Vascular endothelial growth factor (VEGF) is not only essential for vasculogenesis and angiogenesis but is also capable of inducing tissue factor, the prime initiator of coagulation, in endothelial cells. In this study we have analyzed the VEGF-elicited pathways involved in the induction of tissue factor in human umbilical cord vein endothelial cells. Using specific low molecular weight inhibitors we could demonstrate a crucial role of the p38 and Erk-1/2 mitogen-activated protein (MAP) kinases. In contrast, treatment with wortmannin or LY294002, inhibitors of phosphatidylinositol 3 (PI3)-kinase, resulted in a strong enhancement of the VEGF-induced tissue factor production, indicating a negative regulatory role of the PI3-kinase on tissue factor-inducing pathways. Accordingly, transduction with constitutively active Akt led to a reduction of VEGF-induced tissue factor production. Western blot analyses using antibodies specific for phosphorylated p38 showed an enhanced activation of this MAP kinase in human umbilical cord vein endothelial cells when stimulated with VEGF in the presence of wortmannin in comparison to either agent alone. Thus, the negative regulation of the PI3-kinase pathway on endothelial tissue factor activity can be explained at least in part by a suppression of this MAP kinase-signaling pathway. This is the first demonstration of a reciprocal relationship between procoagulant activity and the PI3-kinase-Akt signaling pathway, and it reveals a novel mechanism by which tissue factor expression can be controlled in endothelial cells.
gen monoxide (NO) production and was demonstrated as a potent proangiogenic signaling molecule (19–22). In addition to PI3-kinase activation, VEGF-R2 autophosphorylation has been demonstrated to recruit and activate phospholipase Cγ. Phospholipase Cγ cleaves membrane-bound phosphatidylinositol bisphosphate resulting in soluble inositoltrisphosphate, which causes the release of Ca²⁺ from intracellular storage pools, and diacylglycerol formation, which is the main activator of protein kinases C (PKC). PKC activation is upstream of the Raf/MEK/Erk-signaling cascade and is involved in VEGF effects such as proliferation and vascular permeability (23, 24).

The signaling events leading to VEGF-mediated tissue factor induction are only partially understood. Based on the findings that nuclear factor of activated T-cells and Egr-1 but not NFκB are essential transcription factors for VEGF-induced tissue factor production, evidence for an involvement of Ca²⁺ and MAP kinase-dependent pathways has been provided (15, 16, 25). Using a test system for tissue factor production (2, 16, 26), we show here that inhibition of PI3-kinase activity by wortmannin and LY294002 synergistically enhanced VEGF-induced tissue factor activity. This effect of PI3-kinase inhibitors on VEGF-induced endothelial tissue factor seemed to involve MAP kinases, because the inhibition of PI3-kinase signaling correlated with an enhanced phosphorylation of the p38 MAP kinase and to a certain degree of the Erk-1/2 MAP kinase, two essential mediators of VEGF-induced endothelial tissue factor activity.

MATERIALS AND METHODS

Reagents—Media and other cell culture reagents were obtained from Life Technologies, Inc. Human umbilical cord veins were donated kindly from hospitals in the “Wetterau.” Citrated pooled plasma was obtained from volunteers. Wortmannin, LY294002, PD98059, and SB203580 were obtained from Calbiochem (San Diego, CA). VEGF and placenta growth factor (PIGF) were prepared as described previously (27). Reagents and all general supplies not indicated otherwise were obtained from Sigma.

