Flavopiridol, a Protein Kinase Inhibitor, Down-Regulates Hypoxic Induction of Vascular Endothelial Growth Factor Expression in Human Monocytes

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Abstract

We have investigated the effects of flavopiridol, a novel protein kinase inhibitor that is selective for cyclin-dependent kinases, on hypoxia-induced vascular endothelial growth factor (VEGF) expression in human monocytes. We found that hypoxia induces a time-dependent increase of VEGF mRNA expression and protein levels in human monocytes. Flavopiridol showed a minimal effect on the constitutive levels of VEGF mRNA but completely blocked hypoxia-induced VEGF mRNA and protein expression. The inhibitory effects of flavopiridol on VEGF mRNA induction also occurred in the presence of cycloheximide. The transcriptional activation of either a VEGF promoter-luciferase construct or a hypoxia-inducible factor 1 reporter plasmid was not affected by addition of flavopiridol in transient transfection experiments. In contrast, actinomycin D experiments demonstrated that flavopiridol dramatically decreased VEGF mRNA stability. These data provide the first evidence that flavopiridol can affect gene expression by altering mRNA stability. We propose that flavopiridol may interfere with one or more signaling events, leading to hypoxia-induced, protein kinase-modulated, RNA binding activity. An important clinical implication of our results is that flavopiridol, presently under investigation in clinical trials, might have antiangiogenic as well as direct antiproliferative effects.

Introduction

Mononuclear phagocytes infiltrate tumors after recruitment by chemotactic factors produced by tumor and/or host cells. Macrophages infiltrating the tumor site are a potential source of cytokines and angiogenic factors that may ultimately contribute to tumor progression (1). A feature of many solid tumors that can mediate the tumor-macrophage interaction is decreased oxygen tension. Hypoxic areas of tumor growth are known to contribute to the development of resistance to chemotherapy and radiation therapy. More importantly, hypoxia is a stimulus for the expression of several genes, including angiogenic factors, aimed at counteracting the deleterious effects of prolonged exposure to low oxygen levels. The molecular mechanism by which hypoxia induces gene expression has been, at least in part, elucidated in the last several years (reviewed in Refs. 2–4). Hypoxia increases the stability of certain mRNAs by affecting the function of RNA-binding proteins, which in turn bind to “instability regions” in the 3’ UTR in the mRNA (5). We have reported previously that macrophages can respond to hypoxia by trans-activation of HIF-1 and transcriptional activation of the inducible nitric oxide synthase gene (6). Pharmacological intervention to target macrophage-derived production of angiogenic factors is an attractive possibility with potential therapeutic implications (1).

VEGF is an angiogenic factor produced by normal and transformed cells. VEGF plays an important role in the acquisition of a metastatic phenotype by cancer cells (7). The induction of VEGF mRNA by hypoxia occurs at the transcriptional and posttranscriptional level. Forsythe et al. (8) have demonstrated that a HIF-1 binding site of the VEGF promoter was required for responsiveness to hypoxia of a VEGF promoter-luciferase construct in the human hepatoblastoma cell line Hep3B, indicating that HIF-1 mediates transcriptional activation of the VEGF gene under hypoxic conditions. However, hypoxia-mediated increased stabilization of the VEGF mRNA has also been demonstrated, and putative RNA-binding proteins and RNA instability regions of the VEGF 3’ flanking region have been identified (5, 9–11).

Flavopiridol, a novel protein kinase inhibitor with selectivity for CDKs, has been tested recently in a Phase I clinical trial at the National Cancer Institute (12) and is presently under investigation in Phase II clinical trials at other institutions in the United States and abroad. In vitro studies have demonstrated that flavopiridol acts by competitive inhibition of the ATP-binding site of CDKs (13, 14). Flavopiridol blocks cell proliferation and exerts antitumor activity in vitro (15–17); flavopiridol also induces apoptosis in several human tumor cell lines and in normal and transformed lymphoid cells (18, 19).

