Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways

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Besides being expressed on endothelial cells, vascular endothelial growth factor receptors (VEGFRs) are also functional on subsets of leukemias, resulting in autocrine loops that sustain leukemia migration and proliferation. While recent evidence suggests that VEGF supports hematopoietic stem cell survival via an internal loop, the molecular mechanisms whereby autocrine stimulation of VEGFR-2 (KDR) promotes leukemia growth are not well understood. Here we show on acute myeloid primary leukemias and cell lines that VEGF/KDR autocrine loops operate both internally and externally. First, we demonstrate that KDR is constitutively phosphorylated and located at the nucleus of VEGF-producing leukemias. Treatment with anti-VEGF antibody, which acts externally, blocked KDR nuclear translocation and inhibited nuclear factor κ B (NF-κB; p65 and c-rel) activation. In contrast, a KDR-specific intracellular inhibitor failed to block KDR nuclear translocation, but inhibited the constitutive activation of mitogen activated protein kinase (MAPK)/Erk and the phosphatidylinositol 3-kinase/AKT pathways. Notably, treatment with the anti-VEGF antibody alone had little effect on cell survival, while the internal inhibitor induced leukemia apoptosis, and the 2 drugs produced synergistic effects, together and with chemotherapy, reducing cell survival to a larger extent than either agent alone. Our results demonstrate that internal and external VEGF/KDR autocrine loops regulate leukemia survival via different mechanisms, and suggest that blocking both may have therapeutic potential. (Blood. 2004;103:3883-3889)

Introduction

Vascular endothelial growth factor (VEGF) and its receptors, namely VEGF receptor 2 (KDR) or VEGF receptor 1 (FLT-1), play a crucial role in neovascularization (angiogenesis).[1,2] On endothelial cells (ECs), in response to VEGF stimulation, KDR has been shown to transmit intracellular signals leading to cell proliferation and survival (reviewed in Petrova et al[3]). The signaling pathways activated by VEGF on ECs, downstream of KDR, linked to these particular functions, include several signaling pathways, namely the mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI 3-K) cascades.[4] Besides being expressed on ECs, KDR is also present on subsets of tumor cells such as acute leukemias, where it functions in an autocrine manner, by leukemia-derived VEGF.[5-7] The presence of functional VEGF receptors (VEGFRs) on malignant cells, transmitting signals similar to those on ECs, suggested that targeting such receptor tyrosine kinases, either through the use of neutralizing antibodies[8] or kinase inhibitors,[9] could have clinical potential, blocking both angiogenesis and autocrine VEGF/KDR stimulation of tumor growth.[7] However, the mechanisms whereby such tyrosine kinase receptors, activated in an autocrine manner, protect malignant cells from apoptosis, and promote proliferation, are still not understood.

In addition to acting in a paracrine manner on ECs and in an autocrine manner on malignant cells, VEGF/VEGFRs also act on hematopoietic stem cells (HSCs).[10] In these cells, internal VEGF/VEGFR autocrine loops regulate survival and proliferation.[10]

In the present report we investigated how autocrine VEGF stimulation of KDR-positive leukemia cells promotes their survival. To answer this question, we hypothesized that the regulation of KDR localization might play a role in the process, and asked how external and internal VEGF autocrine loops regulated KDR localization and activation of downstream signaling pathways. Using acute myeloid leukemia cell lines and primary leukemias as a model of autocrine VEGF stimulation, we clearly demonstrate that KDR is constitutively phosphorylated and predominantly nuclear on these cells. Its localization is inhibited by an antibody against secreted VEGF (that acts only externally) and, to a lesser extent, by an internal KDR inhibitor. We demonstrate further that the external blocker affects specifically the nuclear factor κB (NF-κB) pathway, while the internal inhibitor affects the MAPK/Erk and the PI 3-K/AKT pathways, and to a lesser extent also NF-κB. Notably, the 2 agents exert distinct effects on cell survival, as shown by the minor effect of the external antibody at inducing leukemia apoptosis, compared with the strong proapoptotic effect of the internal inhibitor. The combination of both agents, or either drug with chemotherapy, resulted in synergistic proapoptotic effects.

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The results shown here reveal novel aspects of VEGF/VEGFR biology, particularly in what concerns autocrine loops on malignant cells, and may help explain the mechanism whereby tyrosine kinase receptors such as KDR promote cell survival and growth.

Materials and methods

All reagents were obtained from Sigma (St Louis, MO), unless indicated otherwise. The KDR-specific inhibitor was obtained from Calbiochem (Calbiochem/Oncogene Research Products, catalog number 676 480, San Diego, CA). The VEGF neutralizing monoclonal antibody (Ab) 4.6.1 was kindly provided by Genentech (South San Francisco, CA).