Tissue Culture—Human umbilical cord vein endothelial cells (HUVECs) were prepared as described previously (28) were cultured in MCD131 supplemented with 10% fetal calf serum, 4 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (for HUVECs) or RPMI 1640 medium (for monocytes) for 4 h followed by the incubation of cultures with purified recombinant VEGF or PIGF in MCD131 medium containing 10% fetal calf serum, 4 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (HUVECs) or RPMI 1640 medium in the presence/absence of chemical enzyme inhibitors. Assays were carried out with whole cells obtained in suspension, and tissue factor activity was determined as described previously (27). Briefly, cells were removed from the dish by scraping with a rubber policeman. 100 μl of these resuspended cells were mixed with 100 μl of citrated plasma, and clotting times were measured after recalcification with 100 μl of a 25 mM CaCl₂ solution. Tissue factor activity determinations were made using an appropriate range of substrate concentrations. Recombinant Adenoviral Constructs and Infection—The viral expression constructs for the human Akt, a constitutively active form of Akt and green fluorescent protein were described previously (29). A control adenovirus was prepared by cotransfection of the vector pACCMVP-L-pASR+ with pM17, a plasmid containing a deletion in the E1 region, into 293 cells. Large batches of recombinant adenoviruses were grown in 293 cells and purified by two consecutive caesium chloride centrifugation steps. Recombinant Akt adenoviruses were tested for protein expression by Western blot analysis. For infection, HUVECs grown in six-well plates were washed twice with PBS and incubated in PBS for 30 min at a multiplicity of infection of 100 with the recombinant adenoviruses. Thereafter, the cells were washed with PBS and cultivated in HUVEC medium.

FACS analysis—For flow cytometric analysis, confluent HUVECs were detached with 5 mm EDTA in Hanks’ balanced salt solution/25 mM HEPES, incubated with primary un conjugated tissue factor monoclonal antibody TF9 6B4 (30) for 30 min at 4 °C and 10 μg/ml as final concentration, washed twice with PBS buffer (PBS supplemented with 1% bovine serum albumin and 0.1% NaN₃), and then incubated with phosphatidylethanolamine-conjugated goat anti-mouse IgG (Dianova) for 30 min on a rotator. Flow cytometric analysis was performed on a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Isotype-matched control antibodies (Co-IgG) were used to determine the background staining.

Northern Blot Analysis—Total RNA was extracted using the guanidinium thiocyanate method, and Northern blot analysis was performed as described previously (31). Briefly, 10 μg of RNA per lane were electrophoresed on 1.5% agarose gel under denaturing conditions and transferred to a positively charged nylon membrane (Amersham Pharmacia Biotech). Ribosomal RNA was stained on the filters with methylene blue to assess RNA loading and transfer. Filters were hybridized with a 32P-labeled tissue factor cDNA probe, isolated by SalI restriction from plasmid AHTP8 (32). Hybridization was performed in QuikHyb solution (Stratagene) and 40 μl of denatured salmon sperm DNA at 68 °C with 1.5 × 10⁶ cpm/ml hybridization solution for 14–16 h. Blots were washed three times with 20 ml of 2× SSC and 0.1% SDS at room temperature and two times for 15 min each with 10 ml of 0.2× SSC and 0.1% SDS at 55 °C. The levels of specific mRNA were measured using a phosphorimager (Molecular Dynamics). Normalization was performed by comparison with ribosomal 28S RNA.

Western Blot Analysis—For Western blot analysis confluent cultures of HUVECs were serum-starved in MCD131 medium containing 0.5% fetal calf serum for 4 h. 60 min before stimulation with VEGF the PI3-kinase inhibitor wortmannin (100 nM) was added to the medium. After incubation with 50 ng/ml VEGF for 8 min, the cells were washed with PBS, solubilized with 100 μl of SDS sample buffer, and submitted to electrophoresis on 12% SDS-polyacrylamide gels. After transfer to a polyvinylidene difluoride membrane, the proteins were incubated with anti-phospho-Erk-1/2, anti-phospho-p38, or the antibodies directed against the unphosphorylated proteins (New England Biolabs) and detected as described by manufacturer protocol.