An unexpected finding in the Phase I clinical trial (12), in which flavopiridol was administered as continuous infusion to patients with refractory neoplasms, was the appearance of a proinflammatory syndrome including fever, chills, and tumor pain, along with alteration in acute phase reactants, suggesting that flavopiridol might affect gene expression. However, whether flavopiridol plays a role in the regulation of gene expression is presently unknown.

In this report, we show that flavopiridol caused down-regulation of VEGF mRNA and protein expression induced by hypoxia in human monocytes. Flavopiridol did not affect hypoxia-induced transcriptional activation of VEGF but significantly decreased VEGF mRNA half-life. These data provide the first evidence for a novel mechanism of action of flavopiridol in the down-regulation of gene expression. This finding also suggests that flavopiridol may have an antiangiogenic activity that might contribute to its therapeutic potential. Additional studies are warranted to investigate the antiangiogenic activity of flavopiridol in clinical trials.

Materials and Methods

Cells and Reagents. Human peripheral blood mononuclear cells were collected from healthy donors at the Cell Processing Section, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, Maryland. Monocytes

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2 The abbreviations used are: HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; CDK, cyclin-dependent kinase; CHX, cycloheximide; UTR, untranslated region.
were purified by elutriation as reported previously. Monocytes were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mmol/L-glutamine, 20 mmol/L HEPES (all from Life Technologies), and 10% heat inactivated FCS (HyClone Laboratories, Logan, UT). The human THP-1 monocytic cell line and the mouse macrophage cell line ANA-1 were cultured as described (6). Cycloheximide and actinomycin-D were purchased from Sigma. Flavopiridol was obtained from Behringwerke (Marburg, Germany). For experiments under hypoxia, cells were cultured in a modular incubator chamber (Billups Rothenberg, Del Mar, CA) and flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2 at 37°C in a humidified atmosphere (referred to as hypoxic conditions).

Northern Blot Analysis. Total cellular RNA, gel electrophoresis, and blotting were performed as described previously (6). The cDNA probe specific for human VEGF gene was kindly provided by Dr. Marsha Merril (NIH, Bethesda, MD) and was radiolabeled as described (6). The blot was hybridized for 1 h at 68°C in ExpressHyb (Clontech, Palo Alto, CA) with the radiolabeled probes (1 to 2 × 10^6 cpm/ml). The blot was washed four times at room temperature in wash solution 1 (2 × SSC/0.05% SDS) and two times at 50°C in wash solution 2 (0.1 × SSC/0.1% SDS), according to manufacturer’s instruction, and autoradiographed. Densitometry analysis was performed using the UN-SCAN-IT software (Silk Scientific, Orem, Utah). For each sample, results were normalized to the corresponding level of 28S.

ELISA. The content of VEGF protein in culture supernatants of human monocytes was determined by using a commercially available kit following the manufacturer’s instruction (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN).

Transient Transfection of ANA-1 and Luciferase Assay. ANA-1 macrophages were transfected by a modification of the DEAE-dextran method, as described (6). Cells were transfected with plasmid VEGF-P7 containing 1005 bp of the human VEGF 5′-flanking region linked to the luciferase reporter gene, or plasmid p11W, which contained only 47 bp of the VEGF 5′-flanking sequence between −985 and −939, encompassing the HIF-1 binding site at −975 (5′-TACGTGGG-3′), linked to the luciferase reporter gene (both plasmids were kindly provided by Gregg L. Semenza, Johns Hopkins University, Baltimore, MD). Cells were lysed with 1× Reporter Lysis buffer (Promega Corp., Madison, WI), and luciferase activity was assayed according to manufacturer’s recommendations using a Packard LumiCount luminometer. The protein content was determined as described by Bradford, using the Bio-Rad Protein Assay.

