ABSTRACT

The growth of solid tumors and the formation of metastases are dependent on neoangiogenesis. One of the most important factors in inducing the formation of new blood vessels is the vascular endothelial growth factor (VEGF), which acts specifically on endothelial cells. VEGF is expressed and secreted by almost all solid tumors. The molecular mechanisms leading to enhanced production of this angiogenic mitogen are manifold and have been elucidated to some degree. Two VEGF receptors, fms-like tyrosine kinase 1 (FLT-1) and KDR, have been identified almost specifically on human endothelial cells. They are expressed preferentially in the proliferating endothelium of vessels lining and/or penetrating solid tumors, whereas they are almost undetectable by convenient methods in vessels of healthy tissue. However, the underlying mechanisms are not understood. We could show that media conditioned by various cancer cell lines grown under hypoxic conditions were able to up-regulate expression of FLT-1 mRNA and protein but not of KDR mRNA. Furthermore, up-regulation of a shorter mRNA species was observed that most probably codes for the soluble variant of FLT-1. These effects were completely inhibited by VEGF-neutralizing extracellular VEGF receptor domains. The effect could be mimicked by adding recombinant VEGF instead of conditioned cancer cell medium to the endothelial cell cultures. Both mutant VEGF, which activates only KDR, and placenta growth factor, which activates only FLT-1, were able to enhance FLT-1 expression. VEGF-stimulated FLT-1 mRNA expression was inhibited by actinomycin D. These data suggest that VEGF itself is the main factor secreted by tumor cells that is able to enhance the expression of its receptor FLT-1 and of a soluble variant of FLT-1 in endothelial cells.

INTRODUCTION

The growth of solid tumors and the formation of metastases are dependent on neoangiogenesis (1). One of the most important factors in inducing the formation of new blood vessels is VEGF, which acts specifically on endothelial cells (2). VEGF is expressed by almost all solid tumors. The molecular mechanisms leading to enhanced production of this angiogenic mitogen are manifold, including activation of oncogenes such as ras (3, 4), inactivation of tumor suppressor genes such as p53 (5, 6) and VHL (7), activation of protein kinase C by tumor promoters (8), and cellular stimulation by growth factors (8, 9), cytokines (10), and hypoxia (11). Two receptors, FLT-1 and KDR, have been identified almost specifically on human endothelial cells that bind VEGF and mediate proteolytic processes, migration, and proliferation of endothelial cells involved in the formation of new blood vessels (2). The two VEGF receptors have been shown to be expressed preferentially in the proliferating endothelium of vessels lining and/or penetrating solid tumors, whereas they are almost undetectable by convenient methods in the vessels of healthy tissue (12, 13). Thus, expression of both VEGF and its receptors seems to be a prerequisite for tumor angiogenesis. Interference with the VEGF/VEGF receptor signaling system by VEGF-neutralizing antibodies, VEGF antisense or dominant negative VEGF receptors, has been shown in all cases to significantly inhibit tumor growth (14–16) and metastatic spread (14).

In contrast to our knowledge about the various mechanisms by which VEGF is up-regulated, almost nothing is known about the processes responsible for enhanced VEGF receptor expression in tumor vascular endothelial cells. In situ hybridization revealed that during progression from low-grade glioma to glioblastoma, expression of FLT-1 mRNA precedes that of KDR mRNA (17). Abundant expression was also observed for VEGF mRNA in glioblastomas and was mainly attributed to cells in the vicinity of necroses. This indicates that hypoxia is a major stimulus for VEGF expression in glioblastoma (17). The effect of oxygen withdrawal on VEGF receptor expression has also been investigated. Recently, contradictory results have been reported on the effect of hypoxic conditions on VEGF receptor expression in vitro. Although it was shown that hypoxia up-regulates VEGF receptors in bovine retinal endothelial cells (18), no such effect could be observed with HUVECs (19).

To further investigate the mechanisms of endothelial VEGF receptor expression in an in vitro setting as close as possible to the tumor situation, we cultured rat and human brain tumor cells and other tumor cells under hypoxic conditions. Here we show that conditioned media from these tumor cells, when applied to HUVECs and HLMVECs, resulted in increased FLT-1 expression at the mRNA and the protein level. The major factor involved is VEGF itself.

