CONSTITUTIVE AND STIMULATED PRODUCTION OF VEGF BY HUMAN MEGAKARYOBLASTIC CELL LINES: EFFECT ON PROLIFERATION AND SIGNALING PATHWAY

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Release of vascular endothelial growth factor (VEGF) and other candidate angiogenic factors such as basic fibroblast growth factor and transforming growth factor β, may play a role in sustaining neoplastic cell proliferation and tumor growth. We evaluated VEGF expression and synthesis in the two erythro-megakaryocytic cell lines B1647, HEL and one megakaryocytic cell line MO7 expressing erythroid markers. In this study RT-PCR was performed to evaluate VEGF expression and that of its receptor KDR; VEGF production was assayed by Elisa test and western blot analysis; sensitivity to VEGF was tested by thymidine incorporation. VEGF and its receptor KDR were expressed in B1647 and HEL, both as mRNAs and as proteins, while only KDR transcript was found in MO7 cells. Only B1647 and HEL cells showed a strong spontaneous proliferating activity. In fact, measurable amounts of VEGF were present in the unstimulated cell medium, thus suggesting an autocrine production of VEGF by B1647 and HEL cells, but not by MO7, which was inhibited in mRNA-silencing conditions. This production could not be further boosted by other growth factors, whereas it was inhibited by TGF-β1. Finally, analysis of Shc signal transduction proteins following stimulation with VEGF indicated that only p46 was tyrosine phosphorylated. These data indicate that leukemic cells may be capable of autocrine production of VEGF which, in turn, maintains cell proliferation, possibly mediated by Shc p46 phosphorylation.

Vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis and a stimulator of endothelial cell proliferation, differentiation and survival, is involved in the development of blood vessels during embryogenesis and promotes vascular permeability and migration of monocytes through endothelial tissue. It is a homodimeric glycoprotein with a structural similarity to Platelet-Derived Growth Factor (PDGF), and can appear in five isoforms, two bound to the cell surface or to the extracellular matrix (189 and 206) and three soluble (121, 145 and 165) forms that induce proliferation of endothelial cells and *in vivo* angiogenesis (1). Production of VEGF is regulated by growth factors such as Fibroblast Growth Factor 4, PDGF, Tumor Necrosis Factor-α ((TNF-α), Transforming Growth Factor-β (TGF-β)), Keratinocyte Growth Factor, Insuline Growth Factor, Interleukin 1β, Interleukin 6, Thrombopoietin (TPO) and Oncostatin M (2). VEGF binds to tyrosine-kinase receptors identified as VEGFR-1 (fms-like tyrosine kinase1-Flt1), VEGFR-2 (Fetal liver kinase1-Flk1/ Kinase Domain Insert Receptor-KDR), predominantly

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expressed in endothelial cells, and VEGFR-3. Angiogenesis may also be implicated in the pathogenesis of solid (3) and hematologic malignancies (4), since VEGF mRNA has been found to be over-expressed in solid tumors (5), and a role, albeit indirect, has been proposed for it in sustaining the proliferation of leukemic cells (6-7). It has been shown that megakaryocytic cell lines and human megakaryocytes express VEGF transcripts, and that constitutive secretion of VEGF by $CD41^+$ cells is further increased by IL-3 and TPO (8-9).

In this study, we tested the role of VEGF in sustaining leukemic cell growth, by evaluating the constitutive and stimulated synthesis of VEGF, as well as its role in modulating cell proliferation, in the B1647 (10), HEL (11) and MO7 (12) erythro-megakaryocytic cell lines. Lastly, the tyrosine phosphorylation of the signaling cascade Shc protein (a member of the c-Src family) was evaluated following stimulation with VEGF.

MATERIALS AND METHODS

Cell lines

The human erythro-megakaryocytic cell lines B1647 and HEL were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% human serum and 10% Fetal Calf Serum (FCS) respectively (all medium constituents were purchased from BioWhittaker, Walkersville, MD, USA). The human megakaryocytic factor-dependent cell line MO7 was maintained in IMDM supplemented with 5% FCS and 10 ng/mL IL3 (BioSurce, Camarillo, CA, USA). For starvation, cells were washed twice in PBS, resuspended in serum free medium (IMDM without serum or factors) and incubated for 24 hours.

To induce VEGF release, the starved cells were cultured in 24-well plates at a concentration of $1x10^6$ per mL of serum free medium in the presence or absence of the following growth factors: Erythropoietin (Epo, Roche, Monza, Italy) 2U/mL, TPO (R&D systems, Minneapolis, MN, USA) 20 ng/mL, fms-like tyrosine kinase 3 (Flt3, ICN Biomedicals, CA, USA) 100 ng/mL (alone and in combination with TPO), Hepatocyte Growth Factor (HGF, ICN Biomedicals, CA,USA) 20 ng/mL, TGF-β1 (R&D systems, Minneapolis, MN, USA) 5 ng/mL, and incubated for 48 hours at 37 °C, 5% CO_2 and 95% humidified air.

At the end of incubation supernatants were recovered by centrifugation at 1200 rpm for 10 min and frozen for Elisa analysis. Supernatants from non stimulated cells were concentrated five times by Amicon concentrator filter (Millipore Corporation, Bedford, MA, USA) and immunoprecipitated with an antiserum recognizing VEGF (Sigma, Saint Louis, MO, USA) so that the protein released could also be detected by western blot analysis.

