

Imatinib Mesylate (STI-571) Reduces Bcr-Abl-Mediated Vascular Endothelial Growth Factor Secretion in Chronic Myelogenous Leukemia

John M.L. Ebos,^{1,2} Jennifer Tran,^{1,3} Zubin Master,^{1,2} Daniel Dumont,^{1,2} Junia V. Melo,⁴ Elisabeth Buchdunger,⁵ and Robert S. Kerbel^{1,2,3}

¹Molecular and Cell Biology Research, Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario, Canada M4N 3M5; ²Department of Medical Biophysics and ³Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M4N 3M5; ⁴Department of Haematology, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, London, United Kingdom; and ⁵Novartis Pharma AG, Oncology Research, Basel, Switzerland

Abstract

A large and diverse spectrum of oncogenes has been implicated as a contributor to angiogenesis in solid tumors based, in part, on its ability to induce proangiogenic growth factors such as vascular endothelial growth factor (VEGF), and the fact that various anti-oncogenic signaling inhibitor drugs have been shown to reverse such proangiogenic effects both *in vitro* and *in vivo*. Because leukemias are now also considered to be angiogenesis-dependent malignancies, we asked whether a similar paradigm might exist for the *BCR-ABL* oncogene and the Bcr-Abl targeting drug, STI-571 (imatinib mesylate), in the context of chronic myelogenous leukemia (CML) cells. We found that levels of VEGF expression in *BCR-ABL*-positive K562 cells were reduced *in vitro* by treatment with STI-571 in a dose-dependent fashion. Transfection of *BCR-ABL* into murine myeloid 32D and human megakaryocyte MO7e hematopoietic cells resulted in enhanced VEGF expression, which could be further elevated by the exposure to cytokines such as interleukin 3 and granulocyte macrophage colony-stimulating factor. We also found that conditioned media taken from 32D-p210-transfected cells could stimulate human umbilical vein endothelial cells by increasing phosphorylation of VEGF-R2/KDR and the downstream serine/threonine kinase PKB/Akt, an important regulator of endothelial cell survival. Moreover, amplification of *BCR-ABL* in STI-571-resistant cells was associated with elevated VEGF expression levels which could be reversed by treatment with higher concentrations of STI-571. Taken together, our results

implicate *BCR-ABL* as a possible regulator of CML angiogenesis and raise the possibility that STI-571 could mediate some of its anti-CML properties *in vivo* through an angiogenesis-dependent mechanism.

Introduction

The angiogenic switch in tumors is thought to be governed by two major, complementary events: the induction or up-regulation of various proangiogenic growth factors such as vascular endothelial growth factor (VEGF), and the down-regulation or loss of expression of endogenous inhibitors of angiogenesis such as thrombospondin-1 (TSP-1) (1). A growing body of evidence has further implicated both environmental/epigenetic alterations, *e.g.*, tumor hypoxia, as well as genetic changes being responsible for the tumor angiogenic switch (1), and it is thought that such genetic and epigenetic changes may work in a coordinated manner to stimulate an optimal angiogenic response (2). Relevant genetic changes include oncogene activation (3) and tumor suppressor gene mutation/inactivation events (*e.g.*, loss of p53, PTEN, or VHL function), both of which can strongly influence the expression of proangiogenic growth factors and angiogenesis inhibitors (1, 4). With respect to oncogenes, we (5) and Grugel *et al.* (6) first showed in 1995 that activated K- or H-*ras* was a powerful inducer of VEGF expression in transformed rat intestinal epithelial cells, human colorectal carcinoma cells, or mouse 3T3 fibroblasts. Since then, a broad spectrum of other (proto)-oncogenes, including *ERBB-2/HER-2*, the epidermal growth factor receptor (*EGFR*) tyrosine kinase, and *MYC*, among many others, has been shown to cause similar changes (3), including the down-regulation of TSP-1 (1).

An important therapeutic implication of such findings is that many of the anti-cancer signal transduction inhibitor drugs, originally designed to block the activity of specific oncoproteins, may additionally elicit antiangiogenic effects *in vivo*, which could conceivably contribute to their overall antitumor efficacy. Indeed, evidence in support of this possibility was first reported by us with respect to Ras farnesyltransferase inhibitors (Ras, FTIs) (5), Erbitux/C225, an EGFR blocking antibody (7), and Herceptin, the ErbB-2 blocking monoclonal antibody (7) as well as by other groups (8–10).

An intriguing possibility that arises as a result of such findings is that the drug STI-571 (imatinib mesylate) that

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Requests for reprints: Robert S. Kerbel, Molecular and Cell Biology Research, Sunnybrook and Women's College Health Sciences Centre, S-218 Research Building, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5. Phone: (416) 480-5711; Fax: (416) 480-5703.

