

ORIGINAL ARTICLE: RESEARCH

Coordinated deregulation of cellular receptors, proangiogenic factors and intracellular pathways in acute myeloid leukaemia

NURIA BARBARROJA¹, LUIS ARÍSTIDES-TORRES¹, VANESSA HERNANDEZ¹,
CARMEN MARTÍN², GABRIEL DORADO³, ANTONIO TORRES²,
FRANCISCO VELASCO², & CHARY LOPEZ-PEDRERA¹

¹Unidad de Investigación Hospital Reina Sofía, Córdoba, Spain, ²Servicio de Hematología, Hospital Reina Sofía, Córdoba, Spain, and ³Departamento Bioquímica y Biología Molecular, Córdoba, Spain

(Received 30 June 2006; revised 14 March 2007; accepted 14 March 2007)

Abstract

Different signaling routes seem to be simultaneously triggered in leukemia, with distinct and overlapping activities. To analyze if altered signals are coordinated and to evaluate their effect on this disease, we have investigated in acute myeloid leukemia samples (AML) the expression and activation status of procoagulant/proangiogenic tissue factor receptor (TF), angiogenic protein VEGF, its cell surface receptor, KDR, and two intracellular proteins involved in their regulation: extracellular regulated kinase (ERK1/2) and nuclear factor kappa-B (NFκB). Significantly higher mRNA and protein levels of VEGF, KDR, and TF were found in the AML samples versus controls. Enhanced ERK phosphorylation and NFκB activation in most AML samples were also found. In vitro MEK/ERK and NFκB-binding activity blockade suppressed the constitutive expression of TF, VEGF, and KDR. Anti-TF antibody treatment significantly suppressed VEGF and KDR expression as well as ERK activation, suggesting that TF expressed by AML cells may be both a regulatory target and a mediator of tumor-associated angiogenesis. Patients showing parallel activation of the studied proteins trended to exhibit higher incidence of fatal outcome. Our results show a coordinated deregulation of cellular receptors, proangiogenic factors, and intracellular pathways in leukemia cells, which may help to design mechanism-based combinations of single transduction-related therapies.

Keywords: AML, TF, VEGF, ERK, NFκB, Outcome

Introduction

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and overproliferation in the stem cell compartment result in accumulation of nonfunctional cells termed myeloblasts [1]. Many subsets of disease that comprise AML are best defined by specific molecular genetic derangements, which are due to balanced chromosomal translocations, that in turn result in the formation of novel fusion genes at the recombination sites. The gene products of these chimeric genes are often dysfunctional transcription factors that block expression of differentiation-associated genes. Considerable variation of clinical behavior and prognosis of

the disease may be observed in different individuals [2–4].

AML patients may suffer from a disseminated coagulopathy, which can aggravate a pre-existing bleeding tendency due to thrombocytopenia and platelet dysfunction [5]. Systemic hypercoagulability in AML patients is correlated with tissue factor (TF) overexpression by leukemia cells [6–8] and is closely associated with tumor progression [9]. TF significantly participates in thrombosis and tumor-associated angiogenesis and its expression levels have been correlated with the invasive and metastatic potential of many types of hematological malignancies [10]. Moreover, the accumulating data indicate that TF expressed by tumor cells as well as hosts cells initiates, directly or indirectly, signaling events

that support tumor development by distinct mechanisms. By a proteolytic mechanism, TF serves as the cofactor for its physiological ligand factor VIIa in the initiation of blood coagulation. In addition, binding of FVII(a) to cell surface-associated TF results in the production of intracellular signals, e.g., through cytosolic calcium alteration, MAPK phosphorylation and upregulation of multiple genes [10–12]. Furthermore, the cytoplasmic domain of TF also participates in intracellular signaling [13]. Hence, TF-VIIa induced signaling results in the activation, among others, of the pERK 1/2 MAP kinase pathway [14]. The Raf/MEK/ERK signal transduction cascade is responsible for many of the phenotypes observed in a wide array of oncogenic disorders and is constitutively activated in primary acute leukemias, thus indicating a role of this activated kinase in leukemogenesis [15].

Another characteristic feature of TF is its significant participation in tumor-associated angiogenesis. In several models, TF expression mediates the upregulation of the proangiogenic cytokine ‘vascular endothelial growth factor’ (VEGF), a potent angiogenic peptide with biologic effects that induces regulation of hematopoietic stem cell development, extracellular matrix remodeling, and inflammatory cytokine generation [16]. Two VEGF receptors are present on vascular cells (Flt-1 and KDR), and VEGF signaling is mediated by receptor dimerization, leading to autophosphorylation of the cytosolic domains of these receptors. VEGF and KDR have proved to be increased in many types of solid tumors, as well as in some hematological malignancies [17,18]. Moreover, increased expression of VEGF and basic fibroblast growth factor (bFGF) has been correlated with clinical characteristics in leukemia, serving as predictors or poor prognosis [9]. Upregulation of VEGF and angiogenesis can be induced by constitutive activation of oncogenic proteins such as Raf, MEK, or PI3K, acting at various levels of the Ras signaling pathway [19].

The transcription factor nuclear kappa B (NF κ B) has been also studied intensively for its role in controlling the expression of genes involved in immune and inflammatory function. Within the nucleus, NF κ B is involved in the coordinated expression of numerous target genes, including cytokines and chemokines, growth factors, antiapoptotic genes, angiogenic factors and procoagulant molecules. It has also been suggested that NF κ B plays an important role in cell growth and oncogenesis. In hematopoietic malignancies, this is demonstrated by the finding that NF κ B can be constitutively expressed in myeloid leukemic progenitor cells, which confers increased survival to AML blasts [20].

Thus, a number of different signaling routes seem to be triggered simultaneously in leukemia cells, with

distinct and overlapping activities. To analyze if altered signals are coordinated into the same leukemia sample and to evaluate their effect on the behavior of this disease, we have investigated in a subset of AML samples the expression and activation status of TF, VEGF, and two intracellular proteins involved in their regulation: the extracellular regulated kinase (ERK1/2) and the nuclear factor kappa-B (NF κ B). Our results suggest that the simultaneous deregulation of TF and VEGF expression and their inducing signaling pathways might play an important role in the pathogenesis of acute leukemia.

