Platelet-activating factor-induced NF-κB activation enhances VEGF expression through a decrease in p53 activity

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Abstract We investigated the role of p53 in nuclear factor (NF)-κB dependent, platelet-activating factor (PAF)-induced vascular endothelial growth factor (VEGF) expression. Transfected NF-κB subunits in ECV304 cells increased the tumor necrosis factor-α promoter activity, which was completely inhibited by p53. Transfected p53 increased p53RE promoter activity, which was completely inhibited by NF-κB subunits, indicating that cross-regulation occurs between NF-κB and p53. PAF-induced increase in VEGF expression was correlated with decreased p53 activity. These data suggest that NF-κB-dependency of the PAF-induced increase in VEGF expression is due to decreased p53 activity, which is reciprocally regulated by increased NF-κB activity.

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1. Introduction

Neovascularization or angiogenesis is required to sustain growth of primary tumors and metastases. Tumor angiogenesis is mediated by increased production of various angiogenic molecules released by both the tumor itself and nearby host cells [1,2]. Vascular endothelial growth factor (VEGF) is a potent peptide growth factor specific for vascular endothelial cells that promotes neovascularization and increases vascular permeability in vivo [3]. The critical role of VEGF in tumor angiogenesis has been demonstrated as inhibition of tumor growth following treatment with anti-VEGF neutralizing antibodies [4] or by administration of VEGF receptor (flk-1) antagonists [5]. The VEGF promoter contains AP-1, AP-2, SP-1, and HIF-1 binding sites [6,7]. Hypoxia induces VEGF expression through activation of HIF-1 [7] and AP-1 [8,9], whereas tumor necrosis factor (TNF)-α increases VEGF expression via SP-1 [8,10].

Platelet-activating factor (PAF), which is produced by a variety of inflammatory cells, is a potent lipid messenger involved in cellular activation, fertilization, intracellular signaling, apoptosis, and diverse inflammatory reactions [11–14]. We have shown that PAF is a proximal inducer of the transcription factor, nuclear factor (NF)-κB, a pivotal transcription factor that regulates the expression of proinflammatory cytokines and many immunoregulatory molecules [15,16] in response to inflammatory stimuli [17,18] and microbial infection [19]. Recently, we have shown that PAF promotes angiogenesis through the production of NF-κB-dependent angiogenic factors such as interleukin-1, TNF-α, basic fibroblast growth factor (bFGF), and VEGF [20]. Other investigators also have reported that VEGF production and gene expression is compromised by NF-κB inhibition [21,22]. However, NF-κB does not appear to regulate VEGF expression directly, as its promoter does not contain an NF-κB binding site [7].

One mechanism by which NF-κB enhances VEGF expression is to regulate the expression of genes that directly regulate its expression. One candidate gene is the tumor suppressor gene, p53. p53 is one of the most frequently mutated genes in human cancers [23]. Wild-type p53 protein downregulates VEGF promoter activity [24], but the loss of p53 function, via somatic mutations or the expression of viral oncoproteins, contributes to angiogenesis during tumorigenesis [25–27]. Furthermore, NF-κB and p53 cross-regulate mRNA expression. For example, p53 activation by daunomycin is partially regulated by NF-κB [28]. Similarly, NF-κB activation increases p53 promoter activity [29]. In contrast, other groups have reported that NF-κB activation blocks p53 transactivation [30–33]. Thus, it is possible that NF-κB upregulates VEGF expression through modulating p53 activity in PAF-induced angiogenesis. Thus, we investigated this possibility and found that a reciprocal regulation between NF-κB and p53 occurred in PAF-induced VEGF expression.

2. Materials and methods

2.1. Reagents

PAF (1-O-alkyl-2-acetyl-sn-glycercyl-3-phosphorylcholine) was purchased from Sigma Chemical Co. (St. Louis, MO). The PAF receptor antagonist, WEB2170 was a gift from Dr. Rhee, C.K. (College of Medicine, Dankook University, Korea). The NF-κB inhibitor, parthenolide was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). ELISA kit for detecting human VEGF was

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purchased from R&D Systems (Minneapolis, MN). Passive lysis buffer and the luciferase assay kit were purchased from Promega (Madison, WI).

2.2. Antisense oligonucleotides

The following phosphorothioate oligonucleotides were synthesized for use in antisense inhibition of gene expression (Peptron, Korea): p65 antisense (p65 AS) of the 5′ end of the NF-κB gene (5′-GAGGACAGATCGTCCATGTTTAA-3′). A p65 scrambled control (p65 non-sense, p65NS) oligonucleotide (5′-GTACTACTTGCAAGAAAGA-3′) was synthesized to serve as a negative control. The NF-κB AS or NS oligonucleotide includes the ATG initiation codon.