Results and Discussion

Hypoxia Induces VEGF Expression in Human Monocytes. We first sought to determine whether human monocytes exposed to hypoxic conditions expressed VEGF. Human monocytes were treated under either normoxic (21% O_2) or hypoxic (1% O_2) conditions, and the levels of VEGF mRNA expression were evaluated at different time points. In the experiment shown in Fig. 1A, human monocytes cultured under normal oxygen levels expressed low but detectable levels of VEGF mRNA that were significantly augmented by incubation under hypoxic conditions. The levels of VEGF mRNA were already increased after 3 h of incubation in hypoxia, peaked at 6 h (4.5-fold relative to normoxic cells), and were still elevated at 16 h, demonstrating that human monocytes exposed to low oxygen tension do in fact express higher levels of VEGF mRNA. To investigate whether the increase in VEGF mRNA levels was paralleled by an increase in the amount of protein, ELISA experiments were performed on supernatants from monocytes cultured under normoxic or hypoxic conditions for 16 h. In the experiment shown in Fig. 1B, human monocytes expressed low levels of VEGF protein (22 pg/ml) constitutively. Consistent with the Northern blot data, a 3.3-fold increase in the levels of VEGF (73 pg/ml) protein was observed in cells cultured for 16 h under hypoxic conditions. We have observed a donor-to-donor variability in the baseline levels of VEGF mRNA that were slightly inhibited by the addition of flavopiridol (100 nM). Monocytes cultured under hypoxia expressed a 5.8-fold higher level of VEGF mRNA relative to changes in the baseline levels of VEGF mRNA expression, ranging from minimal to low but detectable levels. In three separate ELISA experiments, we have observed a range of baseline expression of VEGF protein from 22 to 120 pg/ml (5.4-fold). These results indicate that fresh human monocytes exhibit a significant donor-to-donor variability in the baseline levels of VEGF expression. However, irrespective of the different levels of baseline expression in human monocytes from different donors, we have consistently observed in each separate experiment a hypoxic induction of VEGF mRNA ranging from 4-fold up to 10-fold above the level expressed in monocytes cultured under normoxic conditions. At the level of VEGF protein, we have observed in each independent experiment a consistent hypoxic induction above the baseline ranging from 2.7-fold up to 4.9-fold (range, 73−352 pg/ml). Thus, hypoxia is a stimulus for the induction of VEGF expression in human monocytes.

Flavopiridol Inhibits Hypoxia-induced VEGF Expression. Because flavopiridol had displayed clinical evidence of a proinflammatory syndrome (12), we sought more direct evidence of flavopiridol modulation of gene expression by testing its effect on the hypoxic induction of VEGF mRNA in human monocytes. In preliminary experiments, monocytes were cultured under normoxic or hypoxic conditions for 16 h in the presence or absence of flavopiridol at a concentration ranging from 100 to 300 nM, based on previous studies where flavopiridol inhibited CDK activity (14). As shown in Fig. 2A, human monocytes cultured under normoxic conditions expressed detectable basal levels of VEGF mRNA that were slightly inhibited by the addition of flavopiridol (100 nM). Monocytes cultured under hypoxia expressed a 5.8-fold higher level of VEGF mRNA relative to
Monocytes cultured under normoxic conditions. However, the addition of flavopiridol dramatically and completely abolished hypoxia-induced VEGF mRNA expression (Fig. 2A, Lane 4). In five independent experiments performed with fresh human monocytes harvested from different donors, we have consistently observed in the presence of flavopiridol a decrease of VEGF mRNA expression ranging from 80 to 99% relative to the levels induced by hypoxia. Similar results were also obtained using the human monocytic cell line THP-1 (data not shown). The inhibition of hypoxia-induced VEGF expression by flavopiridol was not restricted to cells of the myeloid lineage. In fact, flavopiridol also potently inhibited the constitutive and hypoxia-induced expression of VEGF in the human adenocarcinoma DLD-1 cell line (data not shown). The DLD-1 cell line represents an interesting model because of the genetic alteration and constitutive activation of the ras pathway, which has been implicated in the up-regulation of VEGF expression (21). Although the main focus of our investigation was to study the effects of flavopiridol in normal human monocytes, these data indicate that our findings may be extrapolated to tumor cell lines in which the role of oncogenic transformation and genetic abnormalities must be considered.