Materials and methods

Patients

A total of 10 healthy controls and 25 patients with acute myeloid leukemia (AML) diagnosed according to the classification of the French–American–British (FAB) committee classification were studied after informed consent. The AML patients were M0 ($n=2$), M1 ($n=6$), M2 ($n=2$), M3 ($n=6$), M4 ($n=2$); M5 ($n=4$), M6 ($n=2$), and M7 ($n=1$). Blast cells were obtained from bone marrow (BM) aspirates at the time of diagnosis and were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of $10-20 \times 10^6$ cells in RPMI 1640 supplemented with 10% dimethyl sulfoxide (DMSO) and 10% fetal bovine serum (FBS), employing a method of controlled freezing and storage in liquid nitrogen. Cells were thawed at 37°C by progressively adding RPMI 1640 containing 20% FBS and 0.16 KU of deoxyribonuclease I (Sigma–Aldrich, St Louis, MO) per ml. The samples were analyzed by dual color fluorescence-activated cell sorting (FACS) analyses, to check for possible contamination with other leukocytic cell types (monocytes, T-lymphocytes, B-lymphocytes, and neutrophils), using monoclonal antibodies against the appropriate cell markers (CD14, CD2, CD19, and CD15, respectively).

As controls, cells from BM from 10 healthy subjects were used. These were isolated in the same way as the AML blasts. For CD34+ cell selection, the Myltenyi immunoaffinity device (VarioMACS) was used, according to the manufacturer’s directions (Myltenyi Biotech, Auburn, CA). The details of patients examined in the study are indicated in Table I.

Flow cytometry analyses

The presence of TF proteins on the cell surface was estimated by fluorescence-activated cell sorting (FACS; flow cytometry). Cells (1×10^5) were washed twice in phosphate-buffered saline (PBS), 5% FCS,

Table I. Clinical details of AML patients.

No.	Sex	Age (years)	FAB	Blasts (%)	Karyotype	WBC ($\times 10^9/l$)	Disease complications	Patient outcome
1	M	0.6	M0	70	N/A	22.8	Septic Shock CNS Involvement	Exitus
2	M	75	M0	39	45,XY, 7	6.0	Disease progression	Exitus
3	F	75	M1	87	49,XXX,+4,+8/49, XX, +4, +8, +21	81.8	Disease progression	Exitus
4	F	3	M1	84	N/A	N/A	N/A	N/A
5	M	64	M1	89	48,XX,t(1;9) +others/46,XX	86.0	Bilateral pneumonia	Exitus
6	F	71	M1	89	43,X,inv(x),5,7/43,X, inv(x),5,7,8,+1	16.7	Septic shock	Exitus
7	F	91	M1	44	46,XX	9.7	Bilateral pneumonia	Exitus
8	M	46	M1	63	47,XX,+6	3.4		Remission
9	F	72	M2	54	N/A	114.5	N/A	N/A
10	F	68	M2	75	Complex + Mono7	N/A	DVT	Relapse
11	M	61	M3	93	46,XY,t(15;17)	11.1	CID; cerebral hemorrhage	Exitus
12	M	32	M3	84	46,XY, t(15;17)	3.8	ATRA syndrome	Remission
13	F	36	M3	85	46,XX, t(15;17)	0.9	Coagulation disorders	Remission
14	M	49	M3	79	46,XY,t(15;17)	0.7	ATRA syndrome, septic shock	Exitus
15	F	17	M3	60	46,XX, t(15;17), +add(12)	30.1	Coagulation disorders	Remission
16	F	39	M3	90	46,XY,t(15;17)	2.4	Digestive hemorrhage	Remission
17	F	84	M4	65	46, XX	78.8	Disease progression	Exitus
18	F	31	M4	74	46,XX, t(1;11)(p32;q23)	1.0		Remission
19	M	65	M5	76	N/A	93.0	Septic shock	Exitus
20	F	72	M5	85	N/A	156.0	DVT, pneumonia	Exitus
21	F	29	M5	95	48,XX,+8,+8	4.8	Hepatitis C	Remission
22	M	77	M5	81	47,XY,+11,del(12)/46,XY	3.8	Muscle infiltration	N/A
23	M	42	M6	68	46,XY, inv(6)	2.5	Coagulation disorders	Exitus
24	F	71	M6	40	46,XX/46,XX,del(5),7,+8,+others	2.6	Pneumonia	Relapse
25	M	51	M7	85	46,XX, BCR/ABL(-)	16.6	DVT, pneumonia	Relapse

Karyotypic abnormalities are shown for cases where available. FAB, acute myeloid leukaemia subtype, according to the classification of the French-American-British committee; WBC, white blood cell count; N/A, data not available; DVT, deep venous thrombosis; CID, disseminated intravascular coagulation; CNS, central nervous system.

and incubated for 30 min at room temperature with specific monoclonal antibodies to human TF-fluorescein isothiocyanate (FITC)-conjugated (American Diagnostica, Stamford, CT). Negative controls were performed using the same amount of isotype-specific FITC-conjugated IgG. After two washes, cells were resuspended in 0.5 ml PBS. Flow cytometry analyses were performed on a FACSCalibur (BD, Biosciences, San Jose, CA), using the CellQuest software, version 3.2, from the same manufacturer.

RNA isolation and quantitative real-time RT-PCR

Total cellular RNA from blast cells was extracted using the Tri[®] Reagent (Sigma) according to the manufacturer's recommendations. RNA concentration/purity was determined spectrophotometrically (ratio 260/280 nm) and its integrity was verified following separation using 0.8% agarose gel electrophoresis (AGE) containing ethidium bromide. RNA samples were stored at -80°C until use.

The expression levels of the genes encoding the tissue factor (*tf*), vascular endothelial growth factor (*vegf*), kinase insert domain-containing receptor (*kdr*) and glyceraldehyde-3-phosphate dehydrogenase

(*gapdh*) as housekeeping gene, were measured by quantitative real-time RT-PCR using the Light-Cycler Thermal Cycler System (Roche Diagnostics, Indianapolis, IN). The sequence of primers used for the study and expected size of the PCR products were *tf*: (283 bp), forward primer: 5'-CTA CTG TTT CAG TGT TCA AGC AGT GA-3'; reverse primer: 5'CAG TGC AAT ATA GCA TTT GCA GTA GC-3'; *vegf*: (470 bp), forward primer: 5'-CGA AGT GGT GAA GTT CAT GGA TG-3'; reverse primer: 5'TTC TGT ATC AGT CTT TCC TGG TGA-3'; *kdr*: (184 bp), forward primer: 5'-GGG CAT GTA CTG ACG ATT ATG GA-3'; reverse primer: 5'-GAG TGG TGC CGT ACT GGT AGG AA-3'; *gapdh*: (240 bp), forward primer: 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3'; reverse primer: 5'TCC TTG GAG GCC ATG TAG GCC AT-3'.