Immunoprecipitation and western blot analysis

Starved cells ($10 x10^6$) were incubated with or without VEGF (Sigma) 150 ng/mL at 37 °C for 5, 10, 15, 30 min, extracted by cold lysis buffer (50mM TrisHCl pH 7.5, 1mM EDTA pH 8 and 150mM NaCl) plus1% Triton X-100 and a mixture of protease inhibitors (1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 0.15 units/mL aprotinin, 1 μg/mL pepstatin A) for 20 min at 4 °C, and centrifuged at 13,000 g for 20 min (all electrophoresis reagents were purchased from Sigma). The supernatant protein concentration was determined by Bradford assay (13). The clarified supernatant (protein concentration 500 μg) was pre-cleaned for 1 h with 50 μL of Sepharose protein A (3 mg/sample). The pre-cleaned cell lysates were then adsorbed by antisera (anti-KDR- Sigma, anti-VEGF Sigma, and anti-Shc Santa Cruz, California, USA) coupled to protein A-Sepharose (Sigma). Bound proteins were washed several times in lysis buffer and eluted in boiling Laemmli buffer. Eluted proteins were subjected to 6% (for KDR detection), 10% (for Shc detection) and 12% (for VEGF detection) SDS-polyacrylamide gel electrophoresis according to the protein molecular weight. Proteins were then transferred electrophoretically to nitrocellulose (HYBOND, Amersham, Buckinghamshire, England). In order to block non-specific binding, the membranes were incubated in blocking solution (5% low fat milk in 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl–TBS and 0.1% Tween 20) for 1h. Antisera (anti-KDR- Chemicon- Temecula, Ca 92590 USA, anti-VEGF, anti pTyr and anti-Shc Santa Cruz, California, USA) were then added at the same solution, and the incubation was carried out overnight at 4°C. For detection, the filters were washed four times (15 min each wash) with TBS 0.1% Tween 20 and allowed to react for 1h at room temperature with peroxidase-conjugated secondary antibody. The enzyme was removed by washing as above.

The filters were reacted for 5 min with a chemiluminescence reagent (SuperSignal Substrate, Pierce, Rockford, Illinois, USA) and exposed to an autoradiography film for 1-15 min (BIOMAX, Eastman Kodak, Rochester, NY, USA). In order to re-probe, nitrocellulose filters were first stripped of antibody by 62 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol, washed and probed with another antibody (14).

ELISA Test

Enzyme linked immuno-sorbent assay (ELISA; Quantikine, R&D systems, Minneapolis, MN, USA) tests

for VEGF concentration in MO7, B1647 and HEL cell supernatants were performed following the manufacturer's instructions. The lower limit of detection was less than 5 pg/mL. Samples were analysed in duplicate and the results are a mean of three experiments.

Apoptosis

Apoptosis was evaluated from necrotic HEL, B1647 and MO7 cells with an Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Germany). Cells were treated with TGF-β1 (5 ng/mL) for 48 hours and then collected, washed and incubated for 15 min with Annexin-V-fluorescein. Propidium iodide was added to differentiate apoptotic from necrotic cells. Samples were analyzed on a flow cytometer using a 488 nm excitation.

RT-PCR

Total cellular RNA was prepared by the TriPure Isolation reagent method. First strand cDNA was synthesized with 1 μg of total RNA in a reverse buffer containing 100 pmol of oligo dT, dNTPs 0.25 mM, DTT 10 mM, 20 U of Rnase Inhibitor and 50 U of M-MuLV RT. The mixture was incubated for 1 hour at 42 °C, heated for 5 minutes at 94 °C and stored at –20 °C until used. PCR amplifications were carried out in a thermal cycler with cDNA derived from 200 ng of total RNA, reaction buffer 10x, $MgCl_2$ 2mM, dNTP 0.2mM, primers 0.2 μM and 1.25 U of Taq DNA polymerase. Primers were designed and synthesized for VEGF (sense 5'-CGAAGTGGTGAAGTTCATGGATG-3'; antisense 5'-TTCTGTATCAGTCTTTCCTGGTGAG-3') and KDR (sense 5'-AAAACCTTTTGTTGCTTTTTGA-3'; antisense 5'-GAAATGGGATTGGTAAGGATGA-3'). Each cycle included a denaturation step (95°C for 1 min), an annealing step (55 °C for 1 min) and an extension step (72 °C for 1 min). The PCR products were visualized on a 2% agarose gel stained by ethidium bromide.

Sensitivity to VEGF

Cells were washed twice in PBS, re-suspended in serum free medium and incubated for 24 hours. Cell cultures were performed in serum free medium (IMDM) in triplicate, in 96-well microplates at a concentration of 50 x 10^3 per 200 μL per well in the presence or absence of VEGF at different concentrations (10, 50, 100, 150 ng/mL). Antibody against VEGF (Biodesign, Saco, ME) (2 μg/mL) was added to each VEGF concentration in the B1647 and HEL experiments alone.