Next we performed experiments to investigate whether flavopiridol also inhibited the expression of VEGF protein. In the experiment shown in Fig. 2B, human monocytes cultured under normoxic conditions expressed detectable basal levels of VEGF protein (120 pg/ml) that were not significantly affected by addition of flavopiridol (100 nM). In contrast, the hypoxic induction of VEGF protein was decreased, in the presence of flavopiridol, from 2.75-fold (330 pg/ml) to 1.25-fold (152 pg/ml) above the levels present in human monocytes cultured under normoxic conditions, which represents an 86% inhibition of the hypoxic induction above the background levels. In three separate ELISA experiments, the hypoxic induction of VEGF protein ranged from 2.75- to 4.8-fold above the baseline level. In the presence of flavopiridol, the hypoxic induction of VEGF protein was decreased to a range of 1.1–1.4-fold above the levels detected in untreated human monocytes. In conclusion, irrespective of the baseline levels of VEGF expression in human monocytes cultured under normal oxygen conditions, we have consistently observed an inhibitory effect of flavopiridol relative to the hypoxic induction of VEGF above the baseline levels ranging from 80 to 99% at the mRNA level and from 75 to 90% at the protein level. Our data then demonstrate for the first time that flavopiridol is a potent inhibitor of hypoxia-induced VEGF expression in human monocytes.

To test the range of concentrations where flavopiridol was effective, human monocytes cultured under hypoxic conditions were exposed to increasing concentrations of flavopiridol, ranging from 25 to 100 nM. As shown in Fig. 3A, a concentration-dependent inhibition of VEGF mRNA expression by flavopiridol was observed. Flavopiridol at 25 nM inhibited by ~50% the hypoxia-induced expression of VEGF (Lane 3), as assessed by densitometry analysis, whereas flavopiridol at 50 nM caused a 75% inhibition (Lane 4). Complete inhibition was
observed at doses of 100 nM (Lane 3). Of note, flavopiridol did not significantly affect the expression of glyceraldehyde-3-phosphate dehydrogenase, which also is a hypoxia-inducible gene, or the expression of rRNA 28S and 18S. The effective concentration of flavopiridol that inhibited hypoxia-induced VEGF expression (50–100 nM) is slightly lower than the IC_{50} for the inhibition of CDK activity (100–300 nM; Ref. 14). Whether the flavopiridol inhibition of gene expression and CDK activity share a common target and/or mechanism of action remains to be elucidated.

Because flavopiridol can cause apoptosis of normal and transformed human lymphoid cells, we actively investigated whether, under our experimental conditions, apoptosis occurred in human monocytes. Cell viability, as assessed by trypan blue exclusion, was not significantly affected by treatment with flavopiridol at concentrations as high as 300 nM under either normoxic or hypoxic conditions (data not shown). More importantly, apoptosis (as assessed by sub-G1 DNA content using propidium iodide staining and flow cytometry analysis) did not occur in human monocytes cultured under normoxic or hypoxic conditions in the presence of flavopiridol at concentrations as high as 300 nM (data not shown). Because flavopiridol, a known CDK inhibitor, blocks cell cycle progression, the use of fresh human monocytes, which do not proliferate, allows the dissection of novel activities of flavopiridol on gene expression from antiproliferative effects. Cell cycle analysis on cultured human monocytes confirmed that the majority of cells are in G1, with a minimal fraction (<5%) being in S phase (data not shown).

**Protein Synthesis Is Not Required for the Inhibition of VEGF mRNA Expression by Flavopiridol.** Next, we performed experiments to establish whether protein synthesis was required for the down-regulation of hypoxia-induced VEGF mRNA expression by flavopiridol. Human monocytes were pretreated with CHX or medium for 20 min and then were cultured for additional 5 h under either normoxic or hypoxic conditions in the presence or absence of flavopiridol. As shown in Fig. 3B, untreated monocytes cultured under normoxic conditions expressed detectable levels of VEGF mRNA that were significantly augmented by the addition of CHX under normoxic conditions. Flavopiridol decreased the constitutive levels of VEGF mRNA expression and, more importantly, completely blocked CHX-dependent induction of VEGF. In addition, flavopiridol inhibited hypoxia-induced VEGF mRNA expression in the absence or the presence of CHX. In contrast, CHX did not augment the induction of VEGF by hypoxia. These data demonstrate that protein synthesis is not required for the inhibitory effects of flavopiridol on the induction of VEGF expression by hypoxia.