MATERIALS AND METHODS

Cell Cultures and Reagents. HUVECs were purchased from PromoCell (Heidelberg, Germany), HLMVECs were obtained from Clonetics Corp. (San Diego, CA), and HMEC-1 was from Centers for Disease Control (Atlanta, GA). All human endothelial cells were grown in endothelial growth medium-uv (PromoCell) and 5% FCS. The cell line PAEC, kindly provided by Dr. C. H. Heldin, was grown in Ham’s F-12 medium (Gibco, Egggenstein, Germany) supplemented with 15% FCS. For stimulation experiments, cells were washed twice in PBS and grown in basal medium and 1% FCS for 24 h, and then factors and/or conditioned media were added (conditioned medium:basal medium:2:1). For VEGF-neutralizing experiments, VEGF or conditioned media were preincubated with 200 ng/ml rsFLT-1/5 for 15 min before adding to cultures. Rat C6 glioblastoma cells (ATCC CCL-107), human H4 neuroglioma cells (ATCC HTB-148), human A375 malignant melanoma cells (ATCC CRL-1619), and human A549 lung carcinoma cells (ATCC CCL-185) were cultured in DMEM and 10% FCS. The generation of human ARZ-4 renal carcinoma cells, stably transfected with mutated VHL, has been described elsewhere (20). ARZ-4 cells were grown in DMEM, 10% FCS, and G418 (1 mg/ml). Growth of cells under hypoxic conditions was performed for 24 h in serum-free DMEM using GasPak pouches (BBL Microbiology Systems).
Human recombinant VEGF isoforms and PIGF_{152} were expressed with the baculovirus system and purified to homogeneity as described elsewhere: VEGF_{21} (21); VEGF_{165} (21); VEGF_{189} (22); and PIGF_{152} (23). rsFLT-1/5 was expressed and purified as described previously (24). VEGF_{121} (25) and the KDR-specific VEGF_{121} mutant VEGF-L24 were expressed in Escherichia coli. Recombinant human basic fibroblast growth factor was obtained from Saxon Biochemicals (Hannover, Germany). The polyclonal anti-FLT-1 antisera was raised against rsFLT-1/5 in rabbits. The mAb 7A6 was directed against recombinant sFLT-1 containing immunoglobulin-like loop 1-7 as described (24).

Northern Analysis. Total RNA was prepared with the RNeasy total RNA kit (Qiagen, Hilden, Germany). RNA (5 μg/lane) was separated on formaldehyde-containing agarose gels, transferred to nylon membranes, and hybridized to 32P-labeled cDNA fragments as described. The specific cDNA probe for KDR was a human 1.4-kb cDNA fragment (nucleotides 360-1720), and the specific cDNA probe for FLT-1 was a human 1.05-kb cDNA fragment (nucleotides 172-1231; Ref. 26).

FLT-1-specific ELISA Assay. HUVECs were cultured in 6-well cluster plates and treated as described under "Cell Cultures and Reagents." Unstimulated cells were used for each time point as a control. After treatment, cells were washed once with PBS and subsequently lysed in 250 μl of lysate buffer containing 25 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.02 mM leupeptin, and 1% Triton X-100. Lysates were cleared by centrifugation. Ninety-six-well cluster plates (Nunc-Immuno Plate MaxiSorp) were coated with 100 μl/well of the capture antibody (mAb 7A6; 1 μg/ml in PBS) and incubated overnight at 4°C. Wells were washed four times with PBST (PBS containing 1% Tween 20), blocked for 1 h with 200 μl/well PBST, and washed as described previously. Samples (40 μg of lysate protein/well) were added and incubated for 2 h. The plates were washed again with PBST and incubated for at least 1 h with the detection antiserum (100 μg/ml; polyclonal rabbit anti-sFLT-1/5 serum; 1:1000 in PBST). After another four washes, biotinylated antirabbit antibody (100 μg/ml; Vector Laboratories, Burlingame, CA) was added and incubated for another 30 min. Plates were washed, and avidin-phosphatase conjugate (Calbiochem, Bad Soden, Germany) was added (100 μg/ml; 1:2500 in PBST) for at least 20 min. After a final wash (4 × PBST and 1× substrate buffer), the substrate was added [100 μl of p-nitrophenylphosphate/well from a stock of 1 mg/ml in 1× diethanolamine buffer (pH 9.8)]. Absorbance was determined at 405 nm in an ELISA reader. All incubation steps, if not indicated otherwise, were carried out at room temperature in a moist chamber. For calibration, purified rsFLT-1/5 was used.