Cell culture

Two acute myeloid leukemia cell lines, HEL and HL-60 (both previously described as KDR positive[6]), were used as cellular models in most experiments. In addition, we studied 20 primary acute myeloid leukemia cells (obtained from the peripheral blood of patients). Of these, 3 samples were studied in detail (the 3 samples correspond to M1, M2, and M3 leukemia subtypes) and are shown in the present manuscript. All cells were cultured in complete RPMI medium (Gibco BRL, Grand Island, NY), following standard protocols. In the experiments aimed at characterizing the signaling pathways activated by internal or external KDR loops, cells were cultured in serum-free RPMI and exposed to either the antibody 4.6.1 (which acts externally; used at 100 nM) or the KDR inhibitor (an internal inhibitor; used at 70 nM) mentioned in the previous paragraph. In the case of the AKT pathway, cells were incubated in parallel with the inhibitor Ly294002 (50 μM), to allow identification of the phosphorylated forms of AKT. To detect MAPK activation, U0126 (used at 10 μM) was used as the specific inhibitor.

Subcellular protein extraction, immunoprecipitation, and Western blotting

Cytoplasmic extracts were prepared by suspending cell pellets in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.9, 10 mM KCl, 1 mM EDTA [ethylenediaminetetraacetic acid], 0,2% nonidet 40 (NP40), 10% glycerol, 1 mM dithiothreitol (DTT), supplemented with protease and phosphatase inhibitors, and then centrifuged for 2 minutes at 4°C and 12 000 rpm. To obtain nuclear extracts, the pellets resulting from the cytoplasmic extraction were treated with a buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 10 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, and supplemented with protease and phosphatase inhibitors. After 30 minutes on ice, lysates were centrifuged for 5 minutes at 4°C and 3790*g*. Equal protein amounts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were incubated with antibodies raised against KDR, ERK, P-ERK, AKT, p65, p50, c-rel, and phosphotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA). Cytoplasmic and nuclear extracts were used in immunoprecipitation experiments. Lysates were precleared with protein G–Sepharose, supernatants were then incubated with the antiphosphotyrosine antibody for one hour at 4°C and finally incubated with the protein G–Sepharose beads for an additional hour at 4°C. Beads were washed once in lysis buffer containing 500 mM NaCl and twice in lysis buffer containing 150 mM NaCl. Beads were ressuspended in SDS loading buffer before electrophoresis.

Immunofluorescence, confocal microscopy

The different leukemia cells were spun onto glass microscope slides, following standard culture conditions, or the treatment procedures. The cells were fixed in 4% (vol/vol) formaldehyde/phosphate-buffered saline (PBS) for 10 minutes at 4°C and washed in PBS. After permeabilization with 0.1% (vol/vol) Triton X-100 plus 5% (vol/vol) normal serum, the cells were incubated with the primary antibodies for 12 hours at 4°C (rabbit antihuman KDR or FLT-1; Santa Cruz Biotechnology). Then, the cells were washed and incubated with secondary antibodies (Alexa fluor 596; Molecular Probes, Eugene, OR) for 1 hour at room temperature. The samples were mounted in Vectashield and analyzed by fluorescence microscopy (Axioplan Microscope, Zeiss, Germany) and by confocal microscopy. Regarding the latter, sets of optical sections with 0.3-mm intervals along the z-axis were obtained from the bottom to the top of cells using a laser scanning confocal microscope (True Confocal Scanner Leica TCS SP2; Leica Microsystems, Heidelberg, Germany); objectives HCX PL APOCS 63 × 1.4 oil. The relative powers of the laser lines were set in order to have the identical light intensity for the same sample. This was performed using the fieldmaster (Zeiss, Herts, United Kingdom) with the head LM2. Acquisition and image treatment were performed with LSC software (Leica Microsystems). The experiments involving immunofluorescence or confocal microscopy were done using cells from different passage numbers, and repeated at least 3 times.

Electrophoretic mobility shift assay (EMSA)

For EMSAs, 10 μg nuclear extracts were incubated in a buffer containing 10 mM HEPES, 4% Ficoll, 70 nM NaCl, 2 mM DTT, 100 μg/mL bovine serum albumin (BSA), and 0.01% NP40 with ^{32}P-labeled NF-κB probe (sequence: AGTTGAGGGGACTTTCCCAGG). EMSAs were performed following standard methodology. In the case of the supershift experiments, cell extracts were incubated with NF-κB subunit-specific antibodies, to allow identification of the different subunits involved.

Fluorescence activated cell sorting (FACS) analysis

The cells were fixed in 4% paraformaldehyde (PFA) and permeabilized in 90% methanol for 30 minutes at 4°C. Different experimental conditions were tested, as described in "Cell culture": cells alone; cultured in the presence of the MAP/ERK inhibitor, U0126 (used at 10 μM); with PI 3-K inhibitor Ly294 002 (50 μM); in the presence of the external blocker Ab 4.6.1 (100 nM); or treated with the internal KDR inhibitor (70 nM).

After incubation with PBS 1×, 0.1% BSA for 10 minutes at room temperature, the primary antibodies (P-ERK; P-AKT) were added for 30 minutes at room temperature. Then, the cells were washed and incubated with the secondary antibodies (Alexa Fluor 488 or 594) for 30 minutes at room temperature. Finally, the cells were washed and analyzed by flow cytometry.