2.3. Cell culture

ECV304 cells, an immortalized and transformed human vascular endothelial cell line [34], was maintained in Dulbecco’s modified Eagle medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, CAMBREX Co., Walkersville, MD) at 37°C in a 5% CO2 atmosphere. We have already demonstrated that ECV304 cells are of endothelial origin using immunohistochemistry [35]. The human colon cancer cell line, DLD-1, was routinely cultured in RPMI 1640 (GIBCO) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.4. Reporter and expression constructs

The Accl-Nhel fragment comprising –2018 to +50 bp of the VEGF promoter (pLuc 2068) was cloned downstream of the promoterless luciferase reporter vector in pHH 1409 (VEGF-Luc, a gift from Dr. Finkenzeller, G., Molecular Medicine Institute, Germany). Human wild-type p53 and mutant p53 (His175) were cloned in pcDNA3 (a gift from Dr. Lee, H.B. (Chonnam National University, Korea)).

p53RE luciferase reporter plasmid (p53RE-Luc) was a gift from Dr. Lee, J.W. (Baylor College of Medicine, Houston, TX), and the p65 antisense (p65 AS) of the 5′-GCAACGCGAGTCTGTGTTTTT-3′ was a gift from cDNA dilutions. The mean cycle threshold (Ct) values from quadruplicate measurements were used to calculate gene expression, with normalization to β-actin as an internal control. Calculations of the relative level of gene expression were conducted with the complementary computer software (Corbett Research) using a standard curve. cDNA, amplified by PCR (Perkin-Elmer System 2400, Norwalk, CT), was visualized after staining with ethidium bromide.

2.7. Quantitation of cytokines by ELISA

The quantitative determination of cytokines in culture supernatants was performed by ELISA according to the manufacturer’s instructions. Culture supernatants were collected from cells 8 h after treatment with PAF.

2.8. Transient expression assay

Plasmid DNA was prepared using commercial kits (QIAGEN, Chatsworth, CA). ECV304 cells were cultured to 60–80% confluency in 24-well plates. For each well, each plasmid DNA and 1 μl of Lipo-lectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD) were separately diluted in 100 μl of Opti-MEM reduced serum medium (Life Technologies, Inc.), mixed together, and incubated at room temperature for 1 h. Plates were then washed with serum-free medium, 200 μl of Opti-MEM reduced serum medium was added, and the diluted solution was added to the cells. Plates were incubated at 37°C for 8 h, after which time growth medium containing 10% serum was added. After additional 16 h incubation, cells were then left untreated or were treated with 2 μM PAF for 8 h. Cell extracts were prepared using passive lysis buffer. Luciferase assays were performed with the Lumat LB9501 luminometer (Berthold, Germany). 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.

2.9. Statistical analysis

Data are represented as the mean ± S.E. Statistical significance was assessed by the Student’s t-test when two data sets were analyzed, or by ANOVA followed by the appropriate post hoc test for multiple data sets using the StatView statistical software package (version 4.5). All experiments were conducted at least twice. Reproducible results were obtained and representative data were, therefore, shown in the figures.