E-mail: Robert.Kerbel@swchsc.on.ca

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targets the Bcr-Abl kinase in chronic myelogenous leukemia (CML) (11) may likewise function, at least in part, as an inhibitor of angiogenesis as this type of hematologic malignancy, like almost all others, is now thought to be driven, in part, by tumor-induced angiogenesis (12). This is believed to be the result of the induction of enhanced angiogenesis in the bone marrow by leukemic cell-derived growth factors, such as VEGF (13, 14), which in turn stimulate leukemic cell growth and survival factor secretion from the “induced,” activated endothelial cells of the newly formed microvessels in the bone marrow (15). Given that VEGF levels have been shown to be elevated both in the plasma of CML patients (14) and in conditioned media taken from CML cells (16), it is conceivable that VEGF expression could be driven largely by an oncogene, *e.g.*, *BCR-ABL*, especially in the chronic phase of the disease where *BCR-ABL* appears to be the sole oncogenic event (11). As such, suppression of VEGF expression in CML may be an important consequence of STI-571 treatment and if so, could contribute to the net antileukemic efficacy of the drug in CML patients.

The purpose of this study was to address two related questions: (a) Does the *BCR-ABL* oncogene induce/up-regulate VEGF expression in leukemic cells? and (b) If so, would treatment of *BCR-ABL*/VEGF-positive leukemic cells with STI-571 lead to suppression of VEGF expression? Using both murine and human leukemic cell lines, we report affirmative answers to both questions. As such, our results constitute a significant first step toward establishing a possible mechanism by which STI-571 may inhibit tumor angiogenesis, including in CML.

Results

Bcr-Abl Overexpression Leads to VEGF Up-Regulation

As VEGF has previously been shown to be up-regulated, at least in part, by oncogenes such as mutant *RAS* and *ERBB2/NEU* (2, 4–7, 17), we sought to determine whether Bcr-Abl expression can similarly be linked to the secretion of VEGF in both human and murine leukemic cells overexpressing the M_r 210,000 Bcr-Abl protein. We found that VEGF secretion in 32D-p210 (Fig. 1A) and MO7e-p210 (Fig. 1B) cells was 8.59- and 5.19-fold higher ($P < 0.05$), respectively, than in parental cells, thus suggesting that overexpression of the *BCR-ABL* oncogene in cells of hematopoietic origin is sufficient to up-regulate VEGF. Because parental 32D and MO7e rely *in vitro* on exogenous interleukin 3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF), respectively, for normal cell growth and proliferation, p210 transfectants were grown with or without exogenous growth factors as an experimental control. The exposure of 32D-p210 and MO7e-p210 cells to IL-3 and GM-CSF showed a further 1.21- ($P > 0.05$) and 1.38-fold ($P > 0.05$) increase in VEGF secretion, respectively, compared to 32D-p210 and MO7e-p210 cells incubated without supplemental growth factors.

Bcr-Abl-Mediated VEGF Up-Regulation Is Sufficient to Enhance Endothelial VEGF-R2/KDR and PKB/Akt Phosphorylation

To determine whether VEGF secretion in *BCR-ABL* transfectants could effectively activate primary vascular endothelial

cells [human umbilical vein endothelial cells (HUVECs)], we compared the relative potential of conditioned media derived from 32D-p210 cells and their respective parental controls to induce phosphorylation of the VEGF receptor, VEGF-R2/KDR. 32D-p210 conditioned media caused an increase in VEGF-R2/KDR phosphorylation in HUVECs when compared to untransfected controls (Fig. 2A) and could increase the phosphorylation of the downstream serine-threonine kinase PKB/Akt, a