Apoptosis determination

HEL cells were incubated in serum-free medium in the presence or absence of 1 mM etoposide, 70 nM KDR inhibitor, and/or 100 nM Ab 4.6.1, at different experimental times. Cells were washed once in incubation buffer (10 mM Hepes, pH 7.4; 140 mM NaCl, 5 mM $CaCl_2$) and resuspended in incubation buffer plus 0.5 mg/mL propidium iodide (PI; Sigma) and annexin V–fluos (Boehringer Mannheim, Mannheim, Germany) at room temperature for 30 minutes. The percentage of early apoptotic cells (annexin-positive, PI-negative) and late apoptotic cells (annexin- and PI-positive) was determined by flow cytometry (Becton Dickinson, San Jose, CA). Results are shown as the percentage of viable cells (annexin- and PI-negative) at the different experimental times. For statistical analysis, these data were treated with a Student *t* test. A significance interval of *P* value less than .05 was considered significant.

Results

In order to determine how autocrine VEGF stimulation promotes the survival of KDR-positive leukemias, we first hypothesized KDR intracellular localization might be regulated by endogenous VEGF.

As shown by Western blotting, immunofluorescence, and confocal microscopy, untreated, culture-derived HEL cells have a predominantly nuclear KDR expression (Figure 1A-C). In

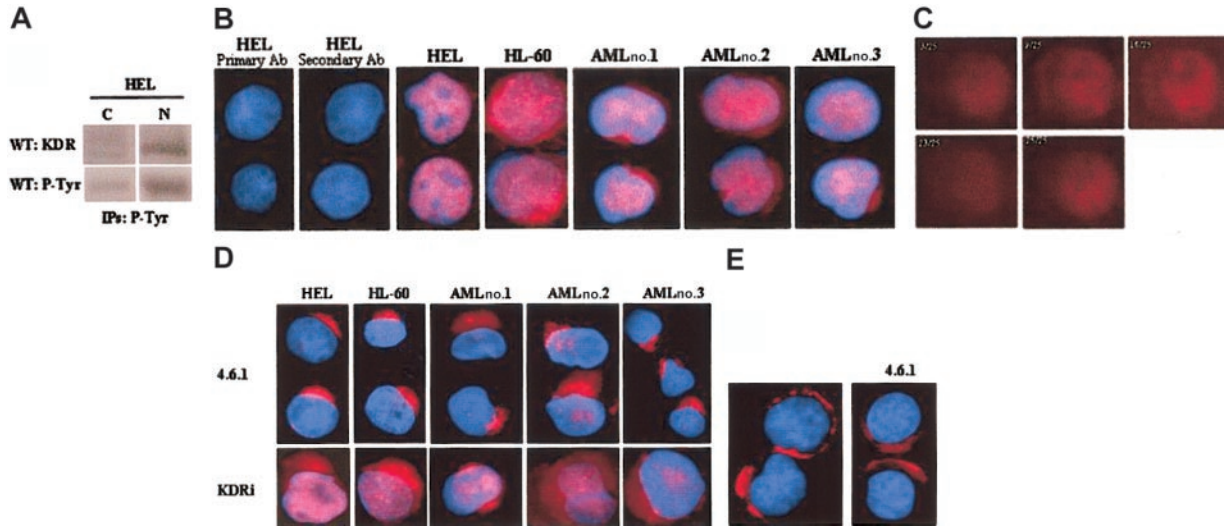

Figure 1. Nuclear KDR expression in primary acute myeloid leukemias and cell lines. (A) Western blot analysis of cytosolic (C) and nuclear (N) protein extracts from HEL, immunoprecipated with phosphotyrosine Abs, and stained against KDR and phosphotyrosine. Note the intense KDR expression, in its phosphorylated form, in nuclear protein extracts of untreated HEL cells. These results are representative of 3 independent experiments. (B) Immunofluorescence analysis of HEL, HL-60 and 3 primary leukemias in serum free medium. These cells were stained for KDR (PE) and DNA (nucleus) (DAPI [4′,6-diamidino-2-phenylindole]). Note the nuclear expression of KDR in all samples shown. This result is seen in most (95%) cells in each experiment, and was obtained at least 3 times. Negative controls are included (primary Ab alone and secondary Ab alone). Original magnification, × 630. (C) Confocal microscopy analysis, demonstrating KDR is predominantly located to the nucleus of HEL cells: intermediate sections, corresponding to the nucleus, have intense staining compared with the most superficial (cytosolic) sections. Confocal microscopy was performed as described in "Materials and methods." Results were obtained at least 3 times in independent experiments. (D) Immunofluorescence analysis of HEL, HL-60, and 2 primary leukemias cultured in the presence of the external blocker Ab 4.6.1 or the internal KDR inhibitor for 12 hours. Cells treated with the external blocker demonstrated a clear shift in KDR (PE) localization, now seen at the cell surface. In contrast, the internal inhibitor has a minor effect in KDR localization (clear nuclear staining is still seen) in all samples. Nuclear staining is shown in blue (DAPI). As above, this result is seen in most (90%) cells in each condition. Original magnification, × 630. (E) Immunofluorescence analysis of HEL cells in culture, stained for FLT-1 (PE) and nuclear staining (DAPI). Note the expression of FLT-1, seen only at the cell surface (cytosol and membrane), even when cells are treated with the external VEGF blocker Ab 4.6.1. Results are representative of at least 95% of cells in culture, and were obtained in 3 independent experiments. Original magnification, × 630.