3. Results

3.1. PAF enhances VEGF expression through the activation of NF-κB

We first examined the role of PAF in VEGF promoter activation, mRNA expression, and protein synthesis in vitro. Treatment of ECV304 cells with PAF resulted in VEGF promoter activation in a dose-dependent manner, which was blocked by the PAF antagonist, WEB2170, the NF-κB inhibitor, parthenolide, or cotransfection with 1×Bz (Fig. 1A). Furthermore, PAF enhanced VEGF mRNA expression (Fig. 1B) and protein synthesis (Fig. 1C), which were blocked by WEB2170, parthenolide, or cotransfection of the cells with 1×Bz, or p65 AS, but not by a p65 NS (Fig. 1B and C). We confirmed the knockdown of NF-κB p65 protein by Western blotting using an antibody against the p65 subunit of NF-κB (data not shown). Cotransfection of the cells with plasmids expressing p65 and p50 subunits of NF-κB increased the luciferase activity (Fig. 2A), as well as VEGF mRNA expression (Fig. 2B), both of which were blocked by parthenolide or cotransfection of the cells with 1×Bz. To complete the data of Fig. 2, the same experiments were performed in DLD-1 cells that lack p53 activity due to a point missense mutation (C→T, Ser-241→Phe) [36]. We obtained similar patterns of luciferase activity (Fig. 2C) and VEGF mRNA expression (Fig. 2D) as seen in Fig. 2A and B. We confirmed that the transfectected p65, p50, 1×Bz plasmids are expressing equivalent amounts of protein by Western blotting (data not shown). These data indicate that NF-κB activity is involved in PAF-induced VEGF expression.
Fig. 1. PAF enhances VEGF expression through the activation of NF-κB. (A) ECV304 cells (1 × 10^5/ml) were transfected with 0.5 μg VEGF-Luc and/or 0.2 μg 1kBz expression vector. The total concentration of DNA was adjusted to 1 μg per transfection with an empty vector. The PAF antagonist, WEB2170 (WEB, 10 μg/ml), or the NF-κB inhibitor, parthenolide (Parthe, 5 μM), was added 30 min prior to PAF treatment. VEGF promoter activity was assayed 8 h after PAF treatment, as described in Section 2. Luciferase activity was normalized by β-galactosidase activity to determine transfection efficiency. All promoter activities are shown as fold activation relative to control. (B and C) Cells (4 × 10^5/ml) were incubated with WEB2170 or parthenolide, 30 min prior to 2 μM PAF treatment, or transfected with 1kBz expression vector, 12 h prior to PAF treatment. ECV304 cells (5 × 10^5/ml) were incubated with p65 AS or p65 NS (1 μM) 5 days prior to PAF treatment. (B) RNA was isolated 4 h after PAF treatment. Real-time RT-PCR was performed, as described in Section 2. The results of agarose gel electrophoresis of RT-PCR are shown (inset), as are the combined quantitative analysis results by real-time RT-PCR. (C) VEGF protein was analyzed by ELISA in the culture supernatants collected 8 h after PAF treatment. *, P < 0.0001 compared with the control group; #, 0.0001 < P < 0.001 compared with the 2 μM PAF-treated group. Values are expressed as mean ± S.E.

Fig. 2. NF-κB increases VEGF promoter activity and mRNA expression. 5 × 10^4 ECV304 cells (A) and DLD-1 cells (C) were cotransfected with VEGF-Luc (0.5 μg) and NF-κB subunits (0.2 μg p65 and/or 0.1 μg p50) and/or increasing amounts (0.1 or 0.2 μg) of 1kBz expression vector. The NF-κB inhibitor, parthenolide (Parthe, 5 μM), was added 8 h prior to luciferase assay. VEGF promoter activity was assayed, as described in the legend for Fig. 1. 8 × 10^5 ECV304 cells (B) and DLD-1 cells (D) were transfected with NF-κB subunits and/or 1kBz expression vector. Real-time RT-PCR was performed, as described in the legend for Fig. 1. *, P < 0.0001 compared with the control group; §, P < 0.0001 compared with the NF-κB subunits (p65 and p50)-transfected group. Values are expressed as mean ± S.E.
3.2. NF-κB and p53 reciprocally inhibit transcription in response to PAF

The interaction between NF-κB and p53 pathways was analyzed using ECV304 cells cotransfected with NF-κB subunits (p50 and p65), p53 (wild or mutated type) and reporter plasmids containing either TNF-α or p53RE promoter. p65/p50 expression increased TNF-α luciferase activity fivefold, which could be completely inhibited by wild-type, but not mutant, p53 (Fig. 3A). Similar results were obtained in DLD-1 cells (data not shown). Likewise, the p53-mediated increase of p53RE luciferase activity was completely inhibited by co-expression of the NF-κB subunits (Fig. 3B). However, cotransfection with IκBα reversed the luciferase activity of the NF-κB subunits in a dose-dependent manner (Fig. 3B). We next compared the capacity of PAF to induce NF-κB activation in cells with different levels of p53 activity. Treatment of ECV304 cells with PAF activated NF-κB, but not in p53-transfected ECV304 cells (Fig. 3C). Likewise, PAF induced significant NF-κB activation in DLD-1 cells. However, PAF failed to induce NF-κB activation in p53-transfected DLD-1 cells (Fig. 3D) indicating that a cross-regulation occurs between NF-κB and p53 in ECV304 cells.

3.3. Decreased p53 activity is associated with a PAF-induced increase of VEGF expression

We next examined whether PAF-induced inhibition of p53 activity is associated with an increase in VEGF production. ECV304 cells were cotransfected with a wild-type p53 expression vector and a p53RE promoter reporter. PAF treatment significantly inhibited p53 promoter activity (Fig. 4A, inset), and the wild-type p53-induced increases in luciferase activity (Fig. 4A). PAF significantly increased the luciferase activity when ECV304 cells were transfected with the VEGF luciferase reporter. Wild-type p53 completely blocked the PAF effect, whereas the mutant form did not (Fig. 4B). Parallel to VEGF luciferase activity, VEGF mRNA expression was similarly regulated by PAF and wild-type p53 (Fig. 4C). PAF increased VEGF mRNA expression in DLD-1 cells, whereas PAF did not show such an effect in p53-transfected DLD-1 cells (Fig. 4D). These data suggest that the level of p53 activity