The RT-PCR was performed on one-step using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Briefly, 100 ng of total RNA was placed into a 20- μl reaction volume containing 150 nM of each primer, 10 μl of SYBR Green PCR master mix, and 0.2 μl of reverse transcriptase. The cDNA was

synthesized at 50°C for 20 min. The cDNA was then exponentially amplified with the following steps: Omniscript and Sensiscript reverse transcriptase deactivation, HotStarTaq DNA Polymerase activation (95°C for 15 min), and PCR (50 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 68°C for 40 s). The temperature transition rate was set at 20°C/s. The fluorescent products were detected at the end of the extension at 68°C. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis from 65 to 90°C. The mRNA samples were analyzed at least in three similar RT-PCR reactions performed in triplicate. Negative controls containing water instead of RNA were run to confirm that the samples were not cross-contaminated. Quantification of relative expression was determined by the standard curve method, according to the manufacturer's directions. The target amount was normalized to the *gapdh* gene and the relative expression was calculated by the ratio of the normalized target values and the calibrator normalized target values.

Western blot analyses

Cytoplasmic and nuclear extracts were prepared by standard protocols [21], and used for Western blotting and EMSA, respectively. Briefly, 5×10^6 blast cells isolated from each sample patient were washed twice in PBS and lysed in NP-40 lysis buffer, containing 10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM Na₂-EGTA, 0.1 mM Na₂-EDTA, 1 mM sodium orthovanadate, 1% of NP-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml aprotinin. The samples were incubated on ice for 10 min, then vortexed for 10 s and finally pelleted by centrifugation (5 min). The supernatant was saved as the cytosolic fraction for Western blotting. The pellet containing the nuclear fraction [22] was resuspended in hypertonic nuclear extraction buffer (20 mM HEPES [pH 7.9]; 420 mM NaCl; 1 mM Na₂-EDTA; 1 mM Na₂-EGTA; 1 mM DTT; 1 mM PMSF; and 10 µg aprotinin/ml). The samples were then incubated on ice for another 20 min with continuous mixing, followed by centrifugation. The peptide concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Following standard protocols [23], IκBα, phosphorylated and nonphosphorylated forms of ERK1/2 MAP kinase, VEGF, and KDR protein levels were determined by Western blotting. An amount of 50 µg of cytoplasmic extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels for ERK and IκBα, 12% gels for VEGF, and 6% gels for KDR analyses, respectively, and then

transferred to nitrocellulose membranes. Immunoblottings were incubated with either monoclonal or polyclonal anti-IκBα, anti-VEGF, anti-KDR, anti-phospho-ERK1/2, anti-ERK1/2 and anti-actin antibodies (Santa Cruz Biotech, Santa Cruz, CA). Immunocomplexes were generated with the appropriate horseradish peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminescence (ECL) (GE Healthcare, Little Chalfont, UK). The protein was quantified using the image analysis software "Intelligent Quantifier" version 2.1.1 (Bio Image, Ann Arbor, MI, USA). Results were calculated in terms of integrated optical density (IOD) and expressed in arbitrary units (AU).

Immunocytochemistry for VEGF and KDR

Cytospins of blast cell preparations were fixed in Bouin's solution (Sigma–Aldrich). The fixative was removed by sequential washing with distilled water, PBS, alcohol iodide, and sodium metabisulfite. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide for 5 min, followed by washing in PBS. The slides were then incubated for 30 min with polyclonal anti-VEGF and anti-Flt-1 antibodies (Santa Cruz Biotechnology) at a dilution of 1:200. This incubation was followed by sequential 15-min incubations with a biotinylated link antibody and peroxidase-labeled streptavidin (Dako, High Wycombe, UK). The color reaction was developed using 3,3'-diaminobenzidine (DAB) as chromogen. Slides were mounted on standard DPX mounting medium. For the negative control slides, normal rabbit immunoglobulin (Dako) was used at the same protein concentration as the antibody.

Electrophoretic mobility shift assays

Nuclear extracts were subjected to assays for NFκB-binding activity employing consensus oligonucleotides 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5' (Santa Cruz Biotechnology, Santa Cruz, CA), using the Digoxigenin electrophoretic mobility shift assays (EMSA) kit (Roche Diagnostics). A total of 25 µg of nuclear protein was incubated with digoxigenin-labeled NFκB oligonucleotide in 4 µl of 5' binding buffer containing 100 mM HEPES at pH 7.6, 5 mM Na₂-EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, 1% (w/v) Tween 20, and 150 mM KCl) together with 1 mg of poly[d(I-C)], and poly L-lysine to a final volume of 20 µl. After 15 min of incubation at room temperature, the protein-DNA complexes were resolved on native 8% polyacrylamide gel in a 0.5' Tris-borate-EDTA buffer system and run at 200 V for 2 h. Gels were transferred to nylon

membranes in a semidry transfer System (Bio-Rad) at 10 V and 300 mA for 30 min. The membranes were dried at 120°C, exposed to UV-light in a transilluminator for 5 min, and incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody. Complexes were detected with CSPD chemiluminescent substrate (Roche Diagnostics) and exposed to Hyperfilm (GE Healthcare) in a film holder for 4–16 h at room temperature. Antibody supershift assays were performed by incubation of the nuclear proteins with 4 mg of polyclonal affinity-purified antibodies (Santa Cruz Biotechnology) against NF κ B p50 (H-119), NF κ B p65 (C-20), and NF κ B p52 (K-27) subunits for 30 min on ice before adding the labeled probe. Specific competition control of unlabeled oligonucleotides at 125-fold excess was added to the binding reaction mixture.

In vitro studies

To examine the involvement of TF expression in the constitutive VEGF expression observed in AML blasts, as well as their relationship with down-stream targets, we then performed experiments in 10 AML blast samples, which were treated with a monoclonal antibody to the human TF protein (American Diagnostica) that rapidly blocks its expression and the initiation of coagulation activation and the intracellular pathways activation induced by the FVIIa-TF complex. The antitissue factor monoclonal antibody TF was administered to cells at a dose of 10 μ g/ml per 5×10^5 cells during 3 h.