addition, phosphorylated KDR was detected predominantly in the nuclear protein fractions of untreated HEL cells, suggesting the autocrine loop might result in KDR activation (Figure 1A). Similar results regarding KDR localization were obtained studying the HL-60 cell line (immunofluorescence staining demonstrating constitutive nuclear KDR is shown in Figure 1B). These results were observed also in 84% (16 of 19) of KDR-positive AML cells analyzed (constitutive nuclear expression in 3 representative AML samples is shown in Figure 1B), corresponding to those leukemias where evidence for autocrine VEGF/KDR loops have been previously described.[6,7]

Next, we asked whether nuclear KDR was in fact the result of autocrine VEGF stimulation. Treatment of the 2 cell lines and primary leukemias with the VEGF neutralizing monoclonal antibody (4.6.1 Ab) decreased KDR nuclear expression, shifting it to the cell surface (immunofluorescence staining for KDR is shown in Figure 1D). Since the VEGF neutralizing antibody used in this study acts only externally, these experiments show that autocrine stimulation of leukemia cells involved the external release of VEGF and its subsequent binding to KDR on the cell surface, leading to its constitutive nuclear localization. The results obtained by immunofluorescence were confirmed by Western blotting of nuclear and cytosolic protein fractions (data not shown).

Next, we asked whether an internal inhibitor of KDR might also regulate receptor localization and activity. To answer this question, we treated the different leukemia cells with a KDR-specific inhibitor (that acts internally), and analyzed KDR localization by Western blotting and immunofluorescence. As shown in Figure 1D, KDR nuclear localization is also reduced by treatment with the internal KDR-specific inhibitor, although the effect is not as striking as with the external VEGF neutralizing antibody. Taken together, these results suggest the external and internal VEGF/KDR autocrine loops regulate the localization of KDR, the former having a stronger effect.

Having demonstrated that KDR internal and external autocrine loops operate on leukemia cells, we asked whether this was a general phenomenon, and whether the other VEGFR present on these cells, FLT-1, could be regulated in a similar fashion. In contrast to what was seen for KDR, FLT-1 expression on HEL cells remains largely cytosolic, in all experimental conditions tested (FLT-1 staining of HEL cells untreated or treated with Ab 4.6.1 is shown in Figure 1E). This was confirmed in the remaining cell line and primary samples tested (data not shown).

Signaling pathways activated by autocrine VEGF

We took advantage of the VEGF neutralizing antibody 4.6.1 (which acts only externally) and of the KDR inhibitor (internal) shown in Figure 1 to regulate KDR localization and activation, respectively, and used them to dissect the signaling pathways activated by autocrine VEGF on leukemia cells.

Since VEGF stimulation of KDR-positive endothelial cells was shown to activate the PI 3-K[11] and the MAPK pathways,[12,13] we decided to investigate a putative role for these pathways in regulating the survival of KDR-positive leukemias, in the context of autocrine VEGF stimulation of KDR-positive leukemias.

Cells treated with the internal KDR inhibitor showed a clear decrease in phosphorylated Erk 1/2 (Figure 2A) and AKT (Figure 2B) levels, this effect being particularly evident in nuclear protein fractions. Total Erk 1/2 levels remained unchanged throughout. In contrast, treatment with the external VEGF blocker Ab 4.6.1 had little effect on either the MAPK or the AKT pathways. As shown in Figure 2A-B, the levels of phosphorylated Erk 1/2, in nuclear or cytosolic protein fractions, showed little variation after HEL cell