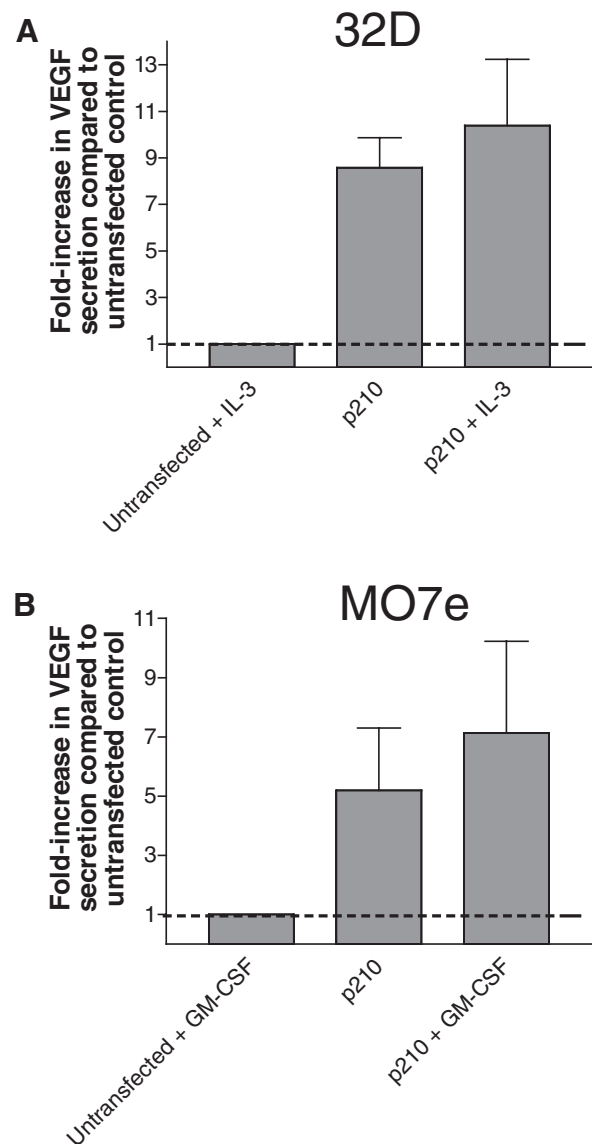


FIGURE 1. Transfection of *BCR-ABL* (p210) into two hematopoietic cell lines results in an increase in VEGF secretion as measured by VEGF ELISA. (A) 32D-p210 and (B) MO7e-p210 cells show an 8.59- and 5.19-fold increase, respectively, in VEGF secretion when compared to untransfected parental controls ($P < 0.05$). The presence of additional growth factors further increased secreted VEGF in 32D-p210 [supplemented with (10 ng/ml) IL-3] and MO7e-p210 [supplemented with (5 ng/ml) GM-CSF] cells by 1.21- ($P > 0.05$) and 1.38-fold ($P > 0.05$), respectively, compared to 32D-p210 and MO7e-p210 with no growth factors. Cells were plated at a density of 5×10^5 cells/ml for 24 h. Graphs are two experiments performed in triplicate. (Error bars, SD.)

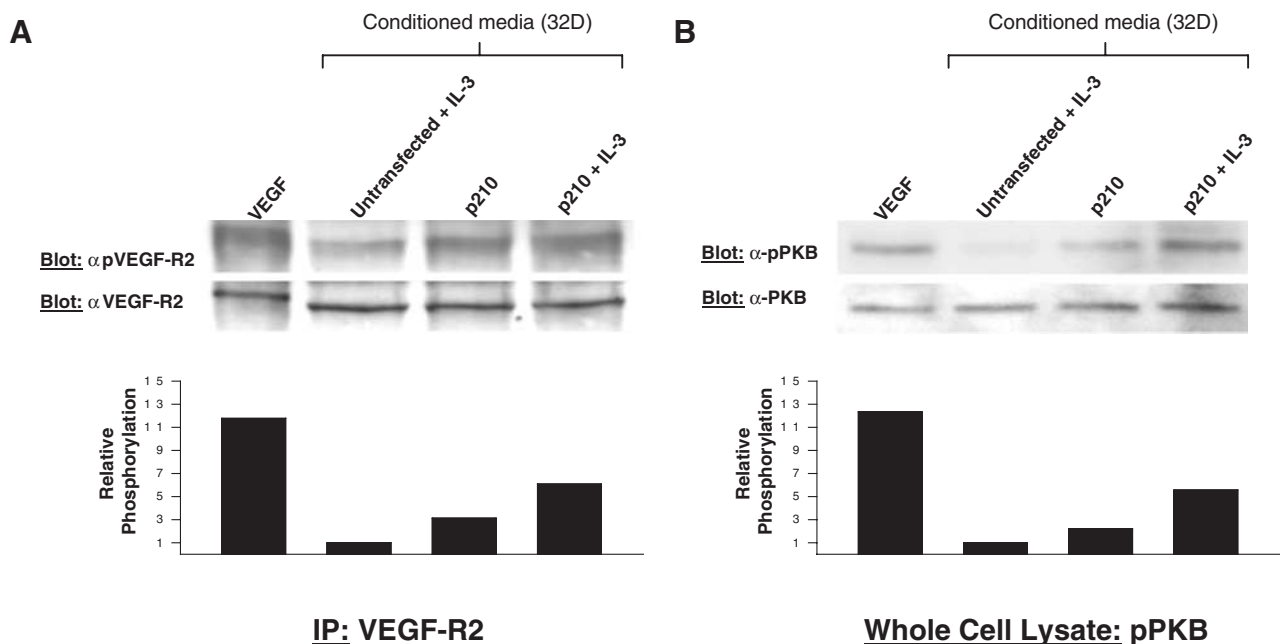


FIGURE 2. Conditioned media from 32D-p210 increase VEGF-R2/KDR and PKB/Akt phosphorylation in HUVECs. HUVECs were treated for 10 min with conditioned media from parental or p210-transfected 32D cells using recombinant VEGF (50 ng/ml) in growth factor-free media as a positive control. Protein lysates prepared from HUVECs treated under these conditions were either **(A)** immunoprecipitated with an anti-VEGF-R2/KDR antibody and Western blotted with an anti-phospho-VEGF-R2/KDR antibody or **(B)** assessed for serine 473 phosphorylation indicating activated PKB. Conditioned media from 32D-p210 transfectants could increase the phosphorylation state of both VEGF-R2/KDR and PKB/Akt 3.14- and 2.19-fold, respectively, with no IL-3, and 6.07- and 5.57-fold, respectively, with IL-3—compared to untransfected parental control (containing IL-3). Reprobing with anti-VEGF-R2/KDR or anti-PKB antibodies revealed equal loading of immunoprecipitates and total protein lysates, respectively, and served as standards for quantification and densitometric analysis of relative phosphorylation. Data are blots and densitometry taken from one representative experiment.

