Nuclear Factor κB Dependency of Platelet-activating Factor-induced Angiogenesis1

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ABSTRACT

This study investigated the mechanisms of platelet-activating factor (PAF)-induced angiogenesis in a mouse model of Matrigel implantation. PAF induced a dose- and time-dependent angiogenic response. Inhibitors of nuclear factor (NF) κB expression or action, including antisense oligonucleotides to the p65 subunit of NFκB (p65 antisense) and antioxidants such as α-tocopherol and N-acetyl-L-cysteine, significantly reduced PAF-induced angiogenesis. In human umbilical vein endothelial cells, PAF-induced mRNA expression and protein synthesis of various NFκB-dependent angiogenic factors, such as tumor necrosis factor-α (TNF-α), interleukin-1α, basic fibroblast growth factor, and vascular endothelial growth factor (VEGF). The PAF-induced expression of the above-mentioned factors was inhibited by p65 antisense or antioxidants. A significant inhibition of the angiogenic effect of PAF was achieved by anti-VEGF antibodies or soluble VEGF receptors such as KDR and flt-1 but not by antibodies against tumor necrosis factor-α, interleukin-1α, or basic fibroblast growth factor. These data indicate that PAF enhances angiogenesis through inducing NFκB activation, which in turn promotes the production of angiogenic factors such as VEGF.

INTRODUCTION

Neovascularization or angiogenesis is required to sustain primary tumor enlargement as well as metastasis. The induction of tumor angiogenesis is mediated by the increased production of various angiogenic molecules released by both tumor and host cells (1, 2). PAF,3 which is produced by a variety of cells involved in inflammatory reactions, is a potent lipid first messenger active in general cell activation, fertilization, intracellular signaling, apoptosis, and diverse inflammatory reactions (3–7). Recent studies have demonstrated that PAF has the capacity to enhance tumor metastasis (8), to induce in vitro migration of human endothelial cells, and promote in vivo angiogenesis (9). PAF-induced angiogenesis may occur via its ability to induce the expression of angiogenic factors such as TNF-α (10) and hepatocyte growth factor (11). Furthermore, a role for PAF has been suggested in neangiogenesis observed in tumors (12–14) and chronic inflammatory disease such as rheumatoid arthritis (15). However, the mechanism of PAF-induced angiogenesis remains largely unknown.

The transcription factor NFκB is normally present in the cytosol in an inactive complex with a class of inhibitory proteins known as IκBs. Phosphorylation of IκBs triggers their degradation and dissociation from NFκB. NFκB subsequently translocates to the nucleus where it transactivates various genes for proinflammatory cytokines and immunoregulatory genes (16, 17). Recent studies have demonstrated that PAF is an inducer of NFκB (18, 19), and we have identified PAF as a proximal mediator in the inflammatory cascade via its ability to activate NFκB (20, 21). Furthermore, several investigators have reported a role for NFκB in angiogenesis (22–24). Therefore, these findings suggest a linkage between PAF-induced NFκB activation and angiogenesis.

In this study, we investigated the role for PAF-mediated NFκB activity during the process of angiogenesis. We found that PAF induced angiogenesis through NFκB activation, which in turn promoted the expression of key effector angiogenic factors, including VEGF.

MATERIALS AND METHODS

Animals. Specific pathogen-free female BALB/c mice were obtained from the Korean Institute of Chemistry Technology (Daejeon, Korea) and were kept in our animal facility for at least 2 weeks before use. All of the mice were used at 8–10 weeks of age.