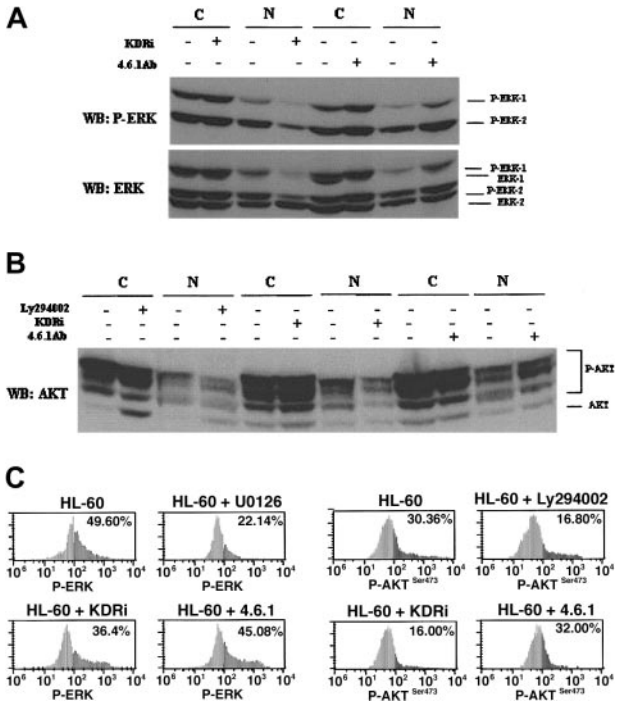

Figure 2. Distinct signaling pathways are activated by internal and external VEGF-KDR autocrine loops: MAPK and PI 3-K. Cells were left untreated or exposed to the internal KDR inhibitor (KDRi) or the VEGF neutralizing antibody 4.6.1 as described in "Materials and methods." (A) Nuclear (N) and cytosolic (C) extracts from HEL cells, probed for total Erk or phosphorylated Erk 1/2. Note the reduction of Erk phosphorylation when cells are treated with the internal KDR inhibitor, while the external blocker 4.6.1 Ab has little effect. These experiments were repeated at least 3 times. (B) Nuclear (N) and cytosolic (C) protein extracts from HEL cells, treated with internal KDR inhibitor, the external blocker 4.6.1 Ab, or the PI 3-K inhibitor LY294 402, and probed for AKT and phosphorylated AKT. Note the decrease in phosphorylated AKT in cells treated with the internal KDR inhibitor, while the external Ab has little effect. These experiments are representative of 3 independent experiments. (C) FACS analysis of HL-60 cells either untreated or treated with Erk inhibitor (U0126), the PI 3-K inhibitor (LY294 402), the internal KDR inhibitor, or the external VEGF blocker Ab 4.6.1; the cells in different conditions were stained against phosphorylated Erk 1/2 or AKT, and analyzed by FACS. The percentage of positive cells was obtained by gating viable events and comparing the staining profile with an irrelevant Ab isotype control (not shown). Note the effect of the internal KDR inhibitor at reducing the levels of phosphorylated Erk in all cells tested, while the external VEGF blocker Ab 4.6.1 has little effect. Percentages indicate the proportion of cells that are stained with Ab against P-ERK or P-AKT. The results shown are representative of 3 independent experiments, and were repeated at least 3 times.

treatment with the external blocker. These results were confirmed by FACS staining of HEL cells, and were also seen on HL-60 cells and 2 primary leukemias (Figure 2C and Table 1). As shown by FACS staining against phosphorylated proteins, the levels of P-Erk 1/2 and P-AKT decreased after treatment with the KDR internal inhibitor, while the external VEGF blocker, Ab 4.6.1, showed little effect (results for the HL-60 cell line are shown in Figure 2C; Table 1 summarizes the results obtained for HEL and the primary leukemia samples).

These results show primarily that external and internal VEGF/KDR loops act via distinct mechanisms. In addition, they also demonstrate that the blockade of internal VEGF signaling results in decreased constitutive activation of MAPK and AKT kinase pathways.

Next, we investigated the effects of blocking the internal or external VEGF loop on the NF-κB pathway. As shown in Figure 3A-B, treatment of HEL or HL-60 cells with the external VEGF blocker reduced the levels of NF-κB (results show a decrease in subunit p65 and, most evidently, c-rel). In contrast, p50 levels remained unchanged throughout the experimental setup (Figure 3A-B). Similarly, NF-κB binding to DNA was also clearly down-regulated by 4.6.1 Ab (Figure 3C, bandshift results shown). Conversely, the internal KDR inhibitor also decreased p65 subunit levels (Western blot results are shown, Figure 3A), but had little effect at the protein level of the c-rel subunit (Figure 3A-B) and in the binding of NF-κB to DNA (Figure 3C). In conclusion, besides exerting a stronger effect at blocking NF-κB than the internal KDR inhibitor, the external VEGF blocker Ab 4.6.1 produces a specific effect, by decreasing the c-rel subunit levels and activity. These results show further evidence of the different strategies produced by the internal and external inhibitors. Also noteworthy is the consistency in results between the 2 cell lines studied. In these experiments, the identities of the NF-κB subunits were confirmed by supershift, using subunit-specific antibodies (Figure 3D).

In order to determine whether the reduction in p65 or c-rel levels, seen after neutralization of VEGF by the external blocker or, to a lesser extent, the internal inhibitor of KDR, were mediated via the proteosome pathway, we coincubated HEL cells with Ab 4.6.1 together with LLNL (an inhibitor of the proteosome pathway). Cotreatment of HEL cells with LLNL and 4.6.1 Ab resulted in a minor recovery in p65 cytosolic levels, while its nuclear levels remained unchanged (data not shown). This result suggests that the decrease in p65 levels upon cell exposure to agents that neutralize the VEGF autocrine loop and reduce nuclear KDR is at least partially mediated via the proteosome pathway.