RESULTS

Protein Kinase C, p38, and Erk-1/2 MAP Kinases but Not NO Formation Are Involved in VEGF-induced Tissue Factor Activity—We have demonstrated previously that treatment of HUVECs with VEGF leads to the induction of procoagulant activity caused by tissue factor production (2). The signaling pathways involved in VEGF induction of procoagulant tissue factor activity were evaluated in the presence of various low molecular weight inhibitors known to interfere with specific signaling events. Treatment with the specific PKC bisindolylmaleimide-based inhibitor GF1109203X, which interacts with the ATP binding site of this family of kinases, reduced VEGF-induced procoagulant activity by −90% (Fig. 1A). However, inhibition of the NO signaling pathway by L-nitro arginine methyl ester at concentrations demonstrated previously to inhibit VEGF-mediated endothelial permeability (33) did not affect VEGF-induced procoagulant activity (Fig. 1B). Furthermore, the addition of either the p38 MAP-kinase inhibitor SB203580 or the specific MEK-1/2 kinase (which is the Erk-1/2 phosphorylating kinase) inhibitor PD98059 reduced the VEGF-induced procoagulant activity by −80–90% (Fig. 1B). Combined inhibition of these two MAP kinases abolished VEGF-induced tissue factor activity completely (Fig. 1B), indicating that at least p38 MAP kinase and the MEK/Erk pathways cooperate.

PI3-kinase/Akt Regulate Endothelial Tissue Factor Activity Induced by VEGF—The PI3-kinase/Akt signaling pathway has been demonstrated to mediate endothelial migration and survival evoked by VEGF (20, 22). Therefore, we tested the ability of PI3-kinase inhibitors wortmannin and LY294002 to modu-
late VEGF-mediated procoagulant tissue factor activity. Unexpectedly, both of these PI3-kinase inhibitors did not reduce but rather enhanced VEGF-dependent procoagulant activity in a synergistic fashion (Fig. 2A). A negative regulatory role of VEGF-induced PI3-kinase activation is suggested by the fact that LY294002 or wortmannin given in the absence of VEGF have little or no effect on tissue factor activity. Next, we examined the role of Akt in PI3-kinase-dependent repression of endothelial procoagulant activity, because Akt kinase is phosphorylated upon stimulation of the VEGFR-2 and functions as a mediator of PI3-kinase signaling (17, 34). HUVECs were transfected using adenoviral gene transfer with a myristoylated Akt, which is constitutively active (29). Western blot analysis of transfected cells with antibodies specific for phosphorylated Akt confirmed the presence of constitutively Akt activation (data not shown). When VEGF was added to the Akt-transduced cells, tissue factor production was inhibited by ~50% in comparison with cells transfected with a control adenovirus without an insert (Fig. 2B). Similar to our observations with the chemical PI3-kinase inhibitors, the myristoylated Akt kinase mutant did not affect basal tissue factor levels in the absence of VEGF (Fig. 2B). Collectively, these data suggest that VEGF-mediated tissue factor activity is induced by the p38 and Erk-1/2 MAP kinases but in parallel is reduced by the activation of the PI3-kinase-Akt signaling pathway.

Synergistic Induction of Tissue Factor Activity by VEGF and Wortmannin Correlates with de Novo Production of Tissue Factor mRNA and Surface Expression of Tissue Factor Protein—To examine whether the observed regulation of tissue factor activity was caused indeed by changes in the expression levels of tissue factor mRNA, Northern blot analysis was performed with a specific 32P-labeled probe for tissue factor. As demonstrated previously, this probe detects two tissue factor transcripts that correspond to two independent splice variants (32, 35). Indeed, VEGF and wortmannin together induced tissue factor mRNA in a more than additive fashion when compared with the addition of each reagent alone (Fig. 3A). The correlation between tissue factor activity and production of tissue factor protein was also confirmed by assessing surface expression using flow-assisted cytfluorimetry (FACS) analysis and a tissue factor-specific monoclonal antibody. This analysis revealed a small induction of tissue factor surface expression by individual treatment with VEGF or wortmannin alone, whereas the combination of these two agents caused a significant increase in surface expression (Fig. 3B). Furthermore, tissue factor pathway inhibitor and thrombomodulin were measured by FACS analysis to exclude the possibility that the observed increased tissue factor activity by the combination of VEGF and wortmannin was also modulated by a decrease in the surface expression of endothelial anticoagulant proteins. Whereas the amount of tissue factor pathway inhibitor on the endothelial cell surface was not influenced by any of the agents, thrombomodulin surface expression was increased rather than decreased by the combination of VEGF and wortmannin (Fig. 3, C and D). In conclusion, the observed increase in procoagulant tissue factor activity by the combination of VEGF and inhibition of the PI3-kinase-Akt-signaling pathway with wortmannin can be explained by the increased production of tissue factor mRNA and surface protein and is not caused by a decrease in surface expression levels of thrombomodulin or tissue factor pathway inhibitor.