Reagents. Water soluble PAF (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), NAC and Vit. E were purchased from Sigma Chemical Co. (St. Louis, MO). PAF antagonist CV 6209 was purchased from WAKO Chemical (Kyoto, Japan). Matrigel, an extract of murine basement membrane proteins, consisting predominantly of laminin, collagen IV, heparin sulfate proteoglycans, and nidogen/entactin, was purchased from Collaborative Research Inc. (Bedford, MA). Human bFGF and mouse recombinant cytokines, such as VEGF, TNF-α, IL-1α were purchased from R & D Systems (Minneapolis, MN). PECA-1 (CD31) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antirabbit IgG-FITC secondary antibody was purchased from Vector Laboratories (Burlingame, CA). Neutralizing antibodies against VEGF, bFGF, and goat IgG were purchased from R & D Systems, and neutralizing antibodies against TNF-α and IL-1α were from Endogen (Minneapolis, MN). Rabbit IgG was purchased from Sigma Chemical Co. Recombinant human flt-1/Fc chimera and KDR/Fc chimera were purchased from R & D Systems. ELISA kits for detecting VEGF and bFGF were purchased from R & D Systems. ELISA kits for detecting TNF-α and IL-1α were purchased from Endogen.

Antisense Oligonucleotides. The following phosphorothioate oligonucleotides were synthesized for use in antisense inhibition of gene expression (Peptron, Korea): p65 antisense of the 5′ end of the NFκB gene (5′-GAAA-CAGATGCCTCGATGTTT-3′) and p65 nonsense (scrambled control) oligonucleotide (5′-GTACTACTCTGACAGCAAGA-3′). The NFκB antisense oligonucleotide includes the ATG initiation codon.

Cell Culture. HUVECs were isolated from human umbilical cord veins and were cultured as described previously (25).

Electrophoretic Mobility Shift Assay. The nuclear extracts were prepared from the cells as described previously (20, 21). To inhibit endogenous protease activity, 1 mM phenylmethylsulfonyl fluoride was added. As a probe for the gel retardation assay, an oligonucleotide containing the immunoglobulin-chain binding site (5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) and containing AP-1 binding site (AP-1, 5′-AAGGCGCTTGTAGACTCAGCCGGAAAG3′) were synthesized. The two complementary strands were annealed and labeled with [α-32P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 µg 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 1 h.
Fig. 1. The angiogenic effect of PAF. A, the Matrigel plugs mixed with indicated concentrations of PAF were injected s.c. On day 6, the Matrigel plugs were excised and used for quantification of angiogenesis by measuring the hemoglobin content in the Matrigel matrix. Hemoglobin was measured by using the Drabkin reagent kit 525 as described in "Materials and Methods." Matrigel containing 100 units/ml heparin and the vehicle alone was used as a control. B, Matrigel was mixed with PAF (1 µg) and injected s.c. The angiogenesis assay was performed at the indicated days. C, the Matrigel plugs mixed with PAF with or without various concentrations of CV 6209 were injected s.c., and angiogenesis assay was performed on day 6. The results were expressed as mg of hemoglobin of Matrigel pellet. * P < 0.0001 compared with control group; **, P < 0.001, and ***, P < 0.0001 compared with PAF-treated group. Values are expressed as means; bars, ±SE.

Fig. 2. Involvement of NFκB activation in PAF-induced angiogenesis. A and B, HUVEC were plated at 1 × 10^5 cells/dish and pretreated with p65 antisense or scrambled control oligonucleotide at indicated concentrations 5 days before PAF (0.5 µg/ml) treatment. C, HUVEC (1 × 10^5 cells/dish) were pretreated with Vit. E, or NAC 30 min before PAF treatment. Nuclear extracts were prepared 1 h after PAF treatment and were incubated with α-32P-labeled xB, AP-1, or CRE oligonucleotide and electrophoresed on a 4% polyacrylamide gel. D, Matrigel containing 64 units/ml heparin was mixed with PAF (1 µg) in the presence or absence of indicated concentrations of p65 antisense or scrambled control oligonucleotide. E, Matrigel containing 64 units/ml heparin was mixed with PAF (1 µg) with or without the addition of Vit. E (5 µg) or NAC (10 µg). The Matrigel plugs were excised and processed on day 6 for quantification of angiogenesis by measuring the hemoglobin content. The results were expressed as mg of hemoglobin/g of Matrigel pellet. * P < 0.001 compared with control group; **, P < 0.001 compared with PAF-treated group. Values are expressed as means; bars, ±SE.
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Histology. The Matrigels were removed and immersed in 4% paraformaldehyde overnight at 4°C. Histological sections were cut at 6 μm and mounted on gelatin-coated glass slides and stained with H&E. Vessel area and the total Matrigel area were then measured.