After 24 and 48 hours' culture the cells were labeled with [methyl-^{3}H] thymidine (ICN Biomedicals, CA,USA) at 1mCi/well for 4 hours, and were assayed for incorporated radioactivity using a multiple cell harvester (Skatron, Oslo, Norway) and a Beta-counter (LKB, Uppsala, Sweden). In order to confirm the possible autocrine VEGF production, an RNA silencing interference mechanism was used.

Preparation of siRNA duplexes

The targeted region was selected from a given cDNA sequence beginning 50 to 100 nucleotides downstream of the start codon. To design target-specific siRNA duplexes, we selected sequences of type AA(N_{19})TT changing the last two nucleotides of the siRNA sense to dTdT (sense siRNA:5'-ccaugaacuuucugcugucdTdT-3', antisense siRNA: 5'-gacagcagaaaguucauggdTdT-3'). A selected siRNA sequence also underwent a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. The siRNA duplex was chemically synthesized by Proligo - Genset Oligos (Hamburg, Germany) (15).

Silencing of the expression of VEGF gene by siRNA

Human cell lines HEL and B1647 were grown at 37°C in IMDM supplemented with 10% FCS for HEL and 5% human serum for B1647. The day before transfection cells were diluted with fresh medium at a concentration of 1x10^6/ml.

On the day of transfection the cells were transferred to a 24-well plate at a concentration of 2.5 x10^5/ml in 200 μl of medium per well, without antibiotics (16). Exogenous delivery of siRNA duplexes to B1647 and HEL cell lines was carried out with the transfection reagent Oligofectamine™ Reagent (Invitrogen, life technologies). For each well 2.5 μl (20 μM) siRNA duplex was mixed with 40 μl of Opti-MEM I. In a separate tube 1 μl of Oligofectamine™ Reagent was added to 6.5 μl of Opti-MEM I, for each well, gently mixed and incubated for 7 - 10 minutes at room temperature. The final solutions were prepared, mixing them gently by inversion and incubating for 20 - 25 minutes at room temperature to allow for formation of liposome complexes. Cells were overlaid with 50 μl of complexes for each well, incubated for 4 hours at 37°C in a CO_2 incubator, and 125 μl growth medium was finally added. After 24, 48, and 72 hours the cells were stained with trypan blue and counted in the light microscope.

RESULTS

VEGF R-2 (KDR) and VEGF mRNA expression and protein

Our first approach to analysing KDR and VEGF in the cell lines was by RT-PCR. We observed that B1647 and HEL cells expressed mRNAs of both KDR and VEGF, while the MO7 cell line expressed KDR- but not VEGF mRNA (Fig.1). For evaluation

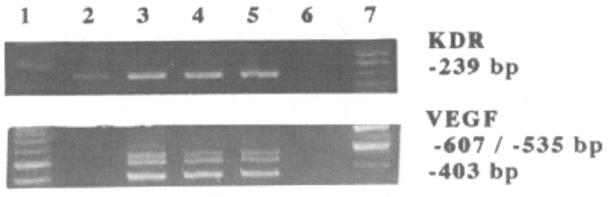


Fig. 1. *Expression of KDR and VEGF mRNA expression in MO7, HEL and B1647 cells by RT-PCR. A faint band corresponding to KDR expression is visible in MO7 while the same cells do not express VEGF mRNA. KDR and its ligand VEGF are expressed in B1647 and HEL cell lines.*
Lane 1: molecular weight marker MWM XIV; Lane 2: MO7 cell line; Lane 3: HEL cell line; Lane 4: B1647 cell line, Lane 5: Positive control for KDR: bone marrow mononuclear cells. Positive control for VEGF: HepG2 (Human hepatocyte cell line), Lane 6: negative control: no mRNA; Lane 7: molecular weight marker MWM IX.

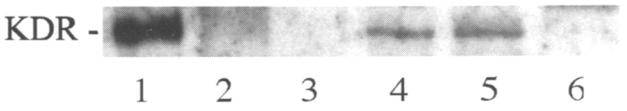


Fig. 2. *KDR protein expression in the three cell lines studied. MO7 cells do not express KDR protein despite the weak expression of KDR transcript. The evidence of KDR in B1647 and HEL cells is confirmed by KDR expression in Human Umbilical Vein Endothelial Cells (HUVECs) used as a positive control. An epithelial cell line (5637) and a lymphoblastic cell line (Jurkat) were used as negative controls.*
Lane 1 : Positive control (HUVECell lysate); Lanes 2-3: Negative controls - lane 2 (5637 cell lysate) lane 3 (Jurkat cell lysate), Lane 4 : B1647 cell lysate; Lane 5 : HEL cell lysate; Lane 6: MO7 cell lysate
Samples were subject to immunoprecipitation and blotting with the same antibody.

of KDR protein, cell lysates were immunoprecipitated with an anti-KDR antibody and immunoblotted with the same antibody. It emerged that B1647 and HEL cells expressed a 210 kDa protein consistent with the presence of KDR. By contrast, the immunoprecipitation of MO7 lysates did not contain the protein, despite expressing KDR transcript (Fig.2).