major regulator of endothelial cell survival downstream of VEGF-R2 activation (Fig. 2B) (18). The ability to induce the phosphorylation of both VEGF-R2/KDR and PKB/Akt suggests that Bcr-Abl-mediated VEGF secretion in 32D-p210 cells is sufficient to activate primary vascular endothelial cells.

VEGF Secretion Is Down-Regulated in K562 Cells Treated With Varying Concentrations of STI-571 in Vitro

The addition of the small molecule antagonist STI-571 has previously been shown to block the ATP binding site of the Bcr-Abl oncoprotein and elicit cytotoxic effects on K562 CML cells (19). K562 expressing endogenous *BCR-ABL* has been previously shown to secrete VEGF (15). To test whether inhibition of Bcr-Abl oncoprotein function by STI-571 could lead to down-regulation of secreted VEGF, we treated K562 cells *in vitro* with STI-571 in a dose-dependent manner, and observed that increasing concentrations of STI-571 lead to a stepwise reduction in secreted VEGF (Fig. 3A). To rule out the possibility that the decrease in VEGF secretion was a consequence of STI-571-inhibited cell growth, [³H]thymidine incorporation measurements were performed to assess cellular proliferation. Although high concentrations of STI-571 (1 and 0.5 μ M) dramatically reduced cellular proliferation over a 48-h period compared to vehicle controls (88.79% and 52.3% reductions, respectively), lower concentrations, *e.g.*, 0.25 and 0.175 μ M of the drug had relatively little effect on cellular proliferation (8.5% and 11.8% reductions, respectively), but could still effectively interfere with VEGF production (56.3%

and 43.3% reductions, respectively) (Fig. 3A). Confirmation of this effect was observed when K562 cells treated with 0.25 μ M STI-571 for 24 h showed a 50% reduction in VEGF secretion, whereas only a 15% reduction was seen in cellular proliferation (Fig. 3B). Notably, cell viability remained unchanged confirming previously demonstrated results by Benjamin *et al.* (20) that the long-term growth capacity of these cells, as shown by colony formation assay, remains unchanged when STI-571 is washed from the cells after 24 h. Verification that STI-571 leads to inhibition of Bcr-Abl phosphorylation was shown by immunoprecipitation followed by Western blotting (Fig. 3C).

Amplification of BCR-ABL in Cells Resistant to STI-571 Can Further Increase Levels of Secreted VEGF

BCR-ABL gene amplification in CML cells has previously been shown to result in resistance to STI-571 both *in vitro* (20–23) and *in vivo* (24). We hypothesized that in STI-571-resistant cells where *BCR-ABL* is amplified, VEGF secretion would also be augmented. To test this, a parental STI-571-sensitive cell line (Baf/*BCR-ABL*-s) was compared to the p210-transfected cell line resistant to STI-571 (Baf/*BCR-ABL*-r1) (21) for their relative levels of secreted VEGF. Baf/*BCR-ABL*-r1 cells maintained in 1 μ M STI-571 (Fig. 4A, Lane 5) were found to secrete comparable amounts of VEGF to the Baf/*BCR-ABL*-s grown in the absence of drug (Fig. 4A, Lane 1) (25.9 and 37.4 pg VEGF/10⁶ cells, respectively; $P > 0.05$). We hypothesized that even though *BCR-ABL* is amplified in the Baf/*BCR-ABL*-r1 cells, the constant presence of STI-571 would limit