Fig. 3. Cross-talk exists between NF-κB and p53 in response to PAF. (A) ECV304 cells (1 × 10^5/ml) were cotransfected with plasmid expressing p65/p50 (0.2 μg/0.1 μg), 0.2 μg wild-type (wt) p53, or mutant (mt) p53 expression vector and 0.5 μg TNF-α-Luc. (B) Cells were cotransfected with 0.1 μg of p53RE-Luc, wild-type p53, NF-κB subunit (p65 and p50) expression vectors, and/or increasing amounts (0.1 or 0.2 μg) of IκBα expression vector. The NF-κB inhibitor, parthenolide (Parthe, 5 μM), was added 8 h prior to luciferase assay. Luciferase assay was performed, as described in the legend for Fig. 1. *, P < 0.0001 compared with the control group; §, P < 0.0001 compared with the NF-κB subunits (p65 and p50)-transfected group; †, P < 0.0001 compared with the wild-type p53-transfected group; ‡, P < 0.0001 compared with the NF-κB subunits and p53-cotransfected group. Values are expressed as mean ± S.E. ECV304 cells (C) and DLD-1 cells (D) were transfected with 0.2 μg wild-type p53 or mutant p53 and treated with 2 μM PAF for 1 h. The nuclear extracts were incubated with α-32P-labeled κB, and electrophoresed on a 5% polyacrylamide gel.
and the magnitude of PAF-induced VEGF mRNA expression are inversely correlated.

4. Discussion

Despite the fact that the VEGF promoter does not contain an NF-κB binding site [7], we and others have reported that VEGF production and gene expression are inhibited by NF-κB inhibition [21–23], suggesting that NF-κB enhances VEGF expression indirectly. In this study, we investigated the mechanisms underlying NF-κB-dependency in PAF-induced VEGF expression and demonstrated that NF-κB activation by PAF results in p53 inactivation, which in turn leads to VEGF expression.

Our results show that a mutually exclusive activation of NF-κB and p53 exists in response to PAF. Such a conclusion is supported by the findings that (i) p65/p50 expression increased TNF-α promoter activity, which was completely inhibited by wild-type p53, and (ii) the p53-mediated increase in p53RE promoter activity was completely inhibited by the expression of the NF-κB p50 or p65 subunits. These data are consistent with the findings of several studies showing mutual transcriptional interference between NF-κB and p53 [6,29–33]. Furthermore, we demonstrated that the interaction between these two factors also occurred in PAF-treated ECV304 cells, suggesting that downregulation of p53 activity through an interaction with NF-κB is a key process in PAF-induced VEGF synthesis.

The mechanism underlying mutual regulation is controversial; both NF-κB and p53 interact with the transcriptional coactivator protein p300 and CREB-binding protein, so both factors may be competing for a limited pool of transactivators [31]. However, inactivation of p53 by the viral transcriptional activator protein Tax is dependent upon NF-κB activity, but not p300 binding or CREB transactivation [33], and the direct interaction between these two transcriptional factors is mediated by their dimerization/tetramerization [37]. Whatever the mechanism for NF-κB and p53 interaction, we here demonstrate for the first time that the mutual interaction between these two proteins appears to play a key role in PAF-induced angiogenesis.

We also demonstrated that PAF-induced inhibition of p53 activity results in an increase of VEGF production. PAF treatment of ECV304 cells cotransfected with a wild-type p53 expression vector and a VEGF promoter reporter increased VEGF luciferase activity and VEGF mRNA expression. PAF also increased VEGF mRNA expression in DLD-1 cells with no p53 activity. How can p53 regulate VEGF expression?
The VEGF promoter contains AP-1, AP-2, SP-1, and HIF-1 binding sites [7,8], and p53 forms a complex with SP-1 to prevent binding to the VEGF promoter [38]. In addition, overexpression of wild-type of p53 reduces VEGF production in HeLa cells through competition between p53 and the AP-1 factor, c-Jun [39]. This competitive inhibition may explain how p53 can inhibit VEGF production despite no binding site in the VEGF promoter. Further studies are required to address whether the same competitive inhibition regulates VEGF expression by PAF.

In summary, we demonstrated that NF-κB and p53 cross-regulation is critical for PAF-induced VEGF expression. PAF may induce NF-κB activation, which in turn downregulates p53 activity, resulting in enhancement of VEGF expression. Given that PAF is the proximal mediator in a variety of inflammatory reactions and a potent angiogenic factor, our data provides evidence to better understand the role of PAF in physiological as well as pathological angiogenesis.

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