The Synergistic Induction of Tissue Factor Activity by VEGF and Wortmannin Is Restricted to VEGFR-2—We have shown previously that VEGF-induced procoagulant tissue factor activity in endothelial cells is mediated mainly by the VEGFR-2 and only to a very small extent by the VEGFR-1 (27). Furthermore, the phosphorylation of the p85/PI3-kinase complex is ~10-fold less with VEGFR-1 in comparison with VEGFR-2-specific VEGF mutants, indicating that the PI3-kinase-Akt-signaling pathway is activated predominately by the VEGFR-2 (34). Therefore, we tested whether VEGFR-2 also mediates the synergistic induction of procoagulant activity by VEGF and inhibition of the PI3-kinase-Akt-signaling pathway. Indeed, the procoagulant effect of the VEGFR-1-specific ligand PlGF was very weak and only additive when used together with wortmannin in HUVECs (Fig. 4A). When PlGF and wortmannin were added to human peripheral blood monocytes, which express only the VEGFR-1 (27), the production of procoagulant activity was not enhanced by wortmannin (Fig. 4B). These findings further support the hypothesis that the PI3-kinase-Akt-signaling pathway functions as a signaling event that is used preferentially by the VEGFR-2 and mediates the synergy between VEGF and wortmannin.

Wortmannin Enhances Phosphorylation of the p38 and Erk-1/2 MAP Kinases—Having shown that activation of the p38 and Erk-1/2 MAP kinases is essential for the VEGF-induced tissue factor production (Fig. 1B), we tested the possibility that a negative regulatory function of the PI3-kinase-Akt-signaling pathway on p38 and Erk-1/2 phosphorylation contributes to the synergistic enhancement of VEGF-induced tissue factor production observed with PI3-kinase inhibitors. Therefore, activation of MAP kinases was assessed by probing with specific
antibodies for the phosphorylated forms of both p38 (Fig. 5A) and Erk-1/2 (Fig. 5B). Both wortmannin and VEGF induced phosphorylation of the p38 MAP kinase, and the combination of both further enhanced the effect of both reagents alone (Fig. 5A). This effect was observed consistently (in all four independent experiments performed). In Fig. 5B the cognate ability of VEGF to induce phosphorylation of the Erk-1/2 MAP kinase is demonstrated. This phosphorylation is mediated by the Erk-1/2 upstream kinase MEK-1/2 (Fig. 5B) as it is inhibited by the MEK-specific inhibitor PD98059. Although wortmannin itself does not induce Erk-1/2 phosphorylation, it can enhance the effect of VEGF (Fig. 5B). This effect is reduced by PD98059 to phosphorylation levels observed with VEGF in combination with PD98059, indicating that the PI3-kinase-mediated inhibitory effect on Erk-1/2 phosphorylation is upstream of MEK-1/2. However, the effect of wortmannin on VEGF-induced phosphorylation of Erk-1/2 was variable. In seven independent experiments wortmannin enhanced the VEGF effect on Erk-1/2 phosphorylation, whereas in three experiments even some slight reduction of the VEGF-induced Erk-1/2 activation was observed.