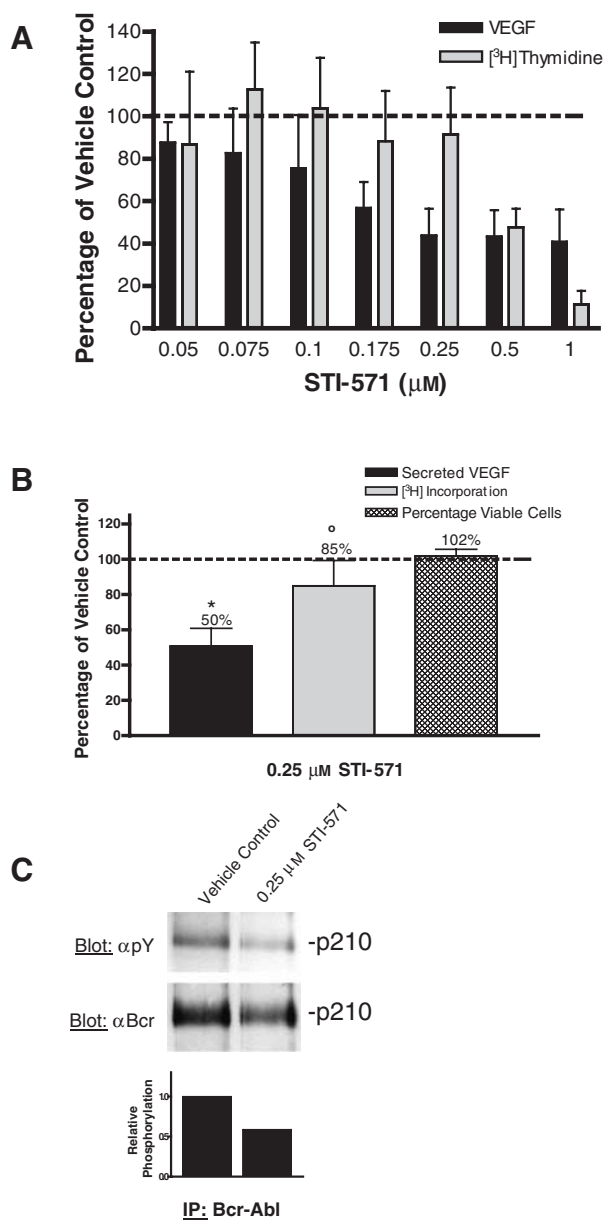


FIGURE 3. VEGF secretion and cellular proliferation in K562 cells treated with STI-571 *in vitro*. **A.** K562 cells (1×10^5 cells/ml) maintained in serum-free media for 48 h were treated with varying concentrations of STI-571 (0.05–1 μM) leading to a stepwise decrease in secreted VEGF as measured by ELISA. Significant reductions in cellular proliferation of 88.79% and 52.3% were observed at treatments of 1 and 0.5 μM STI-571, but not at lower concentrations where 0.25–0.05 μM STI-571 yielded reductions in cellular proliferation ranging within 15% of vehicle control. Each concentration was standardized to a DMSO vehicle control equivalent of 1 μM STI-571. Graphs are two experiments performed in triplicate. **B.** K562 cells (5×10^5 cells/ml) treated with 0.25 μM STI-571 for 24 h in serum-free media yielded a 50% reduction in secreted VEGF and a 15% reduction in cellular proliferation thereby confirming this effect. No significant differences were seen in cell viability as measured by trypan blue exclusion. Graphs are three experiments performed in triplicate. **C.** Cell lysates obtained from triplicate samples taken from **B** were immunoprecipitated for Bcr proteins and immunoblotted with anti-phosphotyrosine antibodies. Blots were then stripped and reprobed with anti-Bcr antibodies. Bcr-Abl (M_r 210,000) phosphorylation compared with protein expression was decreased 42% in STI-571-treated cells as determined by densitometry. (Error bars, SD; *, $P < 0.05$ compared to DMSO control; $^{\circ}$, $P < 0.05$ compared to DMSO control.)

Bcr-Abl phosphorylation to levels comparable to the STI-571-sensitive Baf/*BCR-ABL*-s cells, thus explaining the lack of significant difference in secreted VEGF levels. This was demonstrated to be the case, because when STI-571 was removed completely from Baf/*BCR-ABL*-r1 cells for 24 h and replaced with a vehicle control, Bcr-Abl phosphorylation was increased by 1.8-fold (Fig. 4B, Lane C) and VEGF secretion by 3.6-fold (Fig. 4A, Lane 3), when compared to Baf/*BCR-ABL*-r1 cells maintained in 1 μM STI-571 (Fig. 4A, Lane 5). Interestingly, by reducing the concentration of STI-571 to 0.25 μM in their selection media, Baf/*BCR-ABL*-r1 cells displayed a 1.2-fold increase in Bcr-Abl phosphorylation (Fig. 4B, Lane D) along with a 1.4-fold increase in VEGF secretion (Fig. 4A, Lane 4), when compared to Baf/*BCR-ABL*-r1 cells maintained in 1 μM STI-571 (Fig. 4A, Lane 5). To show that inhibition of Bcr-Abl phosphorylation could lead to down-regulation of secreted VEGF, Baf/*BCR-ABL*-s cells treated with 0.25 μM STI-571 secreted 3.6-fold less VEGF (10.7 pg VEGF/ 10^6 cells; Fig. 4A, Lane 2) than untreated cells with a 0.26-fold reduction in Bcr-Abl phosphorylation (Fig. 4B, Lane B). Similarly, Baf/*BCR-ABL*-r1 cells, normally maintained in 1 μM STI-571, secreted 3.9-fold less VEGF (6.7 pg VEGF/ 10^6 cells; Fig. 4A, Lane 6) and had a 0.21-fold reduction in Bcr-Abl phosphorylation when the concentration of STI-571 was increased to 2 μM (Fig. 4B, Lane F). Our results thus implicate a causal role between increased phosphorylation of the Bcr-Abl oncoprotein and induction of secreted VEGF, which suggests that *BCR-ABL* amplification and the resulting increased protein tyrosine phosphorylation can lead to elevated VEGF production in cells resistant to STI-571.