Immunohistochemistry. Serial sections of paraffin-embedded Matrigel plugs were stained using an immunohistochemistry procedure. Sections were treated with a primary antibody for CD31 (Santa Cruz Biotechnology) diluted 1:200 in PBS and incubated with H&E. Vessel area and the total Matrigel area were then measured.

Quantitation of Cytokines by ELISA. The quantitative determination of cytokines in culture supernatants and cell lysates from HUVEC was performed by ELISA according to the manufacturer’s instructions. Briefly, after pretreatment with antisense oligonucleotides, medium containing 0.5 μg/ml of PAF was added, and culture supernatants and cell lysates were prepared after 3 h. Cell lysates were prepared using 200 μl of radioimmuno-precipitation assay buffer (0.1% SDS, 1% igepal, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulphonyl fluoride).

Statistical Analysis. The data are represented as the mean ± SE. Statistical significance was determined by the Student t test when two data sets were analyzed or, alternatively, by ANOVA followed by the appropriate post-hoc test for multiple data sets with the statistical software StatView (version 4.5). All of the experiments were conducted two or more times. Reproducible results were obtained, and representative data are therefore shown in the figures.

RESULTS

PAF-induced NFκB-dependent Angiogenesis. In the first set of experiments, we determined the in vivo effect of PAF on angiogenesis in a murine model in which Matrigel was injected s.c. Injection of Matrigel containing PAF caused neovascularization in dose- and time-dependent manners (Fig. 1, A and B). Angiogenesis progressively increased from day 2 and reached its maximal at day 6, and the minimal effective dose of PAF was 1 μg/0.2 ml Matrigel. PAF-induced angiogenesis was blocked by the PAF antagonist CV 6209 (Fig. 1C).

PAF is a potent inducer of NFκB in vivo and in vitro (18–21), and NFκB activity has been associated with the process of angiogenesis (22–24). Therefore, it was our hypothesis that PAF may induce angiogenesis via the activation of NFκB. To assess this possibility, the effects of an antisense oligonucleotide to the p65 subunit of NFκB (p65 antisense) and antioxidant treatment (Vit. E and NAC) on PAF-induced angiogenesis were examined. NFκB antisense oligonucleotides have been used as a tool to block the specific activity of NFκB (30, 31). It is well known that ROI are strong inducers of NFκB (32, 33), and we have demonstrated recently that PAF-induced NFκB activation is an exclusively ROI-dependent process (26). We first examined the efficacy of p65 antisense and antioxidants as NFκB inhibitors. As shown in Fig. 2A, p65 antisense inhibited the PAF-induced NFκB activation in a dose-dependent manner in HUVEC cultures, whereas p65 nonsense (scrambled control) oligonucleotide had no significant effect. Moreover, p65 antisense treatment did not
alter PAF-induced AP-1 activation (Fig. 2B), thereby confirming the specificity of the antisense treatment protocol. Antioxidants also significantly inhibited PAF-induced NFκB activation in a dose-dependent manner (Fig. 2C). Both p65 antisense, but not scramble, and antioxidants significantly inhibited PAF-induced angiogenesis in dose-dependent manners (Fig. 2, D and E, respectively), suggesting a critical role for NFκB activity in PAF-induced angiogenesis.

The angiogenic activity of PAF was also evaluated by H&E and immunofluorescence staining for CD31. A significant number of erythrocyte-containing canalized vessels and CD31-positive endothelial cells were observed within the Matrigel in PAF-treated mice but not in Matrigel only control group. Furthermore, both p65 antisense, but not scramble, and Vit. E significantly inhibited the number of PAF-induced increased CD31-positive endothelial cells and canalized vessels (Fig. 3, A and B, respectively). These data indicate that the extent of vessel number and intensity of FITC paralleled the angiogenic pattern measured by hemoglobin contents.