VEGF protein analysis was carried out on supernatants from starved (48 hours) B1647 and HEL cells by Western blot analysis. A 21 kDa band was evident on the nitrocellulose filters, corresponding to VEGF (Fig.3). The identity of the band was confirmed by recombinant VEGF 165, as a positive control. Another band at 18 kDa was observed, possibly corresponding to non-glycosylated VEGF 165. The corresponding medium of MO7 cells was not tested for the presence of protein, as it did not express VEGF transcript.

VEGF production

VEGF production was tested by Elisa assay in cell supernatants both in basal condition and following stimulation (for 48 hours) with Epo, TPO, Flt-3 (alone and in combination with TPO), HGF and TGF-β1 (Fig. 4). B1647 and HEL cells produced 900 pg/mL and 500 pg/mL, respectively. No significant changes were seen in the presence of Epo, TPO, Flt-3, TPO and Flt-3 or HGF, while TGF-β1 caused a significant decrease in VEGF concentration in both cell lines ($p=0.03$). This result could not be ascribed to a reduction in cell number, which remained unmodified over the time of incubation, while the analysis of apoptotic cell death by annexin V and propidium iodide staining revealed no difference between TGF β1 treated and untreated cells (HEL untreated cells: 3.3% and TGF β1 treated: 3.4%, B1647 untreated cells: 1.43% and TGF β1 treated: 1.91%).

Sensitivity to VEGFs

To evaluate the proliferation effect of VEGF, B1647, HEL, and MO7 cells were maintained in serum free conditions in the presence of various concentrations of VEGF (10, 50, 100, 150 ng/mL) for 24 and 48 hours' culture, after which a thymidine incorporation assay was performed.

No response was detected in MO7 proliferation after stimulation with different concentrations of VEGF (Fig. 5A). A dose-dependent proliferation of B1647 cells was evident as early as 24 hours after incubation with VEGF (Figs 5B and 5C). In HEL cell experiments no significant differences were detected between treated and untreated cells after 24 hours of culture (Fig. 5D), while a plateau was reached at 50 ng/mL after 48 hours (Fig. 5E). In both cell lines antibody to VEGF decreased the proliferation to control values at 24 and 48 hours of incubation (Figs 5B, 5C and 5E). In B1647 cell proliferation induced by VEGF at 150 ng/mL could

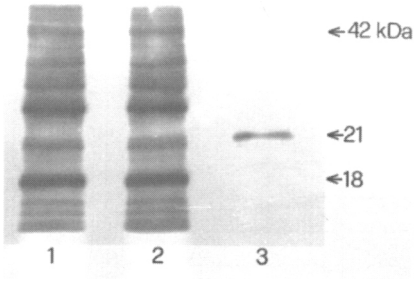


Fig. 3. *Western blot analysis of conditioned medium (CM) from the erythromegakaryocytic cell lines. Twenty-four hours before incubation, the cells were starved of serum to exclude exogenous protein. Supernatants were concentrated five times in order to better detect the protein by western blot technique. Lane 1: B1647 CM Lane 2: HEL CM Lane 3 : rhVEGF 165 (100 ng).*
Samples were subject to immunoprecipitation and blotting with the same antibody.

not be neutralized completely by anti-VEGF antibody.

Small interfering RNAs

Specific VEGF gene silencing was documented by counting the cells stained with trypan blue after 24, 48 and 72 hours of incubation (Figs 6A and 6B). VEGF RNA is able to silence the gene transcription and block B1647 and HEL cell growth. As oligofectamine is a potent transfection vector, in order to introduce siRNA into the cells oligofectamine control was necessary, in case it should have a toxic effect. In both cell lines the serum free and the oligofectamine control curves were similar (Figs 6A-6B). Despite silencing conditions an increase in the HEL cell count was observed after 48 hours, very likely due to the presence of untransfected cells in the culture.

Shc phosphorilation

We also evaluated Shc adaptor protein phosphorylation upon KDR activation with VEGF in HEL and B1647. In mammalian cells Shc is present in three isoforms P66, P52 and P46, produced by alternative translation. By immunoprecipitation of B1647 and HEL cells with an antibody reactive to Shc p66, p52, p46 we found that the B1647 cell line expressed only $p52^{shc}$ and $p46^{shc}$ proteins (Fig. 7, lanes 3 and 4), whereas HEL cells expressed all three isoform proteins (Fig. 7, lanes 1 and 2). By re-