DISCUSSION

This study demonstrates that VEGF can induce signal transduction pathways that regulate endothelial tissue factor production in a positive as well as a negative fashion. Positive regulation was shown to essentially depend on the two MAP kinases p38 and Erk-1/2 by the use of small chemical inhibitors. Negative regulation of tissue factor production occurred via the activation of the PI3-kinase-Akt-signaling pathway as demonstrated with two selective inhibitors of the PI3-kinase and transfection of a constitutively activated mutant of Akt. Although the use of chemical inhibitors is limited by the specificity of the inhibitors, there are several lines of evidence suggesting that the observed negative regulatory effect is indeed caused by the VEGF-induced PI3-kinase activity. First, the wortmannin concentration used (100 nM) is in the range described to be specific for the PI3-kinase (half-maximal at 5 nM), and a dose-response curve for tissue factor induction displayed a very similar response as measured with the PI3-kinase (data not shown). Furthermore, we could confirm our results with a second specific PI3-kinase inhibitor (LY294002). Finally, the use of a constitutively active Akt mutant strongly reduced VEGF-induced procoagulant activity, linking this kinase, which is directly downstream of PI3-kinase, to the negative regulation of tissue factor induction.

To explain the negative regulation of tissue factor production by the PI3-kinase-Akt pathway several alternative explanations are possible. For example, in lipopolysaccharide-activated macrophages inhibition of the PI3-kinase was demonstrated to lead to prolonged NFκB activation (36). Although VEGF has also been reported to induce NFκB activation in endothelial cells (37), this induction is controversial and condition-dependent. For example, VEGF-induced NFκB activation was shown in bovine retina endothelial cells using gel shift assay (37), but no such effect could be observed in HUVECs by assessment of nuclear translocation (16). Furthermore, VEGF-induced tissue
factor induction was demonstrated to be mediated largely by the transcription factor Egr-1. (12, 13, 16). In fact, we failed to demonstrate any effect of VEGF treatment by the use of an NF\(_N\)/\(\beta\)H9260 reporter construct in HUVECs, and in addition, no regulatory role of PI3-kinase on NF\(_N\)/\(\beta\)H9260 activation was observed (data not shown). These data are in accordance to the recent observation that the PI3-kinase-Akt pathway has no influence on NF\(_N\)/\(\beta\)H9260 activation in endothelial cells (38). Therefore, the observed negative regulatory role of the PI3-kinase-Akt pathway on tissue factor production is very unlikely to occur via NF\(_N\)/\(\beta\) modulation.

Another possible negative regulatory role of the PI3-kinase may be mediated by affecting endothelial NO-synthetase expression, because PI3-kinase inhibitors were described to enhance shear stress-induced transcription of this enzyme (39). NO formation was shown previously to be downstream of phospholipase C\(_\gamma\) and PKC but upstream of the Erk-1/2 MAP kinase (24, 40), which is in line with our findings that PKC and MAP kinases are major signaling pathways for VEGF-induced tissue factor production (Fig. 1). However, our findings also show that VEGF-induced tissue factor production is independent on the generation of NO (Fig. 1B). In conclusion, our observations are in favor of the hypothesis that activation of Raf by PKC occurs in HUVECs by a pathway that does not involve NO formation.

Alternatively, the strong enhancement of tissue factor production by chemical inhibition of the PI3-kinase-Akt-signaling pathway can be explained by an inhibitory role of this pathway on p38 and/or Erk-1/2 activation, two kinases shown in this study to be essential for VEGF-induced tissue factor production (Fig. 1B). According to this hypothesis VEGF elicits an anticoagulant pathway by PI3-kinase-mediated suppression of the p38 and/or Erk-1/2 MAP kinases and in parallel procoagulant pathways by p38 and Erk-1/2 MAP kinase activation. In fact, in this study we could demonstrate that the PI3-kinase-signaling pathway inhibits p38 MAP kinase phosphorylation. This is in confirmation of a very recent report showing that the blockade of PI3-kinase-Akt also led to enhanced VEGF activation of p38 and apoptosis (41). In addition, negative regulatory roles of the PI3-kinase-Akt-signaling pathway on the activation of Erk-1/2 were demonstrated in various different cellular systems including differentiated myotubes, a human breast cancer cell line, and also in HUVECs (38, 42, 43). In contrast, a positive regulatory role of the PI3-kinase-mediated Erk-1/2 activation has...
been proposed in HUVECs because inhibition of the PI3-kinase led to a decrease in VEGF-induced Erk-1/2 phosphorylation (44). This controversy observed for the same type of primary cells (HUVECs) is also reflected by our data. Seven experiments supported the hypothesis that PI3-kinase activation in HUVECs is inhibitory, whereas three experiments suggested a rather stimulatory role for the PI3-kinase on Erk-1/2 activation in response to VEGF. Obviously the PI3-kinase effect on this pathway is condition-dependent, which can be explained by genetic and individual (historical) differences of the donor cords from which the umbilical cord vein endothelial cells were isolated. In conclusion, the inhibitory effect of the PI3-kinase pathways on VEGF-induced tissue factor production can be explained at least in part by an inhibitory effect on the p38 MAP kinase.