RESULTS

To investigate whether conditioned media from tumor cells contain factors that are able to enhance the expression of the VEGF receptor FLT-1, we selected C6 cells derived from rat glioblastoma, a highly vascularized and aggressively growing tumor. HUVECs were incubated with conditioned media from C6 cells that were kept under hypoxic conditions for 24 h. As early as 3 h after treatment, a significant increase in the amount of FLT-1 mRNA could be detected, which increased further up to 6 h and remained constant at a high level for 30 h (Fig. 1, upper panel). Interestingly, the expression of a shorter transcript of about 2.7 kb was also enhanced, however, in a more transient manner. When Northern analysis was carried out with a probe for the receptor kinase domain only, the 2.7-kb transcript could not be detected (data not shown). This suggests that the shorter transcript codes for sFLT-1 similar or identical to the one discovered recently (25). To address the question of whether stimulation of FLT-1 mRNA expression by conditioned medium from C6 cells is restricted to HUVECs, we performed similar experiments with HLMVECs. The result, shown in Fig. 1 (lower panel), indicates that these cells, like HUVECs, also show an increase of both FLT-1 and sFLT-1 mRNA on stimulation with rat C6 conditioned medium. It seems, however, that the increase observed for FLT-1 mRNA is more transient than that for HUVECs. Similar data were obtained with two endothelial cell lines, HMEC-1 and PAEC (data not shown).

Next, we investigated whether the effect observed in two different types of human endothelial cells was confined to a stimulating activity in conditioned medium from rat C6 cells or whether similar results could also be obtained with conditioned media from various human tumor cell lines grown under hypoxic conditions. Therefore, conditioned media from hypoxic human neuroglioma cells (H4), human malignant melanoma cells (A375), human lung carcinoma cells (A549), and human renal carcinoma cells transfected with a truncated mutant of the VHL tumor suppressor gene (ARZ-4) were used to stimulate HUVECs for 4 h. As shown in Fig. 2, increased amounts of the 7.5-8-kb mRNA for the full-length receptor and of the shorter 2.7-kb transcript for its soluble variant were obtained in all cases. Similar results were observed when HLMVECs instead of HUVECs were used as target cells (data not shown).

Preliminary attempts to purify the factor(s) responsible for the enhanced expression of FLT-1 and sFLT-1 mRNA indicated that this factor might be VEGF itself: chromatographic separation of proteins from the conditioned media revealed that the activity responsible for the stimulation of FLT-1 and sFLT-1 mRNA expression showed a chromatographic behavior similar to that of VEGF (data not shown). We therefore decided to use rsFLT-1/5, which binds VEGF with high affinity (24), to neutralize VEGF activity in conditioned media from C6 and H4 cells. Incubation of HUVECs for 6 h with these condi-
VEGF up-regulates FLT-1

Amounts of KDR mRNA. Similar results were obtained with conditioned media from other hypoxic tumor cells used in this study. This might be due to the fact the HUVECs already express high levels of KDR mRNA under normal culture conditions, in contrast to FLT-1 mRNA, which is present only in small amounts.

To confirm the stimulating effect of conditioned media from hypoxic C6 and H4 cells as well as that of VEGF165 on the expression of FLT-1 mRNA at the protein level, we used an ELISA that was developed on the basis of the mAb 7A6 recognizing the first amino-terminal Ig-like loop of FLT-1 (24). Cell lysates were prepared from HUVECs after incubation for 8 h with conditioned media from hypoxic C6 and H4 cells or VEGF165 and VEGF121. As shown in Fig. 7, both conditioned media as well as VEGF are able to significantly enhance the amount of FLT-1. Similar data were obtained when conditioned media from stimulated HUVECs were investigated for the presence of the soluble receptor variant (data not shown). Thus, the effects observed at the mRNA level could be confirmed at the protein level.