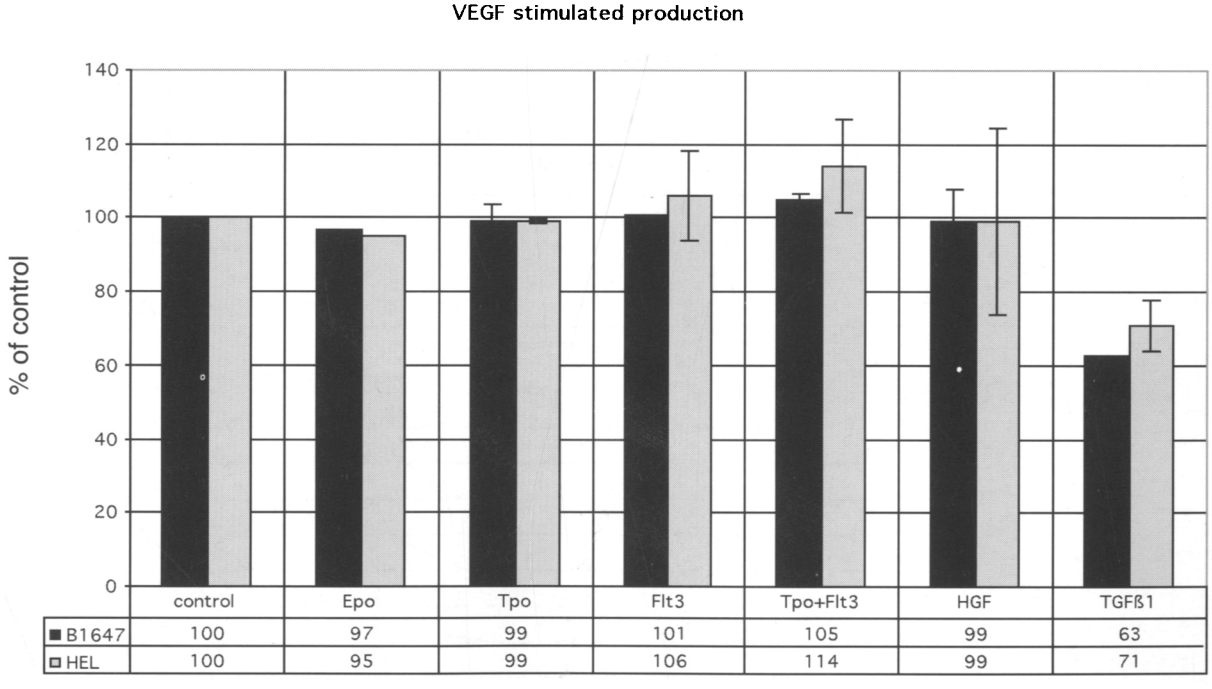
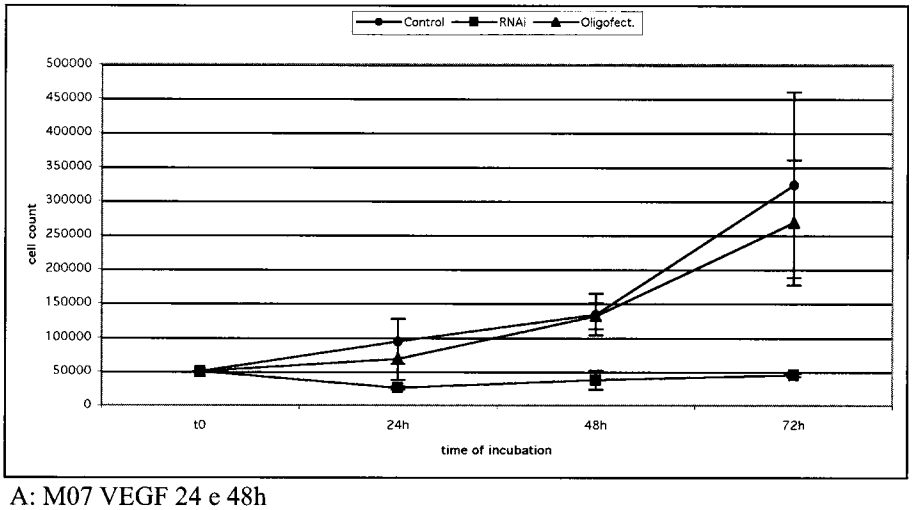
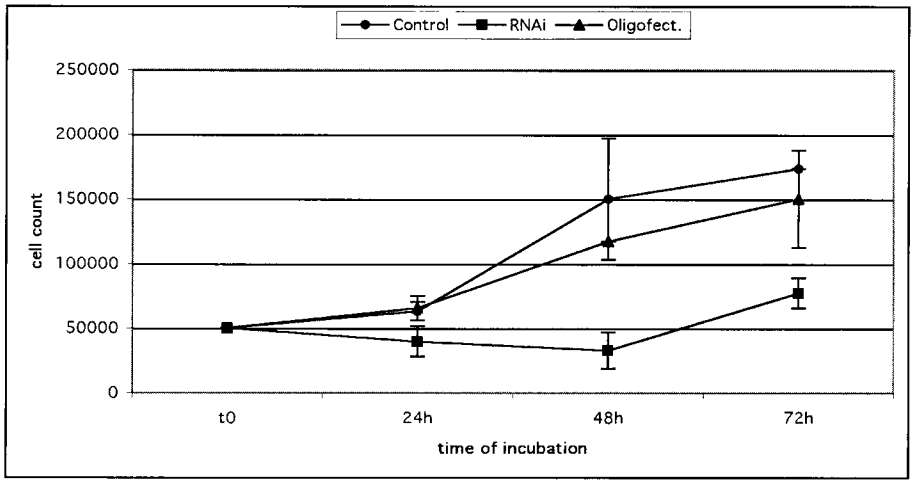