There are numerous reports suggesting that VEGFR-2 is the major if not exclusive signaling VEGF receptor for endothelial cell functions including angiogenesis. This hypothesis has been derived also from studies using receptor-specific homologues such as the PI3K that binds to VEGFR-1 but not to VEGFR-2 (45), VEGF-E, which binds selectively to VEGFR-2 but not to VEGFR-1 (46), or receptor-specific mutants (34). In terms of tissue factor induction in endothelial cells, VEGF-E was found to have a similar bioactivity as VEGF (also designated as VEGF-A itself) (46). In contrast, tissue factor was only slightly induced by PI3K (maximally at 2–3-fold) in comparison with similar concentrations of VEGF (maximally 25–80-fold) (27). On top of these findings is our present observation that VEGF-2 signaling is essential for the observed synergy between VEGF and PI3-kinase inhibitors in tissue factor induction. This is in line with the observation that VEGFR-2-mediated signaling is the major inducer of the PI3-kinase-Akt pathway (34), a finding that can be also explained by the presence of a repressor sequence in the juxtamembrane domain of the VEGFR-1, which inhibits VEGF-dependent PI3-kinase activation (47). Furthermore, in the same study only a marginal activation of P38 and Erk-1/2 was observed with VEGFR-1-specific VEGF mutants. In conclusion, a VEGFR-2-dependent PI3-kinase and Akt-activation pathway, which can suppress MAP kinase activities and subsequently also tissue factor production, fits best with the data provided in this study.

In conclusion, our finding that a PI3-kinase-dependent pathway is a negative regulator for the MAP kinase-mediated tissue factor production defines a novel regulatory principle in endothelial cells. We propose that in vascular endothelial cells under physiological conditions, continuous PI3-kinase activation by plasma components such as insulin and insulin-like growth factors occurs, leading to the suppression of tissue factor production. In tumors, however, blood flow and perfusion are impaired, and the supply with growth factors is suboptimal (48, 49). Consequently, levels of PI3-kinase activation may be reduced in tumors. In addition, tumor-produced cytokines such as VEGF can elicit specific signaling events that are not induced by serum components, leading to tissue factor production. Thus, the striking observation that tumor endothelium is one rare exception of tissue factor expression on the endothelium may be explained by diminished PI3-kinase activity together with increased P38 and Erk-1/2 MAP kinase activity in comparison to the physiological conditions.

Our hypothesis that PI3-kinase activity is a suppressor for endothelial activation and tissue factor production is supported by preliminary experiments, in which wortmannin was applied to animals bearing tumors producing high amounts of VEGF. In these animals hemorrhagic necroses occurred that were restricted to the tumors2 and similar to that observed with systemic TNF application or targeting of tissue factor to the tumor endothelium (50). This is in line with a recent in vitro observation that wortmannin can also enhance the production of endothelial tissue factor protein when co-applied together with TNF and VEGF (51). Collectively, these observations give novel insights in the regulatory mechanism controlling endothelial activation and tissue factor production under physiological and pathological conditions. Further studies that address PI3-kinase induction in activated quiescent endothelial cells are currently under investigation.

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An Inhibitory Role of the Phosphatidylinositol 3-Kinase-signaling Pathway in Vascular Endothelial Growth Factor-induced Tissue Factor Expression

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An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression.

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Katja Issbrücker’s name was printed incorrectly. The correct spelling is shown above.

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