The effect of both the MEK1 inhibitor PD98059 (that block ERK1/ERK2 activation), and the NF κ B inhibitor SN50 (which inhibits NF κ B binding activity) were also evaluated in the same subset of AML samples. The PD98059 was administered to cells at a dose of 20 μ M per 5×10^5 cells during 3 h. The SN50 was used at a dosage of 50 μ g/ml per 5×10^5 cells for the same period of time. The cells were then harvested and processed to evaluate the presence of *tf*, *vegf*, and *kdr* mRNA (gene expression), as well as TF, VEGF, and KDR peptides (protein synthesis), as well as both ERK and NF κ B activation.

Clinical outcome

Intensive therapy administered to AML patients consisted of an induction phase with anthracycline and cytarabine, followed by one cycle of consolidation using the same drugs and, in patients younger than 60 years, by autologous or allogenic bone marrow transplantation. Patients with acute promyelocytic leukemia (APL) received regimens containing all-trans retinoic acid (ATRA) and idarubicin-based chemotherapy for induction and consolidation, as

well as ATRA-based maintenance. Overall, 10 patients treated with intensive chemotherapy reached complete remission, 2 showed induction death, and 3 relapsed. The probability of remaining in remission 2 years after beginning the treatment was 70%. These data reflect in part the high proportion of APL patients (6, favourable cytogenetics) in our series.

Statistical analyses

All data are expressed as mean \pm SEM (standard error of mean). Statistical analyses were carried out using the software package Sigma Stat (Systat, San Jose, CA). Before comparing two data groups, a normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric test (paired Student's *t*-test). If the normality and/or equal variance test was violated, a comparison was made by a nonparametric test (Mann–Whitney rank sum test). Correlations were assessed by the Pearson product-moment correlation tests. Differences were considered significant if $p < 0.05$.

Results

TF, VEGF, and KDR expression in AMLs cells

The *tf* mRNA levels were found significantly increased in preparations of total RNA of blast samples from all AML patients (0.48 ± 0.09) compared to healthy controls [0.025 ± 0.005 , $p < 0.001$; Figure 1(A)]. Cell surface-associated TF protein was also detected in samples from these patients and their levels were significantly increased in patients with AML (particularly in those of M3 and M5 subtype; mean $47.8\% \pm 3.7\%$ positive cells) compared with the levels observed in normal CD34+/CD33+ cells [mean $6.6\% \pm 1.7\%$, $p < 0.001$; Figure 1(B)]. To determine whether acute leukaemia cells were also a source of angiogenic factors and could therefore stimulate angiogenesis *in vivo*, we used real time-RT-PCR to study the mRNA expression of the *vegf* gene and the gene *kdr* encoding its receptor in leukemic blast samples (Figure 2). The *vegf* gene was expressed in all leukemia samples [Figure 2(A)], although the extent of *vegf* and *kdr* mRNA expression varied among the different samples tested. Western blot analyses of cytosolic extracts showed that AML cells also synthesized increased levels of both VEGF and KDR proteins [Figure 2(B)].

Constitutive ERK activity in AML cells

Blasts from the 25 patients with AML were analyzed with regard to their constitutive ERK 1/2 activity, by

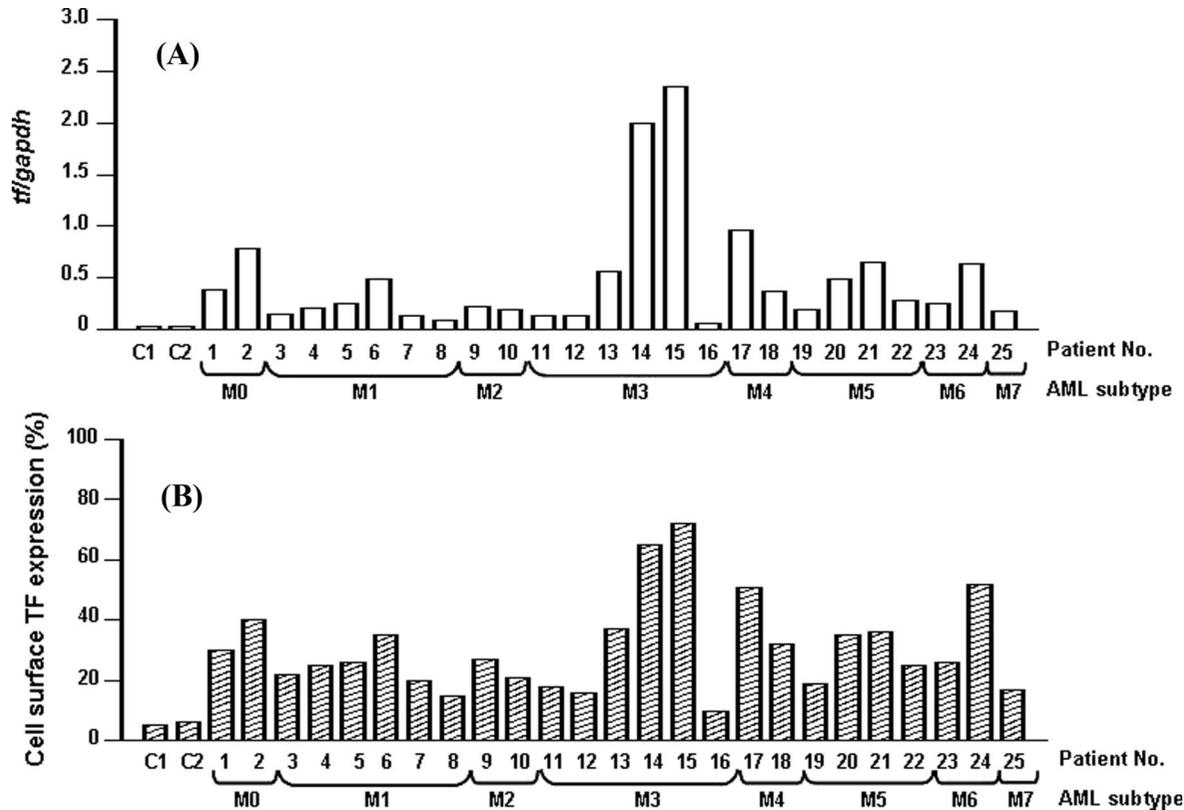


Figure 1. Tissue factor expression on blast cells from AML patients. The *tf* mRNA relative expression levels and cell surface TF presence in blast cells from AML patients and healthy controls. (A) RT-PCR real time was performed with total RNA, using primers corresponding to human *tf* cDNA sequences, as indicated. The relative mRNA amount was quantified as the ratio of the mRNA expression of the targeted gene to that of *gapdh*. (B) The presence of the TF protein on the cell surface was analyzed in intact cells by flow cytometry using specific monoclonal antibodies.

means of Western blot. Mean levels of phosphorylated forms of ERK1/2 MAP kinases, as measured by densitometry, were significantly higher in AML patients (2736.7 ± 134.5 arbitrary units; AU of integrated optical density, IOD) compared with that of controls (993.9 ± 95.7 AU of IOD) ($p < 0.002$; representative panels are shown in Figure 3).