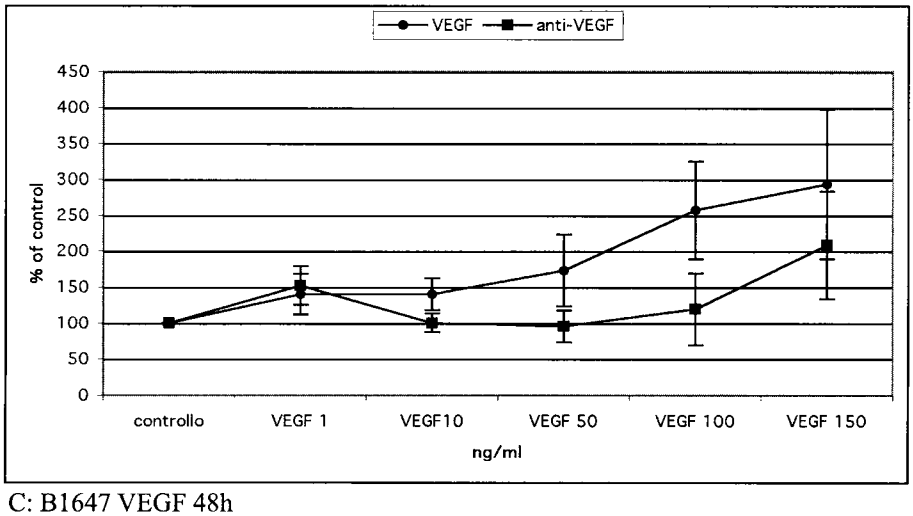
Fig. 4. *VEGF production in supernatants from stimulated and unstimulated B1647 and HEL cells (ELISA test) after 48 hours of incubation. Data are expressed as a percentage of the control (supernatant from unstimulated cells) and represent the mean of three experiments. No factors were able to induce VEGF production in the two cell lines. TGF β1 caused a significant decrease in VEGF synthesis in both cell lines (p=0.03).*



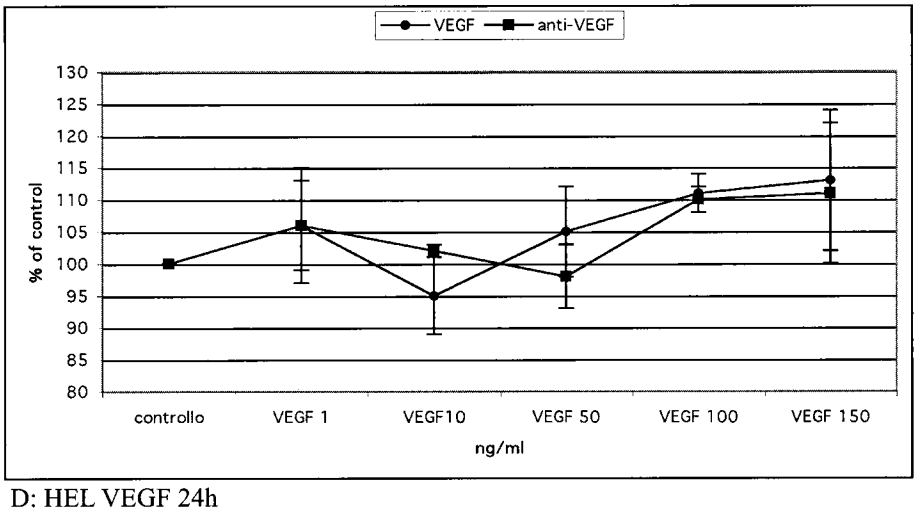
A: M07 VEGF 24 e 48h



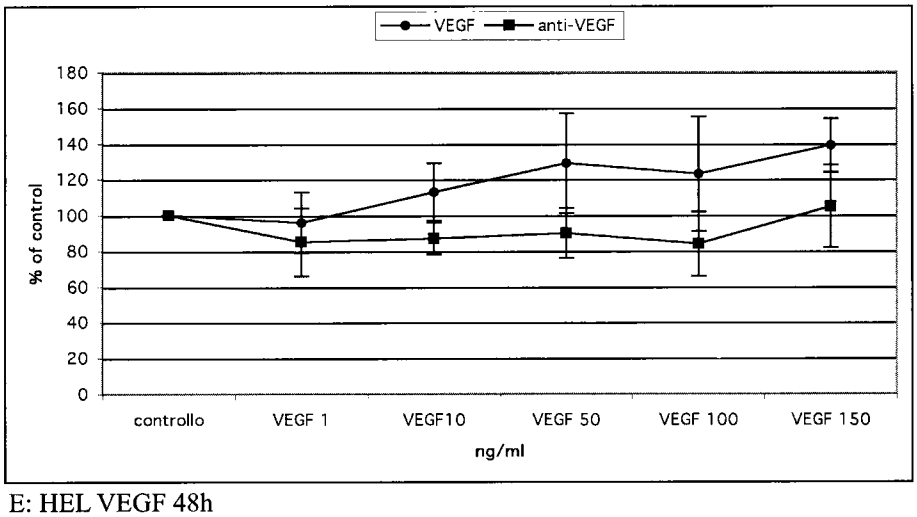
B: B1647 VEGF 24h



C: B1647 VEGF 48h



D: HEL VEGF 24h



E: HEL VEGF 48h

Fig. 5. *Effect of different VEGF concentrations on MO7 (panel A), B1647 (panels B-C), and HEL (panels D-E) cells. After 24 and 48 hours' incubation the proliferation was evaluated as ^{3}H-TdR incorporation and measured as counts per min (cpm) expressed as a % of the control. Compared to MO7 cells, 50 ng/mL ($p=0.02$) and 10 ng/mL ($p=0.006$) was sufficient to increase B1647 cell proliferation after 24 and 48 hours respectively; 50 ng/mL was needed to stimulate HEL cells significantly after 48 hours ($p=0.003$).*
Data represent a mean of three experiments + SEM.

