egr-1/Sp-1. The nuclear extracts from H1299 cells stably expressing wt p53 were immunoprecipitated with p53 antibody, and the resultant immune complexes were subjected to immunoblot analysis with either Egr-1 or Sp-1 anti-

Fig. 5. Physical interaction between p53 and Egr-1 or Sp-1 in response to PAF. (A) H1299 cells (8×10^5) stably expressing wt p53 were treated for 1 h with PAF. The nuclear extracts were immunoprecipitated with control (normal) IgG or p53 antibody and the resultant immune complexes were subjected to immunoblot analysis with Egr-1 or Sp-1 antibodies. (B) H1299 cells were treated with PAF for the indicated times. (C) H1299 cells (4×10^5) were transfected with wt p53 or mt p53 expression vector, and then treated with PAF for 1 h. The nuclear extracts were incubated with α - ^{32} P-labeled Egr-1 or Sp-1 oligonucleotide, and then electrophoresed on 5% polyacrylamide gel. A 50-fold excess of cold (Egr-1 or Sp-1) or irrelevant CRE (cAMP response element) oligonucleotide was added as a competitor. One representative data from three independent experiments is shown.

body. As is shown in Fig. 5A, the nuclear extracts immunoprecipitated with p53 antibody were stained with Egr-1 or Sp-1 antibody, and were inhibited by the addition of PAF. Additionally, the results of the gel mobility shift assay demonstrated that the treatment of H1299 cells with PAF resulted in the mobilization of Egr-1 and Sp-1 to the nucleus (Fig. 5B). The blockage of Egr-1 or Sp-1 mobilization via the addition of the cold competitor, but not via the addition of an irrelevant motif, CRE, was considered reflective of the specificity of its binding. This mobilization was inhibited by transfection with wt p53, but not mt p53 (Fig. 5C). These data demonstrate that p53 forms a protein complex with Egr-1 and Sp-1, and inhibits their binding to the VEGF promoter to prevent transcriptional activation.

Discussion

VEGF is the most important directly acting angiogenic protein [26-28], and p53 has been identified as a repressor of VEGF transcription [29-31]. We have also demonstrated previously that PAF induces VEGF expression via the downregulation of p53 activity [12]. In this study, we have elucidated the mechanism by which p53 inhibits the PAF-mediated transcription of VEGF in an Egr-1 and Sp-1-dependent manner in the H1299 NSCLC cell line.

With regard to the mechanism responsible for the PAF-mediated repression of VEGF expression, it appears unlikely that p53 directly modulates the transcriptional

regulation of VEGF via binding to the VEGF promoter, because the VEGF promoter harbors no NF- κ B binding sites [20, 21]. Consistent with this rationale, our ChIP assay also demonstrated no binding of p53 to the VEGF promoter; this suggests that p53 must suppress VEGF expression via a less direct mechanism. In order to determine the principal element(s) in the VEGF promoter relevant to the role of p53 in PAF-mediated VEGF expression, we generated a series of VEGF promoter deletion mutant plasmids. We determined that nucleotides -414 to +50 within the VEGF promoter are critical for p53-mediated transcriptional regulation. This region has been previously determined to contain binding sites for Egr-1 and Sp-1 [20, 21].

Egr-1 has been implicated previously in the upregulation of VEGF in ischemic stress [32], in duodenal ulceration [33], and in response to prolactin [34]. In addition to Egr-1, Sp-1 has also been implicated as an important factor in the induction of VEGF in response to certain stimuli, including oxidative stress [35], IL-1 [36], and TNF- α [37]. These findings are reflective of positive roles for Egr-1 and Sp-1 in the regulation of VEGF expression. This notion was further bolstered by our findings that Egr-1 and Sp-1 enhanced VEGF luciferase activity, and that VEGF transcriptional activity occurring in response to PAF was inhibited by treatment with either Egr-1 or Sp-1 siRNA (Fig. 3).

Recently, a physical interaction has been observed to occur between p53 and Egr-1 [38] or Sp-1 [18, 39]. Consistent with these reports, our immunoprecipitation and immunoblot analysis verified that p53 was capable

of binding to both Egr-1 and Sp-1. Furthermore, we observed in this study that PAF induced the mobilization of Egr-1 and Sp-1 to the nucleus, and that this phenomenon was blocked by the transfection of the cells with wt p53. These data show that p53 reduces the levels of Egr-1 and Sp-1 in their free forms, thereby blocking the binding of Egr-1 and Sp-1 to the VEGF promoter, and ultimately resulting in the downregulation of VEGF expression. Therefore, it appears likely that when the levels of p53 are reduced in response to PAF, the levels of free-form Egr-1 and Sp-1 are increased, thus resulting in enhanced VEGF production.

Recently it has been reported that the physical interaction of phosphorylated Sp-1 with NF- κ B was observed in vascular smooth muscle cells [40], and this interaction enhanced target promoter transactivation. Similarly, Stein et al. [41] reported that c-Fos and c-Jun are capable of physically interacting with NF- κ B. This complex exhibits enhanced DNA binding and subsequent biological function. Therefore, it is possible that physical interaction between NF- κ B and Sp-1 occurred in H1299 cells as well in response to PAF, and this complex lead to upregulation of VEGF expression through enhancing its binding to VEGF promoter. The possible involvement of such interaction can not be excluded. Regarding the effect of PAF on Sp-1, Taheri et al. [42] reported that PAF directly activates Sp-1 by phosphorylation through ERK MAPK signaling. Our unpublished data also demonstrates that PAF activates ERK, suggesting that PAF is able to

activate Sp-1 in H1299 cells via ERK activation, which, in turn, may modulate the physical interaction of Sp-1 with p53 or NF- κ B. Further studies are required to address these issues.

In summary, the findings of this study demonstrate, for the first time, the formation of a protein-protein complex between p53 and Egr-1/Sp-1 *in vivo* as a possible mechanism underlying the PAF-mediated upregulation of VEGF expression. Considering that PAF is generated by a variety of inflammatory cells, as well as tumor cells [43-45], our results contribute significantly to our current understanding of the mechanisms underlying the angiogenesis observed in certain pathological situations, most notably inflammation and cancer [46].

Abbreviations

PAF (Platelet-activating factor); VEGF (Vascular endothelial growth factor).

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