**Effects of NFκB-dependent Angiogenic Factors on PAF-induced Angiogenesis.** The critical role for NFκB activity in PAF-induced angiogenesis derived from the Matrigel implies that specific angiogenic molecules under the transcriptional regulation by NFκB may be involved in the process. Thus, we examined whether various known NFκB-dependent angiogenic factors such as TNF-α (34), IL-1α (35), bFGF (36), and VEGF (37–40) were regulated by PAF.

We first questioned whether PAF is able to induce mRNA expression and protein synthesis of the angiogenic factors, and, if so, p65 antisense treatment can block the PAF-dependent regulation of angiogenic factor expression. Treatment of HUVEC cultures with PAF resulted in the mRNA expression of all of the cytokines examined (Fig. 4A). Both p65 antisense oligonucleotide, but not scrambled control oligonucleotide, and antioxidants blocked the PAF-induced mRNA expression of the cytokines (Fig. 4, B and C, respectively).

Likewise, treatment of HUVEC with PAF induced the synthesis of the cytokine proteins (Fig. 5). The TNF-α, IL-1α, and bFGF proteins...
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were detected in the culture supernatants, but VEGF protein was detected in only cell lysate. Both p65 antisense and antioxidants inhibited cytokine synthesis (Fig. 5, A and B, respectively), indicating that PAF-induced mRNA expression and protein synthesis of these cytokines occurs via a NFκB-dependent process.

The extent of the involvement of these cytokines in PAF-induced angiogenesis was subsequently analyzed through the use of neutralizing antibodies. We performed preliminary experiments and used a concentration of antibody that resulted in the neutralization of 50% maximal angiogenic effect of each cytokine in Matrigel (maximum angiogenic effect of TNF-α was 50 ng; IL-1α, 50 ng; bFGF, 1 ng; and VEGF, 10 ng). Fig. 6 showed that using antibodies against TNF-α, IL-1α, or bFGF showed very little inhibitory effect on PAF-induced angiogenesis. In contrast, anti-VEGF antibody inhibited the greatest angiogenic effect. To additionally clarify the effect of VEGF, the angiogenic effect of soluble VEGF receptors, sKDR and sflt-1 on PAF-induced angiogenesis was subsequently analyzed through the use of neutralizing antibodies. We performed preliminary experiments and used a concentration of antibody that resulted in the neutralization of 50% maximal angiogenic effect of each cytokine in Matrigel (maximum angiogenic effect of TNF-α was 50 ng; IL-1α, 50 ng; bFGF, 1 ng; and VEGF, 10 ng). Fig. 6 showed that using antibodies against TNF-α, IL-1α, or bFGF showed very little inhibitory effect on PAF-induced angiogenesis. In contrast, anti-VEGF antibody inhibited the greatest angiogenic effect. To additionally clarify the effect of VEGF, the angiogenic effect of soluble VEGF receptors, sKDR and sflt-1 on PAF-induced angiogenesis was examined. sKDR or sflt-1 chimera almost completely inhibited the angiogenic effect of PAF at a concentration as low as 1 ng/0.2 ml Matrigel, indicating that VEGF is the most potent effector molecule in PAF-induced angiogenesis.