Positive correlations among ERK activity and VEGF, KDR and TF expression

The Pearson's relational statistic test detected a significant rank correlation between the titres of pERK MAP kinase and that for mRNA of *tf* [$r = 0.59$; $p = 0.0015$; Figure 4(A)] and *vegf* [$r = 0.58$; $p = 0.002$; Figure 4(B)], as well as with both protein levels of TF ($r = 0.862$; $p < 0.05$) and VEGF ($r = 0.701$; $p < 0.05$). There was also a significant positive correlation between TF and VEGF protein levels ($r = 0.776$; $p = 0.02$).

Constitutive NF κ B binding activity in AML cells

Among the 25 AML samples analyzed, in 13 cases (52%), constitutive NF κ B DNA-binding activity

could be demonstrated, while 12 cases showed minor or no NF κ B DNA-binding activity (representative panels are shown in Figure 5). The specificity of the NF κ B DNA-binding activity was shown in a competition assay, by adding a 100-fold molar excess of unlabeled oligonucleotide (not shown).

Antitissue factor antibody and MEK/ERK and NF κ B-binding activity blockade effects in the constitutive expression of TF and VEGF and down-stream targets

The possible link between TF and VEGF expression in AML cells and with downstream signals was investigated both by administering to a subset of AML samples a rapidly neutralizing monoclonal antibody to TF and through the blockade of the MEK/ERK module by using the specific inhibitor PD98059.

The effect of administration of TF antibody on TF, VEGF, and KDR expression is illustrated in Figures 6(A) and 6(B). The anti-TF monoclonal antibody significantly repressed the *tf*, *vegf*, and *kdr* gene expression and inhibited the peptide synthesis of TF, VEGF, and KDR proteins. Significant inhibition in ERK activation was further observed

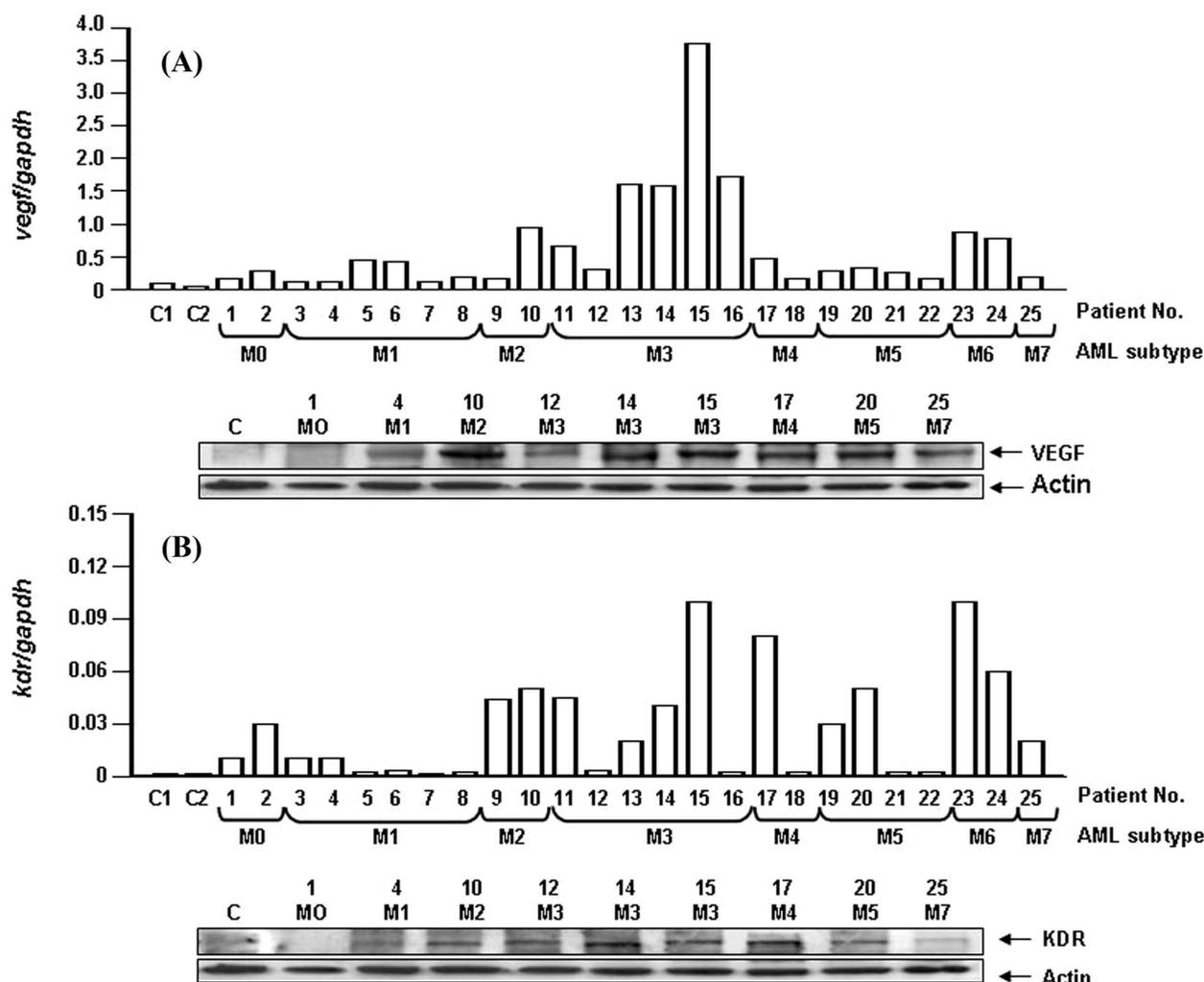


Figure 2. VEGF and KDR expression on blast cells from AML patients. The bar graphs show the relative gene expression levels for the mRNA of *vegf* (A) and *kdr* (B), respectively, of 25 AML patients and two samples from healthy controls. The panels below the bar graphs are representative Western blots of various samples from AML patients and healthy controls, obtained as described in the Material and Methods.