probing the same nitrocellulose membrane with an antibody reactive to phosphotyrosine-containing proteins, in both cell lines only p46^{shc} proved to be phosphorylated following stimulation with VEGF at a concentration of 100 ng/mL for 5 min. However no difference was detected between stimulated and unstimulated cells, a finding that is most likely due to autocrine VEGF production in these cell lines, despite 24 hour cell starvation.

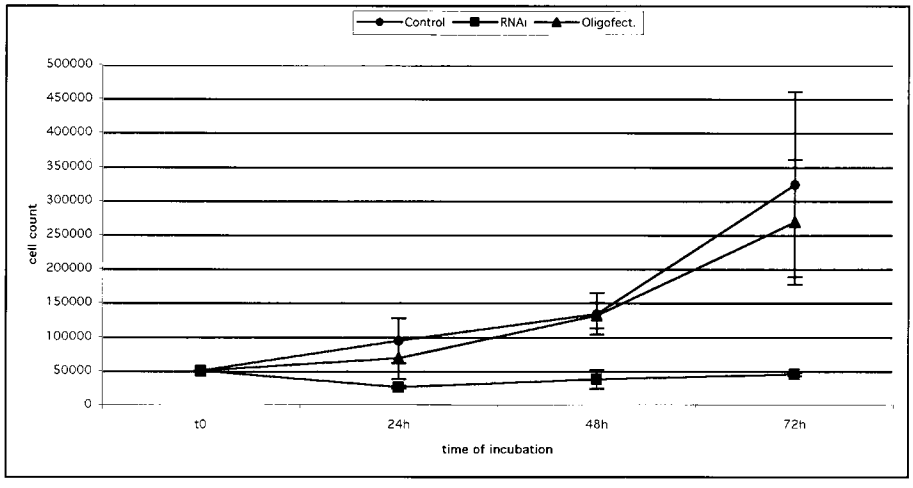
DISCUSSION

In normal hematopoiesis VEGF is produced by human megakaryocytes and released by these precursor cells and platelets (8, 17). Our study indicates that leukemic cell lines of the erythro-megakarycocytic phenotype, such as HEL and B1647, also constitutively produce VEGF and express KDR. This is not the case in all instances, a fact shown by the lack of VEGF mRNA and KDR protein in MO7 cells.

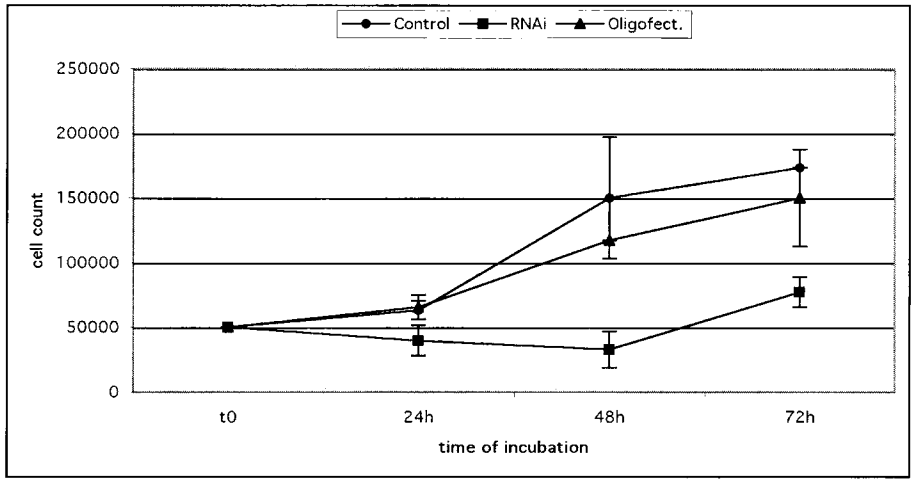
The same VEGF–R isoform was first demonstrated in HEL cells (18), but no data was previously available in other cell lines.

In a previous study we demonstrated that proliferation of B1647 cells was not modulated by hemopoietic cytokines, while in the presence of TPO the transcripts of specific erythroid proteins, such as β and γ globins increased (10). In that work we observed that only B1647 and HEL proliferated in the presence of VEGF in the culture medium and while a maximum stimulating dose was not found in B1647, a plateau in the proliferation curve of HEL cells was observed at the concentration of 50 ng/mL VEGF. These results are in agreement with the observation that only these two cell lines, but not MO7, actually produce KDR protein.

The suggestion that VEGF may have a role in sustaining leukemic cell growth is further reinforced by the observation that proliferation was inhibited by the antibody to VEGF in thymidine incorporation and the number of cells was reduced in the RNA interference experiments because of the block on autocrine production. In our setting this last method (19-20) stressed the role of VEGF gene in regulating B1647 and HEL cell growth.