Internal and external VEGF/KDR autocrine loops regulate leukemia survival

Both NF-κB and PI 3-K signaling pathways have been linked to cell survival and antiapoptotic mechanisms.[14-17] Hypothesizing that nuclear KDR, as maintained by internal and external VEGF/KDR autocrine loops, might play an important role in promoting leukemia survival, we followed a similar approach to the one described in Figures 1 and 2, by treating HEL cells with the external VEGF blocker Ab 4.6.1 or the internal KDR inhibitor, and determining their effects on cell survival. Cells treated with either agent undergo death by apoptosis, as determined by annexin V

Table 1. FACS results of HEL cells and 2 primary leukemias, untreated or treated with pathway-specific inhibitors, the external VEGF blocker Ab 4.6.1 or the internal KDR inhibitor, and stained against phosphorylated Erk 1/2 or AKT

Cell line	Treatment	P-ERK, %	P-AKT, %
HEL			
	Untreated	43.72	31.22
	KDRi	24.22	12.50
	4.6.1	42.00	30.94
	U0126	12.78	ND
	Ly294002	ND	10.18
AML no. 1			
	Untreated	51.12	15.09
	KDRi	35.78	0
	4.6.1	52.40	16.07
	U0126	20.17	ND
	Ly294002	ND	0
AML no. 3			
	Untreated	32.00	21.00
	KDRi	18.99	4.50
	4.6.1	34.00	22.08
	U0126	2.26	ND
	Ly294002	ND	7.00

Note the effect of the internal KDR inhibitor at reducing the percentage of cells with phosphorylated Erk 1/2 or AKT, whereas the external VEGF blocker has little effect. This result is consistent with the Western blot results (in the case of the HEL cell line), and in the different leukemias analyzed. The results shown are representative of 3 independent experiments.

ND indicates not detected.

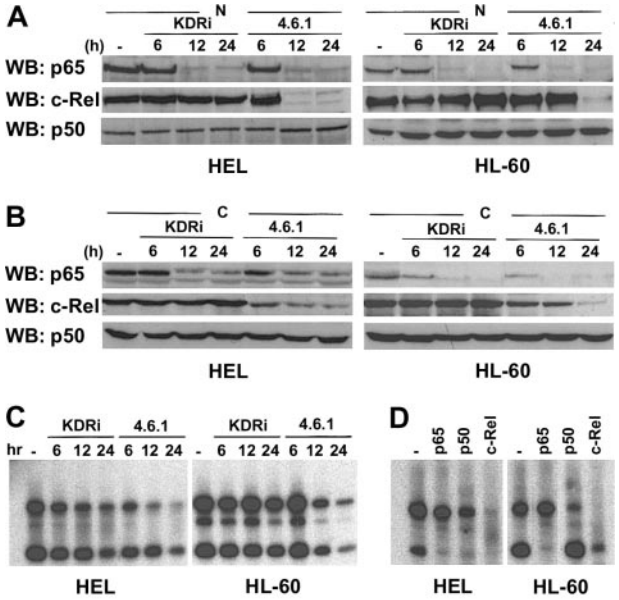

Figure 3. Distinct signaling pathways are activated by internal and external VEGF-KDR autocrine loops: NF-κB. (A) Nuclear (N) protein extracts from HEL and HL-60 cells, untreated or treated with the external VEGF blocker Ab 4.6.1 or the internal KDR inhibitor (KDRi) for 24 hours, and probed using Western blotting against the p65 and p50 NF-κB subunits. Note the decrease in nuclear p65 and c-rel levels in cells treated with the Ab 4.6.1, while those exposed to KDRi show a clear decrease in p65 levels but sustained c-rel expression. The results are consistent for the 2 cell lines. These results are representative of 3 independent experiments, and were repeated at least 3 times. (B) Cytosolic (C) protein extracts from HEL and HL-60 cells, untreated or treated with the external VEGF blocker Ab 4.6.1 or the internal KDR inhibitor (KDRi) for 24 hours, and probed using Western blotting against the p65 and p50 NF-κB subunits. Note the decrease in cytosolic p65 and c-rel levels in cells treated with the Ab 4.6.1, while those exposed to KDRi also show a clear decrease in p65 levels but sustained c-rel expression. p50 subunit levels did not change in any of the conditions tested, and in either cell line. All the results were consistent for the 2 cell lines. These results are representative of 3 independent experiments, and were repeated at least 3 times. (C) Note the decrease in DNA (NF-κB) binding activity of nuclear protein extracts from HEL or HL-60 cells treated with the 4.6.1 Ab, while those treated with KDRi show little effect (after 12 or 24 hours). The results shown are representative of 3 independent experiments, and were repeated 3 times. (D) Supershift assay of nuclear protein extracts from HEL or HL-60 cells, coincubated with p65-, p50-, or c-rel–specific antibodies, to demonstrate the identity of the NF-κB subunits seen throughout the experiments described earlier.

staining (Figure 4A), although 4.6.1 Ab alone shows a weaker effect. More important is the fact that the 2 agents showed synergistic effects (Figure 4A). Cells treated with both the internal and external KDR/VEGF blocker undergo cell death by apoptosis significantly more than with either agent alone.

In addition, cells pre-exposed to either treatment are also more sensitive to the proapoptotic effects of chemotherapy (etoposide was used; Figure 4B, results obtained with the 4.6.1 Ab are shown), suggesting a synergistic effect between the 2 types of drugs, which may have therapeutic implications. Taken together, these results demonstrate that although internal and external VEGF/KDR autocrine loops exert their effects through distinct mechanisms, the blockade of either pathway induces leukemia apoptosis and renders them more sensitive to chemotherapy. Consequently, these data also suggest that autocrine KDR may promote apoptosis resistance and leukemia survival.