Discussion

Our results represent an important first step in determining whether STI-571 treatment blocks CML growth, at least in part, by suppressing a *BCR-ABL*-induced mediator of angiogenesis such as VEGF. First, we found that transfection of the *BCR-ABL* oncogene into two hematopoietic cell lines, murine 32D cells and human MO7e cells, resulted in the induction of secreted VEGF. These findings confirm and extend a study by Janowska-Wieczorek *et al.* (16) who reported induction of VEGF in murine FL5.12 cells after *BCR-ABL* gene transfection and are similar in principle to others employing a number of other oncogenes such as mutant *RAS*, *ERBB-2/NEU* in various model cell systems involving immortalized fibroblasts or epithelial cells (5). Furthermore, our finding that stimulation of HUVECs with conditioned media from 32D-p210 cells could enhance the phosphorylation of both of VEGFR-2/KDR and the downstream serine-threonine kinase PKB/Akt, a major regulator of endothelial cell activation, suggests that the expression of *BCR-ABL* may be sufficient to stimulate vascular endothelial cells. Second, the amplification of the *BCR-ABL* gene in STI-571-resistant variants, at least in the case of Baf/*BCR-ABL* cells, was associated with an elevated VEGF secretion. Third, treatment of a variety of VEGF-positive *BCR-ABL*-expressing cell lines, *e.g.*, human K562 cells, and Baf/*BCR-ABL* transfectants, both STI-571-resistant and -sensitive cells, resulted in the down-regulation of VEGF expression. This latter observation is similar in nature to results obtained with other signaling

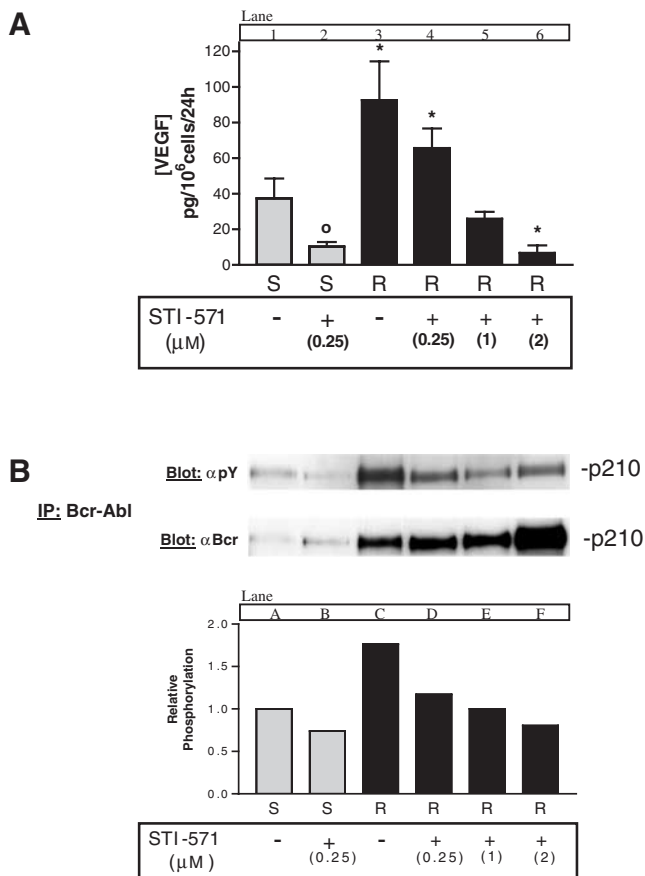


FIGURE 4. STI-571-resistant cells expressing amplified *BCR-ABL* secrete elevated levels of VEGF. STI-571-sensitive Baf/*BCR-ABL*-s (S) cells and the derived Baf/*BCR-ABL*-r1 cells resistant to 1 μM STI-571 (R) were incubated for 24 h (5×10^5 cells/ml) and evaluated for relative levels of (A) VEGF secretion (Lanes 1–6) and (B) Bcr-Abl phosphorylation (Lanes A–F) on treatment with STI-571. Baf/*BCR-ABL*-s cells (gray bars) treated with 0.25 μM STI-571 exhibited both a decrease in VEGF secretion and Bcr-Abl phosphorylation when compared to cells treated with vehicle control. Baf/*BCR-ABL*-r1 cells (black bars), normally maintained in 1 μM STI-571, showed both elevated VEGF secretion and Bcr-Abl phosphorylation when STI-571 was reduced to 0.25 μM or removed completely. Treatment of Baf/*BCR-ABL*-r1 cells with 2 μM STI-571 led to both a decrease in VEGF secretion and in Bcr-Abl phosphorylation compared to control. VEGF secretion data are two experiments performed in triplicate. Immunoblots and densitometry were taken from one representative experiment. (Error bars, SD; °, $P < 0.05$ when compared to VEGF levels of untreated Baf/*BCR-ABL*-s cells; *, $P < 0.05$ when compared to VEGF levels of Baf/*BCR-ABL*-r1 cells maintained in 1 μM STI-571.)