C6 C6 H4 H4 VEGF is able to bind to and activate both receptors, FLT-1 and KDR, in endothelial cells. To address the question of which of the two

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Fig. 2. Up-regulation of FLT-1 and sFLT-1 mRNA by conditioned media from various tumor cells in HUVECs. Serum-starved HUVECs (passage 5) were incubated with conditioned media from hypoxic tumor cells for 4 h. The tumor cells are C6 (rat glioblastoma), H4 (human neuroglioma), A375 (human malignant melanoma), A549 (human lung carcinoma), and ARZ-4 (human renal carcinoma transfected with mutant VHL). Northern analysis was performed as described in “Materials and Methods.”

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Fig. 3. Inhibition of FLT-1 and sFLT-1 mRNA expression by VEGF-neutralizing rsFLT-1/5. Serum-starved HUVECs were incubated for 6 h with conditioned media from hypoxic C6 and H4 cells with or without the addition of 200 ng/ml rsFLT-1/5. Northern analysis was performed as described in “Materials and Methods.” The first two lanes represent controls.

To confirm conclusively that VEGF is able to up-regulate FLT-1 and sFLT-1 mRNA in endothelial cells, we incubated HUVECs with recombinant human VEGF165 for various time periods. The results presented in Fig. 4 reflect a similar picture as obtained with conditioned media from hypoxic C6 and H4 cells with or without the addition of 200 ng/ml rsFLT-1/5. Northern analysis was performed as described in “Materials and Methods.”

<table>
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Fig. 4. Up-regulation of FLT-1 and sFLT-1 mRNA by VEGF165 in HUVECs. Serum-starved HUVECs (passage 5) were incubated with VEGF165 (5 ng/ml) for various times. Northern analysis was performed as described in “Materials and Methods.”
receptors mediates the effect of VEGF to up-regulate the expression of FLT-1 and sFLT-1, we used recombinant PIGF_{152} (27), which binds exclusively to and specifically activates KDR, and a recombinant VEGF_{121} mutant (VEGF-L2), which binds exclusively to and specifically activates KDR. Recombinant PIGF_{152} causes a weak but significant increase of FLT-1 mRNA (data not shown) and a concomitant increase in the amount of FLT-1 protein (Fig. 7) as well as that of sFLT-1 protein (data not shown). This increase is rather small and might be explained by the low expression of FLT-1 in HUVECs. The KDR-specific mutant VEGF-L2 greatly enhanced FLT-1 and sFLT-1 mRNA levels (data not shown) and also increased the protein levels of the full-length receptor (Fig. 7) and its soluble variant (data not shown). This indicates that stimulation of either FLT-1 or KDR is able to cause an increase of both FLT-1 mRNA and protein and, in addition, an increase of mRNA and protein of sFLT-1.

**DISCUSSION**

The two VEGF receptors, FLT-1 and KDR, have been shown to be expressed preferentially in proliferating endothelium lining and/or penetrating solid tumors, whereas they are almost undetectable by convenient methods in the vessels of healthy tissue surrounding the tumors (12, 13). A prerequisite for tumor angiogenesis, besides the expression of the angiogenic VEGF by tumor and stromal cells, is the expression of the VEGF receptors FLT-1 and KDR in endothelial cells.

Thus, we have addressed the question by which mechanism(s) tumor cells induce the expression of VEGF receptors, in particular the receptor FLT-1, in endothelial cells. It could be shown that conditioned media from rat C6 glioblastoma cells and three human tumor cell lines derived from neuroglioma, melanoma, and renal carcinoma are all able to induce enhanced expression of FLT-1 mRNA and that increased message is translated into FLT-1 protein in human endothelial cells. The increase of FLT-1 mRNA was observed not only in HUVECs but also in HLMVECs, indicating that this effect is not restricted to one endothelial cell type. Preliminary attempts to purify the factor(s) responsible for FLT-1 up-regulation revealed that the factor(s) exhibited a chromatographic behavior similar to that of VEGF. Using a recombinant extracellular domain of human FLT-1, we were able to neutralize the stimulating factor(s) from the conditioned tumor cell media. This soluble receptor fragment, sFLT-1/5, has been shown to bind the VEGF isoforms VEGF_{121}, VEGF_{165}, and VEGF_{189} with high affinity (24). In accordance with these results, all of these three recombinantly produced human VEGF isoforms caused an increase of FLT-1 mRNA and protein in human endothelial cells. Thus, VEGF itself is the main factor secreted by the tumor cells and is able to up-regulate its own receptor, FLT-1.