Discussion

VEGF and its receptors, namely VEGFR-2/KDR, have been implicated in neovascularization (angiogenesis) processes; their importance may be greater in the context of tumor angiogenesis, since most tumors are known to produce VEGF,[18] and activated (angiogenic) endothelium is believed to express increased levels of KDR.[19-22] As shown in numerous experimental models, tumor cell–derived VEGF acts in a paracrine manner on the endothelial cells, resulting in the expansion of the tumor vasculature and the growth support of the growing tumor mass.

Besides endothelial cells, subsets of tumor cells such as acute leukemias express functional KDR,[6,23,24] which supports tumor growth independently of angiogenesis, through autocrine stimulation by leukemia-produced VEGF. As a result, the growth of leukemia is regulated by both paracrine and autocrine VEGF loops, as demonstrated in preclinical models of the human disease.[7]

Autocrine VEGF/VEGFR loops are not unique to malignant cells, as shown by the recent data on their importance in regulating hematopoietic stem cell (HSC) survival.[10] The existence of "private" (internal) VEGF/KDR autocrine loops on such cells suggests these receptors may not be exclusively membrane bound, as previously thought. Relevant to the present study is the fact that external/paracrine VEGF stimulation of HSCs did not induce cell survival or proliferation, demonstrating the importance of "private" autocrine loops in this particular system.[10]

In the present study we demonstrate for the first time that as a result of autocrine VEGF stimulation, KDR is expressed not only at the cell surface of leukemia cells; rather, it is predominantly nuclear and constitutively activated (phosphorylated). This was observed and studied in detail in 2 cell lines and 3 primary (patient) samples, with consistent results. We demonstrate further that KDR nuclear accumulation is impeded by treating the leukemia cells with a neutralizing monoclonal antibody against secreted VEGF (which acts externally) and, to a lesser extent, by an internal KDR-specific inhibitor. The use of these agents suggested that, in contrast to what was shown for HSCs,[10] the VEGF/KDR autocrine loops that operate on leukemia act not only internally, but rather require also VEGF to be exported, and the subsequent internalization and nuclear accumulation of the receptor. This interaction of VEGF with its receptors, resulting in KDR nuclear accumulation, is mediated specifically via KDR, as shown by the minor effect

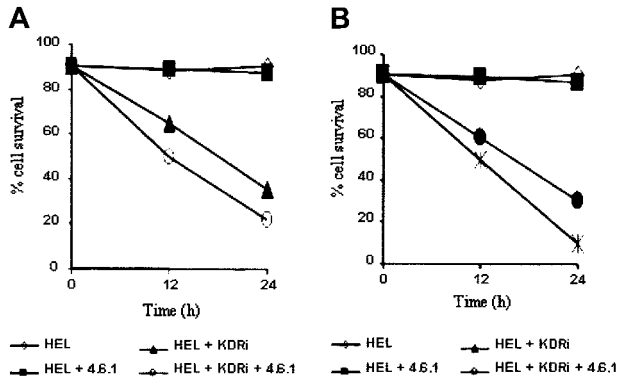

Figure 4. Blockade of internal VEGF-KDR autocrine loop induces leukemia apoptosis: synergistic effects with the external blocker and with chemotherapy. (A) HEL cells were left untreated (in serum-free conditions), treated with the Ab 4.6.1 or/and with the internal KDR inhibitor (KDRi), stained with annexin V and PI, and analyzed by FACS. Results show the percentage of viable cells (nonapoptotic, annexin V–negative) cells in the different conditions. As shown by the data, treatment with the internal KDRi has a clear effect at reducing cell viability, in contrast to those treated with the 4.6.1 Ab. Importantly, the 2 agents had synergistic effects and reduced cell viability significantly ($P < .05$) more than either agent alone. These results are representative of 3 independent experiments, and were repeated 3 times. (B) Combination of chemotherapy (etoposide) with the 4.6.1 Ab results in synergistic effects, decreasing cell visibility significantly ($P < .05$). Results are shown as the percentage of viable (annexin V–negative) cells. These results are representative of 3 independent experiments, and were repeated 3 times.

seen in cells treated with a monoclonal neutralizing antibody against FLT-1 (data not shown).

Taken together, these results may have fundamental clinical relevance, namely when trying to target secreted VEGF or internal KDR for therapeutic purposes.