DISCUSSION

PAF has been shown recently to induce in vitro migration of human endothelial cells and promote in vivo angiogenesis (12). In these studies, PAF was implicated as a mediator of angiogenesis by its capacity to induce expression of angiogenic factors such as TNF-α (10) and hepatocyte growth factor (11). Furthermore, a role for PAF has been suggested in neoangiogenesis observed in tumors (12–14) and chronic inflammatory disease such as rheumatoid arthritis (15). Consistent with these findings, we observed an angiogenic effect of PAF in this study in which Matrigel was used as a vehicle. Importantly, we demonstrated that the activation of NFκB appears to be essential to the angiogenic activity of PAF. This was substantiated by the findings that NFκB inhibitors such as antisense oligonucleotide to the p65 subunit of NFκB and antioxidants (Vit. E and NAC) significantly inhibited the angiogenic effect of PAF (Fig. 2). In recent studies, NFκB antisense oligonucleotides have been used as a tool to block the specific activity of NFκB (30, 31). Additionally, we demonstrated recently that PAF induced NFκB activation via generation of ROI (26). These two tools (p65 antisense and antioxidants) were used in the current study to inhibit NFκB activation. Our data are the first to show that PAF induces angiogenesis through activating NFκB in a ROI-dependent manner. Limited data exist concerning the possible contribution of NFκB to angiogenesis. An arachidonic acid metabolite, 12(R)-hydroxy-5,5,14-eicosatrienoic acid (22), oxidative stress (23), and hypoxia (24) have been reported to be associated with angiogenesis through the activation of NFκB. It is not clear whether there is a basic mechanism common to these mechanisms. However, given that ROI are potent inducers of NFκB (32, 33) and arachidonic acid metabolites, oxidative stress, and hypoxia cited above have ability to generate ROI (22, 40, 41), any conditions and molecules including PAF that are capable of generating ROI appear to induce angiogenesis through the activation of NFκB.

The PAF-induced NFκB-dependent angiogenic factors used in this study included TNF-α, IL-α, bFGF, and VEGF. In response to PAF, VEGF protein was detected only in cell lysate. This implies that VEGF induced by PAF is the cell-associated VEGF 189 isoform (42). Among the angiogenic factors, VEGF was found to be the most potent. Anti-VEGF antibodies or soluble VEGF receptors inhibited nearly all of the PAF-induced angiogenesis. VEGF is a potent peptide growth factor, specific for vascular endothelial cells, which promotes neovascularization and increases vascular permeability in vivo (43, 44). The critical role of VEGF in in vivo tumor angiogenesis was evidenced by experiments showing inhibition of tumor growth after treatment with anti-VEGF neutralizing antibodies (45) or by blocking signals provided by the VEGF receptors (46). Several mediators including hypoxia (47), ROI (48), and proinflammatory cytokines such as TNF-α, and IL-1α (49) have been shown to induce VEGF gene expression. Although the role of PAF in the regulation of VEGF expression has not been documented, the fact that PAF is released from the hypoxic cells (50), is an inducer of ROI generation (32, 51), and is an inducer of proinflammatory cytokine expression (18, 20, 21, 52) supports the idea that PAF may be the initial inducer of VEGF.

It was well-established that the transcription factor NFκB is essential for TNF-α, IL-1α, and bFGF expression, but it is not known whether NFκB regulates VEGF expression. Although VEGF promoter does not contain the NFκB binding site (53), many investigators have reported that VEGF production and gene expression are inhibited by NFκB inhibition (34, 37–39). For example, VEGF promoter activity is significantly decreased in cancer cells transfected with mutated IκBα, which blocks NFκB activation (39). In this study, we also observed that blocking of NFκB activity resulted in the inhibition of VEGF production and gene expression. More importantly, we have observed a significantly increased (4-fold) luciferase activity in the human endothelial cell line ECV304 when the cells were transfected with VEGF luciferase promoter-reporter and plasmids expressing p65 or p50 subunit of NFκB (data not shown). These observations suggest that VEGF promoter may contain NFκB-like binding site. Studies are required to define the NFκB binding site(s) in regulatory regions of VEGF gene.

Our present data demonstrated that: (a) PAF induced mRNA expression and protein synthesis of various angiogenic factors such as TNF-α, IL-1α, bFGF, and VEGF, which is inhibited by both p65 antisense and antioxidants; and (b) PAF-induced angiogenesis was significantly inhibited by blocking VEGF, which strongly suggests that PAF exerts its angiogenic effect through expressing NFκB-dependent angiogenic factors. In conclusion, this study demonstrated that PAF enhances angiogenesis via the activation of NFκB, which in turn promotes the expression of angiogenic factor(s) such as VEGF.
Because PAF is a key inducer of NFκB in a wide range of cell types and/or organ systems, it is possible this is a major pathway leading to angiogenesis in inflammatory and tumorogenic processes.

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