**Flavopiridol Decreases VEGF mRNA Half-Life.** The stability of mRNA plays an important role in the hypoxic induction of VEGF expression by flavopiridol.
expression. To test whether flavopiridol affected VEGF mRNA half-life, we assessed the levels of VEGF mRNA after addition of actinomycin D. The human monocytic THP-1 cells were treated under hypoxic conditions in the presence or absence of flavopiridol (50 nM). Actinomycin D was added after 16 h of incubation, and cells were harvested at different time points based on the predicted half-life of VEGF mRNA from previous studies (5). In THP-1 cells treated under hypoxic conditions, the half life of the VEGF mRNA was ~4 h (Fig. 4C). In contrast, the half life of VEGF mRNA in THP-1 cells treated with hypoxia in the presence of flavopiridol was dramatically reduced to ~2 h. Similar results were consistently observed in at least three separate experiments. The half-life of the VEGF mRNA that we have observed in THP-1 cells is longer than that reported in the literature for other cell types (1.5–2 h; Ref. 5). However, to the best of our knowledge, there is no data available on the half-life of VEGF mRNA in human monocytes cultured under hypoxic conditions. This discrepancy in the half-life of VEGF mRNA between human monocytes and other cell types may be due to a “tissue specific” regulation present in cells of the myeloid lineage. These data demonstrate that flavopiridol destabilizes hypoxia-induced VEGF mRNA expression and decreases VEGF mRNA half-life, providing the first evidence that flavopiridol can control gene expression by acting at a posttranscriptional level. The VEGF mRNA 3′-UTR contains instability regions rich in adenylate-uridylate (AU) sequences, which have been demonstrated to mediate the rapid turnover of multiple cytokine mRNAs. A hypoxia-inducible protein complex that binds to the AU-rich element in the 3′-UTR of the VEGF mRNA has been described recently (10). Interestingly, the tyrosine kinase inhibitor genistein, although at relatively high concentrations (500 μM), blocked the hypoxia-induced stabilization of VEGF 3′-UTR transcripts and inhibited hypoxia-induced protein binding to the VEGF 3′-UTR (5). Although the mechanism by which flavopiridol inhibits hypoxia-induced stabilization of VEGF mRNA remains to be elucidated, our results are consistent with a model in which flavopiridol might prevent the binding of a hypoxia-induced RNA-binding protein to elements of the VEGF mRNA 3′-UTR. Flavopiridol is a protein kinase inhibitor and might interfere with the phosphorylation of the hypoxia-induced RNA trans-activating factor. This possibility is consistent with our data indicating that protein synthesis was not required for the inhibitory effects of flavopiridol to occur, making it less likely that flavopiridol might block the expression and/or production of the RNA binding protein. Studies are ongoing to identify the target of flavopiridol in the hypoxia-induced signaling cascade.

In this study, we report that flavopiridol, a known CDK inhibitor, potently inhibits VEGF expression in human monocytes. More importantly, flavopiridol blocks the expression of VEGF induced by hypoxia, a component of many solid tumors, which represents a pathophysiological stimulus for the induction of angiogenesis in vivo. The identification of molecular target(s) by which pharmacological agents inhibit hypoxic induction of VEGF expression may lead to the successful manipulation of VEGF production. Macrophages are a suitable target of a novel therapeutic strategy aimed at decreasing tumor progression and metastasis (1). Our data may have important therapeutic implications in particular because flavopiridol is presently under investigation in several Phase I and II clinical trials. The potential for antiangiogenic activity of flavopiridol, which is implicit in these experiments, warrants further investigation in future clinical trials.

References


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