The mechanisms of KDR transport and subsequent nuclear accumulation remain largely undefined, although we have preliminary evidence that suggests the PI 3-K pathway may be involved in this mechanism (S.C.R.S. and S.D., unpublished data, December 2003). Other membrane tyrosine kinase receptors are internalized after ligand binding, namely the EGF receptor.[25,26] In the EGF system, the internalization and subsequent nuclear accumulation of the receptor appears to be mediated by activation of different signaling pathways[26] and transport/motor proteins, although consensus as to which factors regulate its cellular localization is still lacking.[27] Regarding KDR, it was recently shown on endothelial cells that this receptor interacts with surface proteins, namely caveolin, and may be internalized upon VEGF binding.[28,29] In addition, a recent report also using endothelial cells as a model showed KDR expression is up-regulated[30] and locates to the nucleus of cells exposed to shear stress.[31] These observations remain to be confirmed in the leukemia system, since here we are dealing with cells under "autostimulation" (autocrine) VEGF/KDR loops. In addition, the precise role of the internal and external autocrine loops in regulating KDR transport and nuclear accumulation remain to be dissected. The experiments done with shear stress suggest the internalization and subsequent nuclear accumulation of KDR may identify activated endothelial cells, and perhaps be the result of a global prosurvival program. Once in the nucleus, whether KDR recruits other signaling partners or acts as a transcription factor itself is still under scrutiny. In addition, the functional role of nuclear KDR is also not known, although we have preliminary evidence suggesting it may regulate nuclear NF-κB levels and activity (S.C.R.S. and S.D., unpublished data, December 2003), suggesting its role in promoting cell survival.

Having described the importance of the external and internal VEGF/KDR autocrine loops in regulating KDR nuclear localization and activation, we next investigated the signaling pathways activated by one or the other process. For this, we used the VEGF neutralizing Ab 4.6.1, which acts externally, and a KDR-specific intracellular inhibitor, and demonstrated that the signaling pathways inhibited by one or the other agent differ. Cells treated with the internal KDR inhibitor reveal a lower phosphorylation rate of Erk 1/2 and AKT, identifying such pathways as downstream of phosphorylated KDR. This inhibitor, in turn, had a minor effect on NF-κB. In contrast, the antibody 4.6.1 had little or no effect on either the MAPK/ERK or the PI 3-K/AKT pathways, but clearly affected NF-κB (p65, rel) levels and DNA-binding activity. These results suggest the stimulation of external or internal VEGF/KDR autocrine loops may result in the activation of distinct signaling pathways. Moreover, given the crosstalk between the 2 VEGFRs, resulting for instance in the formation of heterodimers, the role of FLT-1 in the process of VEGF signaling pathway activation should also be considered. However, as mentioned earlier, we found no evidence that FLT-1 affected KDR localization on HEL cells (data not shown), and no evidence of FLT-1 activation of downstream signaling pathways on leukemia cells (data not shown, and Dias et al[32]). Taken together, the data presented here highlight a mechanism whereby internal and external VEGF/KDR autocrine loops, by regulating distinct signaling pathways, may lead to different cellular functions.

Proof of principle of the importance of autocrine VEGF/KDR loops in regulating leukemia survival was obtained by treating leukemia cells with the monoclonal antibody 4.6.1 or with the KDR tyrosine kinase inhibitor, and determining cell viability by annexin staining (which identifies apoptotic cells). Interestingly, the internal KDR tyrosine kinase inhibitor was more effective at inducing cell death by apoptosis than the external blocker, suggesting that the distinct blockade of signaling pathways may have different effects on cell viability. The striking proapoptotic result obtained with the internal blocker is somewhat surprising, given the established role for NF-κB (shown here to be strongly inhibited by the external blocker, the antibody 4.6.1) in mediating cell survival,[14,33] while treatment with the antibody had a minor effect in cell viability. Nevertheless, it could be argued that the KDR inhibitor, since it blocks the activation of several downstream signaling pathways, namely MAPK and PI 3-K/AKT (which are also involved in cell survival), may act differently from the external blocker, and also lead to cell death by apoptosis. Notably, the 2 agents used in combination exerted synergistic effects and decreased cell viability to a greater extent than either drug alone. This result awaits confirmation in vivo, but suggests that blocking both internal and external VEGF/VEGFR pathways may be essential to achieve clinical remission in subsets of acute myeloid leukemias.

Importantly, exposure of leukemia cells to either treatment also increased their sensitivity toward the effects of chemotherapy, as shown by an increase in apoptosis when cells are exposed to the external blocker prior to the cytostatic drug etoposide, compared to the effects of either drug alone. Importantly, the internal blocker exerted similar synergistic effects with etoposide (data not shown).

Taken together, the results shown here demonstrate the feasibility of blocking autocrine VEGF/KDR loops on malignant cells as a means of inducing cell apoptosis. Alone or in combination with cytostatic agents, anti-VEGF or anti-KDR approaches have clear therapeutic potential for the treatment of subsets of leukemia. A recent report on the potential of blocking KDR signaling for the treatment of subsets of leukemia showed a partial response in 7 of 43 patients[34]; this study used an internal KDR tyrosine kinase inhibitor, SU5416, as a therapeutic agent. Although this inhibitor is not specific for KDR, but rather has a broad spectrum of tyrosine kinase inhibition, the results are nevertheless encouraging. Whether the clinical responses and disease outcome would be the same having targeted both the internal and the external VEGF/KDR loops remains to be tested.

Ongoing studies are focusing on the regulation of KDR transport on malignant cells, its function once in the nucleus, and the mechanisms whereby distinct signaling pathways may be activated inside the cell, either through autocrine or paracrine VEGF stimulation.

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