A: B1647



B: HEL

Fig. 6. *Silencing of VEGF expression in B1647 (panel A) and in HEL (panel B) cell lines. After 24, 48, 72 hours from transfection with 2.5 μl (20 μM) VEGF siRNA duplexes, trypan blue stained cells were counted.*
The growth relative to the cells treated with the transfection agent (Oligofectamine) is reported. Bars represent the means of three experiments + SEM.

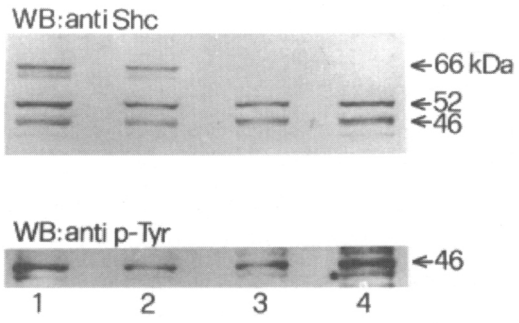


Fig. 7. *Involvement of Shc proteins in the KDR signal pathway in HEL (1-2) and B1647 (3-4) cell lines. Lysates were immunoprecipitated with anti Shc protein and immunoblotted with the same antibody (upper panel) while Shc phophorylation was detected after stripping (lower panel). Lanes 1 and 3: immunoprecipitates from unstimulated cells; Lanes 2 and 4: immunoprecipitates from cells stimulated with 100 ng/mL VEGF for 5 min. WB: western blotting.*

The possible role of VEGF in leukemic proliferation is consistent with three more observations: a) myeloblast and immature myeloid cells, but not erythroid precursors and lymphoid cells, co-express VEGF and its receptor (21); b) antiVEGF antibodies promote the formation of CFU-GEMM and BFU-E colonies in methylcellulose cultures of bone marrow cells from patients with myelodysplastic syndromes, by inhibiting TNFα and IL1β production from BM mononuclear and stromal cells (21); c) anti leukemic activity by agents such as AS_2O_3 (7) inhibits VEGF production by leukemic cells and induces endothelial cell apoptosis, thereby reducing the release of leukemic cell stimulating cytokines, including VEGF.

In our experimental conditions cytokine stimulation failed to increase VEGF protein secretion by B1647 and HEL cells, while MO7 cells did not produce VEGF, which is consistent with the lack of the transcript. This is in contrast with the observation of Bobik et al. that TPO caused a time-and dose-dependent increase in the levels of VEGF released by the megakaryocytic cell line CMK, and the c-mpl expressing UT7 cells. On the other hand, our data indicate that TGF-β, a multifunctional polypeptide that regulates the proliferation and differentiation of various types of cells, has a strong and direct inhibitory effect on the synthesis of VEGF. In fact the evident inhibition of thymidine incorporation in the presence of TGF-β observed in B1647 and HEL cells did not correspond to a decrease in the number of cells in culture at the time of supernatant collection (data not shown). This is in contrast with the suggestion by Pertovaara et al. that the angiogenic effect of TGF-β1 on endothelial cells is partly mediated by a paracrine induction of VEGF in other surrounding cell types (22) as well as with the alleged TGF-β driven increase in VEGF expression by osteoblasts and osteoblast-like cells (23-24). Western blot analysis of VEGF production in B1647 and HEL supernatants revealed bands at different molecular weight besides the 21 kDa band corresponding to $rhVEGF_{165}$: 42 kDa, 37 kDa, 30 kDa and 18 kDa. As also reported by Mohle et al, 18 kDa band corresponds to non-glycolysated $VEGF_{165}$ whereas 30-37 kDa bands were found by other authors in rat pituitary tumor cell line GH_3 (25). VEGF mediates its pleiotropic action by binding to KDR with an intrinsic protein tyrosine kinase activity. The first step in signal activation is dimerization of receptor KDR after binding with VEGF and this process is followed by tyrosine autophosphorylation of kinase domain and cytoplasmic protein "target". We studied Shc, one of these target proteins called "adaptors" because of their capacity for interacting with proteins of a multimolecular complex (26). Three different isoforms of Shc, 46 kDa, 52 kDa and 66 kDa, are known: the 46 and 52 kDa isoforms are expressed ubiquitously, whereas the 66 kDa isoform is predominantly found in cells of epithelial origin (27-28). In our experiments only p46 was phosphorylated after the binding of VEGF with its receptor KDR although B1647 cells also expressed p52 and HEL cells expressed both p52 and p66. It could be of interest to investigate the role of p52 and p66 in the VEGF induced proliferation of the erythro-megakaryoblastic cell lines, because p52 and p66, but not p46, have been found to be associated with activation EGFR-Src complex in the human carcinoma A431 cell line (29).

In conclusion, our findings support the relationship between angiogenesis and leukemic growth: a) VEGF is produced by some of the leukemic cell lines, and TGF-β is the only modulator of VEGF synthesis; b) VEGF regulates cell proliferation that is inhibited by antibodies to

VEGF; c) VEGF binding to KDR transduces a proliferative signal through the phosphorylation of $p46^{shc}$.

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