Figure 3. Constitutive ERK activation in blasts from AML patients. Cytoplasmatic extracts were subjected to Western blot analyses using antiphospho-tyr-204-ERK 1/2 antibodies. The same blots were then stripped and reprobred with anti-ERK 1/2. The panels are representative of 13 AML patients and one healthy control.

[Figure 6(C)]. No changes were promoted after anti-TF administration in the constitutive NF κ B binding activity of AML blast cells (not shown).

The treatment with PD98059 (20 μ M) prevented the constitutive ERK1/2 activation found in the AML samples tested; mean levels of phosphorylated forms of ERK1/2 MAP kinases, as measured by densitometry, were significantly lower in PD98059-treated

AML patients (865.2 ± 66.9 AU of IOD) compared to values in AML patients without MEK1-inhibitor treatment (3256.2 ± 284.2 AU of IOD) [$p < 0.001$; representative panels are shown in Figure 7(C)]. The inhibitory effect of PD98059 occurred without altering cell viability and also had no effect on total cellular levels of ERK1/2. In addition, at the concentrations that preclude ERK activation, this

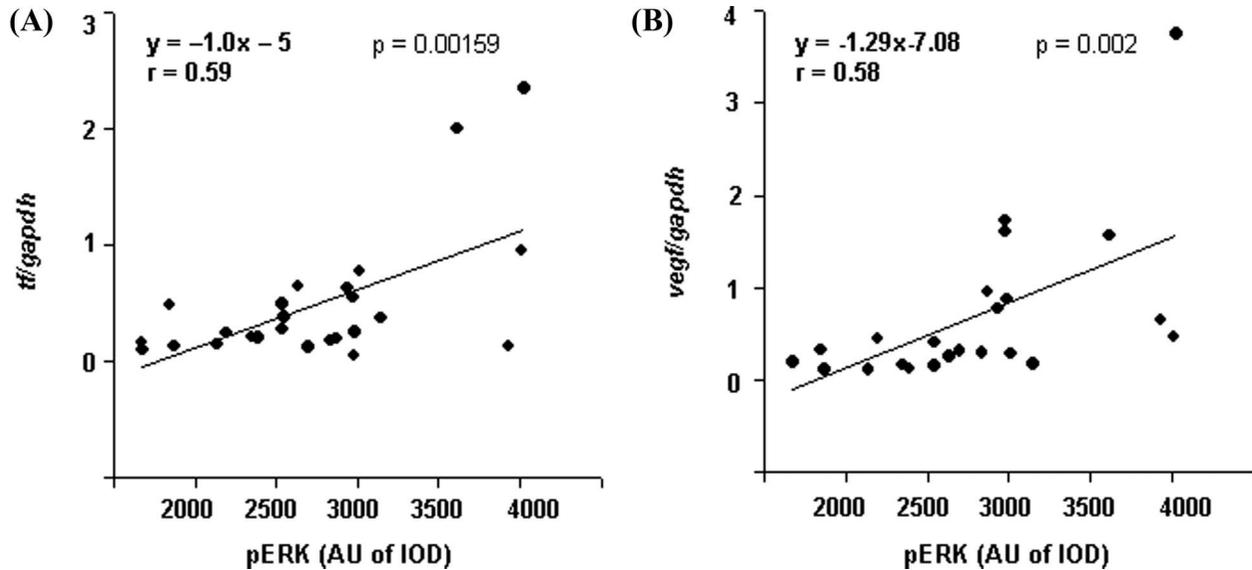


Figure 4. Positive correlations among ERK activity, TF, and VEGF expression. Correlations were assessed by Pearson product-moment correlation. Differences were considered significant if $p < 0.05$.

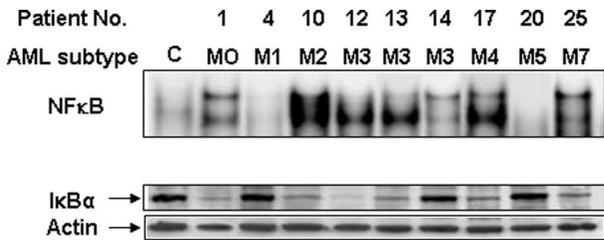


Figure 5. NF κ B binding activity in blast cells from AML patients. Representative EMSA of nine AML patients and one healthy control done using digoxigenin-labeled NF κ B consensus oligonucleotides, as described in Material and Methods. The proteins binding to the NF κ B consensus motif were characterized as members of the NF κ B family, based on the interaction with antibodies directed against proteins of the NF κ B family. Cytoplasmic extracts were subjected to Western blot analyses using I κ B α and anti-actin antibodies. The panel is representative for three independent experiments with similar results.

inhibitor also prevented the constitutive mRNA gene expression and protein synthesis of TF and VEGF found in basal AML cells [Figures 7(A) and 7(B)]. Since the NF κ B binding to the promoter of the TF gene is required for induction of gene transcription, we examined whether PD98059 might exert its effect through inhibition of NF κ B translocation. Treatment of the cells with 20 μ M PD98059 largely abolished the NF κ B binding activity in all the samples tested. The Figure 7(C) illustrates a representative EMSA of three AML patients. In addition, suppression of NF κ B binding activity by using the specific inhibitor SN50 also impaired angiogenesis, in part through downregulation of the angiogenic molecules TF, VEGF, and KDR (Figure 8). These

data provide a further evidence for the essential role of NF κ B in AML angiogenesis.

Outcome of the disease among AML patients with coordinated alteration in the expression, and/or activation of the analyzed proteins

Among the AML patients analyzed, 12 suffered fatal outcome in the following six months after diagnosis, mainly due to coagulopathy, septic shock, or pneumonia (Table I). Both TF and VEGF were found elevated and simultaneously expressed in blast cells from 10 of those patients, and correlated with both increased pERK and NF κ B activity.

In keeping with these observations, we suggest that the coordinated deregulation in the expression and activation of those proteins, found in approximately 55% of patients with AML, may be partly responsible for their more aggressive leukemia phenotype and/or for their poor outcome. Nevertheless, larger clinical trials will allow a definite assessment of the prognostic value of the coordinated deregulation in the expression of the analyzed proteins in AML.