inhibitors such as Ras farnesyltransferase inhibitors (5), EGF receptor blocking drugs (7, 8), or ErbB-2/Neu inhibitory drugs, *e.g.*, Herceptin/trastuzumab in epithelial cell malignancies (25, 26). Subsequent studies in mice have provided evidence that these *in vitro* results could be recapitulated *in vivo*, suggesting that such drugs may indeed have “collateral” antiangiogenic effects, in addition to direct inhibitory tumor cell properties (7, 8, 26). Whether this is also the case for STI-571 and CML *in vivo* remains to be established.

It is important to note that in STI-571-sensitive tumors other than CML, this tyrosine kinase inhibitor may suppress angiogenesis by other mechanisms. Because the drug targets platelet-derived growth factor receptor (PDGFR) activity, it may also

block the recruitment of pericytes, which express high levels of this receptor, potentially interfering with vessel maturation during the angiogenic process (27). Second, STI-571-mediated blockage of PDGFRs, expressed by certain types of tumors, *e.g.*, gliomas (28–31), or rare cases of *BCR-ABL*-negative myeloproliferative disorders with PDGFRB fusion genes (32, 33), could potentially alter the production of proangiogenic stimulators, such as VEGF, and/or perhaps cause the up-regulation of endogenous inhibitors such as TSP-1. Similarly, given the ability of STI-571 to interfere with signaling through the Kit receptor tyrosine kinase, treatment of tumors expressing mutant Kit, *e.g.*, gastrointestinal tumors (GIST), with STI-571 could result in similar changes in their angiogenic regulatory molecule profile, consequently inhibiting GIST angiogenesis.

With respect to CML and GIST, it is probable that the major effect of STI-571 treatment is direct tumor cell killing and/or inhibition of cellular proliferation. Nevertheless, our results raise the possibility that an indirect mechanism involving inhibition of angiogenesis may exist. In this regard, we found that cytostatic drug concentrations, *e.g.*, 0.25 μM STI-571 when testing K562 cells, did not cause any detectable change in cell viability, but nevertheless could still effectively reduce VEGF expression.

An additional, important point when evaluating the possibility that Bcr-Abl can act as a mediator of VEGF secretion in CML is that a major microenvironmental (epigenetic) factor known to mediate VEGF expression in solid tumors—hypoxia—would presumably be irrelevant in the context of liquid hematologic malignancies such as CML. In this regard, our results showing that cytokines/growth factors could augment expression of VEGF may represent an alternative situation where epigenetic changes can cooperate with tumor cell oncogenic alterations to drive tumor angiogenesis in a maximal fashion (2).

Of interest as well was our finding that *BCR-ABL* gene amplification in STI-571-resistant Baf/*BCR-ABL*-r1 cells was associated with elevated VEGF secretion, but only when STI-571 was reduced or removed entirely from the growth media—further implicating a causal connection between Bcr-Abl phosphorylation (which is increased when STI-571 is removed) and the secretion of VEGF. In addition, the possibility that resistance to STI-571 via changes in *BCR-ABL* (*i.e.*, amplification or mutation) may lead to changes in the angiogenic properties of CML cells—a concept analogous to our previous work showing that acquired resistance to C225 resulted in elevated VEGF secretion (25)—may provide a rationale for combining STI-571 with specific antiangiogenic agents in patients who have become less responsive to STI-571 treatment.

In summary, our results implicate the *BCR-ABL* oncogene as a possible inducer of angiogenesis in CML through the induction of VEGF expression, and suggest the possibility that STI-571 may function to block CML growth, at least in part, by blockade of the oncoprotein-driven angiogenesis. *In vivo* experiments to examine this hypothesis are currently underway.