Brogi et al. (19) have shown that conditioned media from transformed myoblasts and nontransformed smooth muscle cells are able to up-regulate the VEGF receptor KDR but not FLT-1 in HUVECs and HMECs. It was also reported that the effect on KDR expression could not be inhibited by VEGF-neutralizing antibodies and thus excludes VEGF from being involved in KDR up-regulation (19). We have also investigated the effect of conditioned media from five hypoxic tumor cell lines on the expression of KDR mRNA but were unable to detect significant changes. At least two explanations for this discrepancy are apparent: (a) the ^{125}I-labeled VEGF binding assay used by Brogi et al. (19) might not have been sensitive enough to detect an increase of the FLT-1 receptor, which is expressed in HUVECs only at very low levels as compared to KDR; and (b) the tumor cell lines used in our experiments do not secrete the factor(s) responsible for KDR up-regulation, as do transformed myoblasts and normal smooth muscle cells.

However, the question still remains as to why conditioned media from tumor cells that cause up-regulation of FLT-1 are unable to up-regulate KDR in human endothelial cells in culture. At least two explanations are obvious: (a) the receptor is already expressed at a high level in HUVECs and can not be up-regulated further. Endothelial cells expressing only low levels of KDR will be a prerequisite to investigate this problem further; and (b) endothelial cells in culture might not be the appropriate assay system, because regulation of KDR expression might require interaction of tumor cells and stromal cells and/or cell-cell contact and thus tissue integrity.
Furthermore, our experiments show that the expression of sFLT-1 that has been described recently (28) is considerably enhanced at the mRNA and protein level on stimulation of human endothelial cells with conditioned media from various tumor cells. Similar to the expression of FLT-1 mRNA and protein, the expression of sFLT-1 could also be completely abrogated when neutralizing rsFLT-1/5 was added to the endothelial culture medium. Thus, at least at this level, FLT-1 as well as sFLT-1 expression seems to be up-regulated by VEGF in a comparable fashion. The functional role of FLT-1 is not very clear at present. It has been shown that FLT-1-deficient mice formed endothelial cells but assembled these cells into abnormal vascular channels (29). This suggests that FLT-1 might be involved in endothelial cell-cell and/or cell-matrix interaction. Furthermore, it has been reported that stimulation of FLT-1 in HUVECs contributes to the expression of tissue factor, and thus FLT-1 might be involved in procoagulant activity (30). As far as sFLT-1 is concerned, its physiological and/or pathophysiological role is even less understood. One obvious possibility is that in the tumor situation, sFLT-1 could associate with and neutralize angiogenic VEGF. However, additional investigations will be required to determine quantitatively whether the amounts of sFLT-1 secreted by endothelial cells are sufficient to effectively interfere with the angiogenic potential of VEGF. Thus far, we have been able to identify sFLT-1 in the sera of cancer patients.

The enhanced expression of FLT-1 and sFLT-1 is most probably due to transcriptional activation, because the addition of actinomycin D completely abolished the effect. To address the question of which of the two VEGF receptors mediates the effect of VEGF to up-regulate the expression of FLT-1 and sFLT-1, we stimulated HUVECs with either PIGF (27), which binds exclusively to and specifically activates FLT-1, and a recombinant VEGF_{121} mutant, VEGF-L2, which binds exclusively to and specifically activates KDR. Both receptor-specific agonists were able to induce the expression of FLT-1 and sFLT-1, indicating that both FLT-1 and KDR-mediated signaling, which has been shown to reach the nucleus (30,31), are able to provoke the effect.

ACKNOWLEDGMENTS

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Note Added in Proof

While this work was under review, C. Kremer et al. (Cancer Res., 57: 3852—3859, 1997) published results showing that VEGF up-regulates its receptor KDR in a cerebral slice culture system.

REFERENCES


5 B. Barleon and D. Marmé, unpublished results.
Vascular Endothelial Growth Factor Up-Regulates Its Receptor 
\textit{fms}-like Tyrosine Kinase 1 (FLT-1) and a Soluble Variant of 
FLT-1 in Human Vascular Endothelial Cells

Bernhard Barleon, Gerhard Siemeister, Georg Martiny-Baron, et al.


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