Discussion

In the last few years, it has become clear that the processes of tumor angiogenesis, metastasis, and invasiveness are highly dependent on components of the blood coagulation cascade. One of the key proteins in coagulation is the TF. Such protein significantly participates in thrombosis and tumor-associated angiogenesis and its expression levels have been correlated with the invasive and metastatic

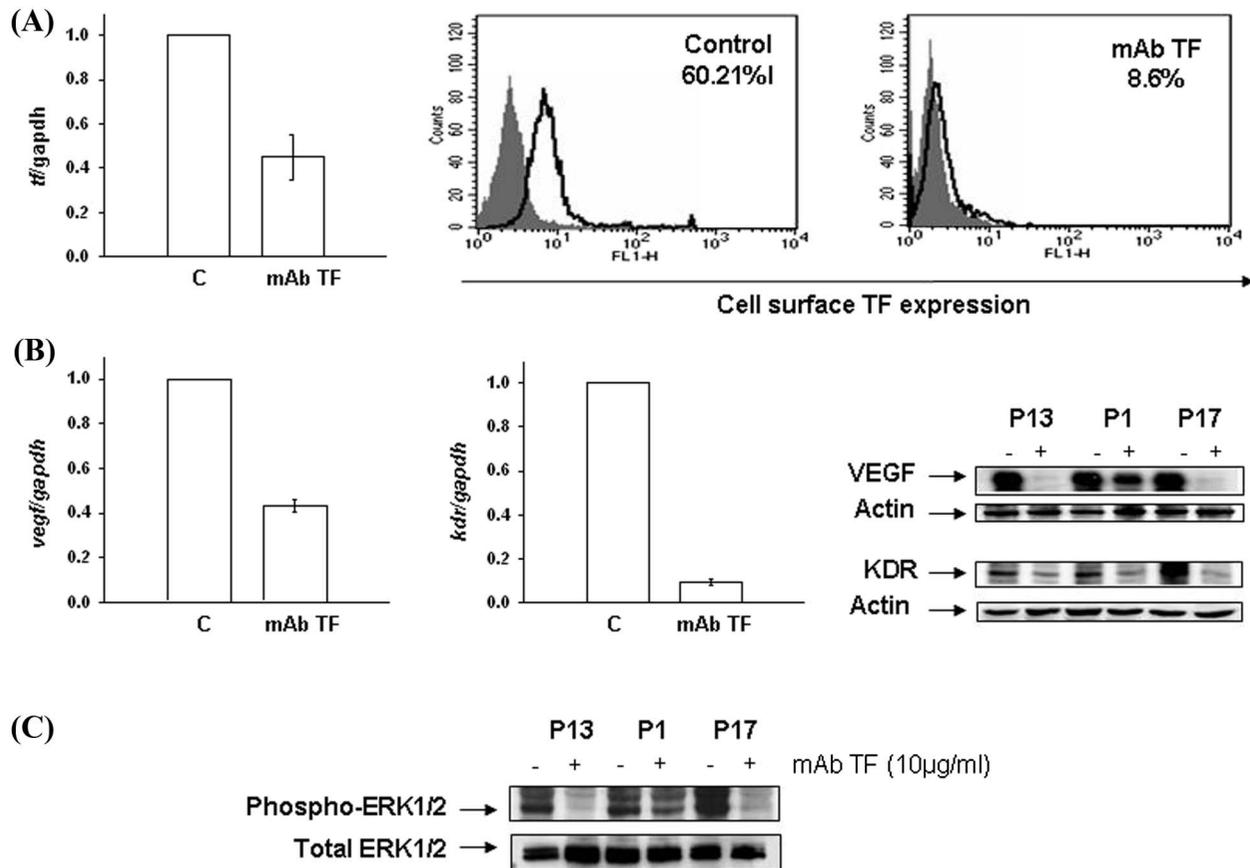


Figure 6. Anti-tissue factor antibody effects in the constitutive expression of TF and VEGF and down-stream targets. Anti-tissue factor monoclonal antibody TF was administered to blast cells at a dose of 10 $\mu\text{g/ml}$ per 5×10^5 cells during 3 h. Cells were then harvested and processed to quantitate *tf*, *vegf*, and *kdr* mRNA gene expression and protein TF, VEGF, and KDR synthesis, and both ERK and $\text{NF}\kappa\text{B}$ activation as described in Material and Methods. (A) mRNA relative gene expression of *tf* and cell surface TF presence in mAb TF-treated blast cells and controls. Significant differences at $p < 0.05$ versus control cells. Histograms show cell surface TF presence on AML blast cells from a representative subject, by flow cytometry. (B) Relative mRNA gene expression levels of *vegf* and *kdr*. The bar graphs show the mean mRNA levels \pm SEM of *vegf* and *kdr* of 10 treated AML samples. The panels on the right hand side of bar graphs are representative Western blots of various samples from AML patients treated as described in Material and Methods. (C) pERK activation in blasts from AML patients after mAb TF treatment. The panels are representative of three AML patients.

potential of many types of haematological malignancies. Indeed, upregulation of VEGF and angiogenesis has been demonstrated in haematological neoplasias. However, the origin of constitutive TF and VEGF expression, as well as their association with other molecular markers relevant to the development of these neoplasias, remains unknown.

In the present study, we found significantly higher *vegf* and *tf*-mRNA, as well as VEGF and TF protein levels in the majority of the samples analyzed, when compared with that of controls, with a positive correlation between them. The extent of VEGF and TF expression varied among the different samples tested, but was higher in all subtypes of the AMLs FAB classification. Our results are also in agreement with a recent work [24] showing that VEGF expression tends to be lower in undifferentiated leukemia cells. Besides, such data further correlated with

previous studies, showing that both TF and VEGF are coexpressed under similar conditions and in some solid tumors [11]. In that way, some transcription factors, including the specificity protein-1 (SP-1), the activator protein-1 (AP-1) and the nuclear factor kappa B ($\text{NF}\kappa\text{B}$), have been shown to be involved in the regulation of both TF and VEGF, offering a potential mechanism for their co-regulation and protein co-localization in many tumors [16]. Concomitantly, fresh leukemia samples also expressed the VEGF receptor KDR, thus suggesting the possibility of an autocrine pathway in which the tumor cells may stimulate their own growth after VEGF exposure [25]. The coexpression of both VEGF and VEGF receptors also found in lymphoma and multiple myeloma, coupled with their direct effects on tumor cell survival, migration, and proliferation, further points to the pivotal role of