Materials and Methods

Cell Lines

K562 CML cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The murine

myeloid cell line 32D is dependent on IL-3 for growth and proliferation (34, 35); cells were routinely cultured in RPMI 1640 supplemented with 10% FCS and supplemented with 10% WEHI-3B-conditioned media as a source of IL-3. For experiments, purified murine IL-3 (Sigma, Canada Ltd., Oakville, Ontario) was used at a concentration of 10 ng/ml in place of WEHI media. MO7e is a human megakaryocytic leukemia cell line requiring GM-CSF for proliferation (36); cells were cultured in RPMI 1640 supplemented with 10% FCS and 5 ng/ml GM-CSF (Sigma). 32D and MO7e cells, both parental and p210 transfectants, were a kind gift from Dr. Brian Druker (Oregon Health Sciences University, Portland, OR). Baf/*BCR-ABL-r1* cells, a murine pro-B lymphocyte cell line expressing amplified *BCR-ABL* and resistant to 1 μ M STI-571, were published elsewhere (21). K562 and all p210-transfected cells were maintained in RPMI 1640 supplemented with 10% FCS. HUVECs were maintained as previously described (37). Cell cultures were incubated at 37°C and 5% CO₂ in a humidified incubator.

Reagents

STI-571 was provided by Novartis Inc., Basel, Switzerland. A stock solution of 10 mM was prepared by dissolving the compound in DMSO and was stored at -20°C.

Antibodies

Commercially available antibodies were used as follows: anti-Bcr antibody (α Bcr) (Oncogene Inc., San Diego, CA), anti-phospho-VEGF-Receptor-2/KDR (Tyr951) antibody (α pVEGF-R2) (New England Biolabs, Beverly, MA), anti-KDR antibody (α VEGF-R2) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphoserine 473 PKB/AKT (α PKB) (New England Biolabs), anti-PKB/AKT (α PKB) (New England Biolabs), and anti-phosphotyrosine antibody 4G10 (α pY). Secondary antibodies were anti-rabbit IgG HRP and anti-mouse IgG HRP (Promega, Madison, WI).

Immunoprecipitation and Western Blotting

Cells were placed in lysis buffer [20 mM Tris (pH 7.5), 137 mM NaCl, 100 mM NaF, 10% glycerol, 1% NP40, 1 mM Na₂VO₄, and supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin] for 30 min at 4°C and then centrifuged at 15,000 rpm for 10 min. Cell lysates were quantified using Bradford reagent (Bio-Rad, Hercules, CA) and standardized using known amounts of BSA (Life Technologies, Inc., New York, NY). Coimmunoprecipitation was carried out as described previously (38) using 1 mg of total protein precipitated with 2 μ g of antibody and recovered with protein A-Sepharose beads (Sigma). Eluted proteins were resolved on 7.5% SDS-PAGE gels and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) by electrophoretic transfer. Before immunoblotting, membranes were blocked in either 5% nonfat milk in TBST buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20] or 5% BSA in TBST (for phosphorylation blotting). Immobilized proteins were subjected to immunoblot analysis after extensive washings and membranes were developed using a chemiluminescent reaction [enhanced chemiluminescence (ECL), Amersham Corporation, Piscataway, NJ].

Densitometric Analysis

Immunoblot exposures were subjected to densitometric analysis (Personal Densitometry, SI; Molecular Dynamics, Sunnyville, CA) and quantified using Image QuantNT ver.4.2a (Molecular Dynamics). For all figures, densitometric quantification represents a ratio of phosphorylated protein to total protein with graphs comparing these ratios. To ensure that quantification was performed in a linear range, at least three immunoblot exposures of varying intensity were analyzed with only nonsaturated blots subjected to quantification.

Proliferation Assays

The effect of STI-571 on cellular proliferation in K562 cells was determined by [³H]thymidine incorporation. Cells were plated in 96-well plates and incubated for 24–48 h in the presence of STI-571 or DMSO vehicle control. [³H]thymidine (2 μ Ci/well) was added for the last 4–5 h of incubation. Radioisotope incorporation was quantified using a scintillation counter. For all experiments, pooled data represent an average of at least 15 samples.

Measurement of Human and Mouse VEGF Protein Level in Conditioned Media

Cells were incubated for 24 or 48 h in the presence of STI-571 or DMSO vehicle control, after which conditioned media were collected and cellular debris removed by centrifugation. Samples were kept at -70°C until VEGF quantification. Cell number was determined immediately after media recovery by trypan blue exclusion using a hemocytometer and used to normalize for VEGF secretion levels. Levels of secreted VEGF were assessed using a commercially available VEGF ELISA kit (R&D Systems, Inc., Minneapolis, MN) following manufacturer's instructions.

Endothelial Cell Stimulation

To assess both VEGF-R2/KDR and PKB/Akt phosphorylation, HUVECs were stimulated with conditioned media from 32D (parental and p210-transfected cells) for 10 min using recombinant human VEGF (50 ng/ml) as a positive control.

Statistical Analysis

The results (means \pm SD) of VEGF secretion in p210 transfectants and cells treated with STI-571 were subjected to statistical analysis by ANOVA, followed by the Student-Newman-Keuls test using the GraphPad Prism software package v.30 (GraphPad Software Inc., San Diego, CA). The level of significance was set at $P < 0.05$.

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