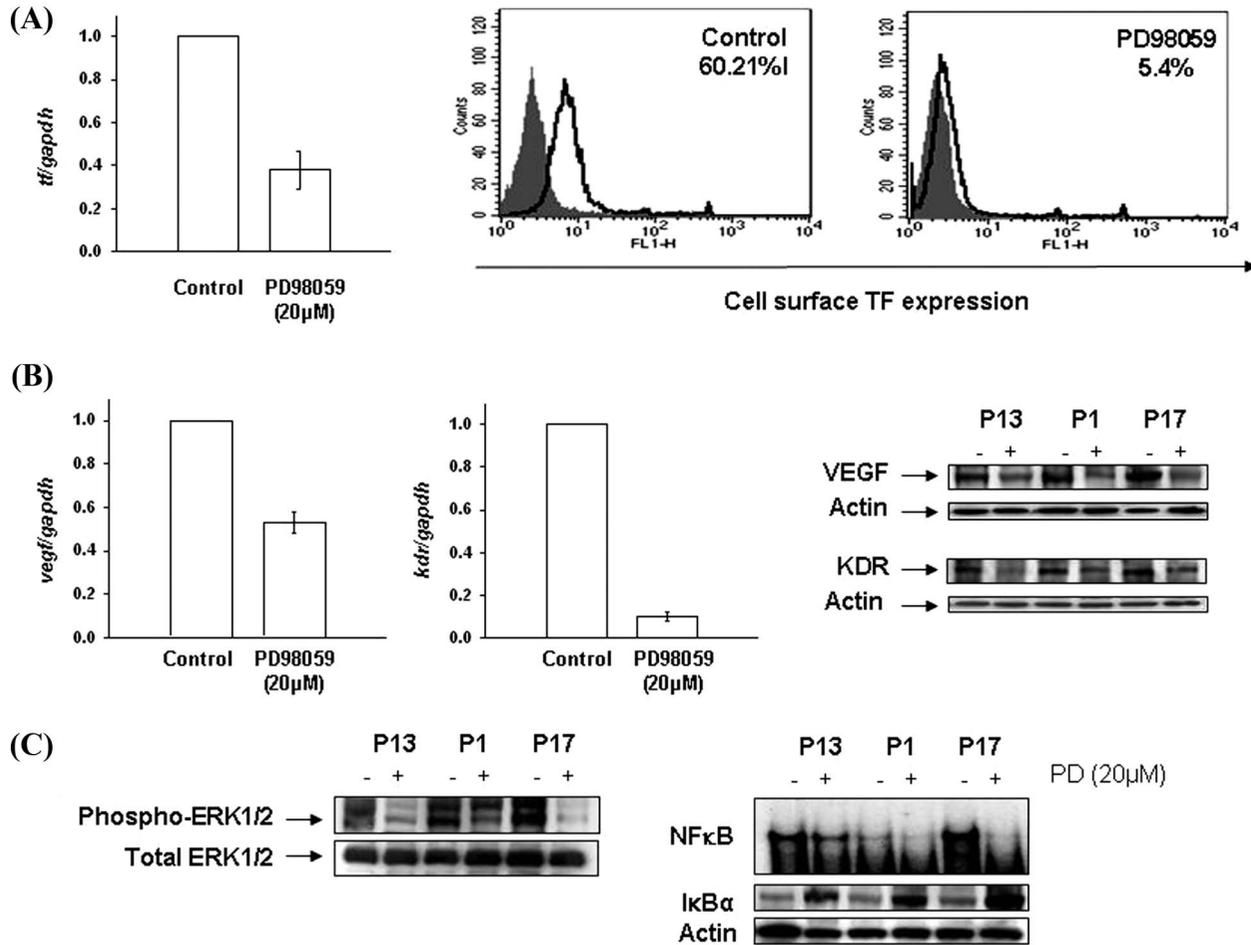


Figure 7. MEK/ERK blockade effects in the constitutive expression of TF and VEGF and down-stream targets. PD98059 (PD) was administered to cells at a dose of 20 μ M per 5×10^5 cells during 3 h. Cells were then harvested and processed to quantitate *tf*, *vegf*, and *kdr* mRNA gene expression, protein TF, VEGF and KDR synthesis, and both ERK and NF κ B activation as described in Material and Methods. (A) Relative *tf* mRNA gene expression levels and cell surface TF presence in mAb TF-treated blast cells and controls. Significant differences at $p < 0.05$ versus control cells. Histograms show cell surface TF presence on AML blast cells from a representative subject, by flow cytometry. (B) Relative *vegf* and *kdr* mRNA gene expression levels. The bar graphs show the mean levels \pm SEM of *vegf* and *kdr* mRNA of 10 treated AML samples. The panels on the right hand side of bar graphs are representative Western blots of various samples from AML patients treated as described in Material and Methods. (C) ERK activation and NF κ B binding activity in blasts from AML patients after PD treatment. Panels are representative of three AML patients.

autocrine VEGF loops in the pathogenesis of these malignancies [26].

The accumulating data indicate that the TF expressed by tumor cells as well as host cells may initiate direct or indirect signaling events that support tumor angiogenesis. In this study, the possible link between TF and VEGF expression in AML cells and the related down-stream targets was investigated by administering a rapidly neutralizing monoclonal TF antibody to a subset of AML samples. The anti-TF monoclonal antibody treatment significantly repressed *tf*, *vegf*, and *kdr* gene expression, as well as inhibited their peptide synthesis, thus suggesting a significant participation of the TF in tumor-associated angiogenesis, as previously demonstrated for solid tumors (17). Previous studies have shown

that binding of FVII(a) to cell surface associated TF results in the production of intracellular signals through cytosolic calcium alteration, mitogen activated protein kinase (MAPK) phosphorylation, and upregulation of multiple genes. In our hands, the inhibition of ERK activation after anti-TF treatment of AML cells further suggests that the TF expressed by cancer cells appears to act as both a regulatory target and as an important mediator of oncogene-driven tumor growth and neovascularization.

Our results also suggest that the constitutively activated ERK pathway found in leukemic blasts further sustained VEGF and TF overexpression in AML cells. Several groups have recently provided substantial evidence that the MEK/ERK signaling module is frequently deregulated in myeloid

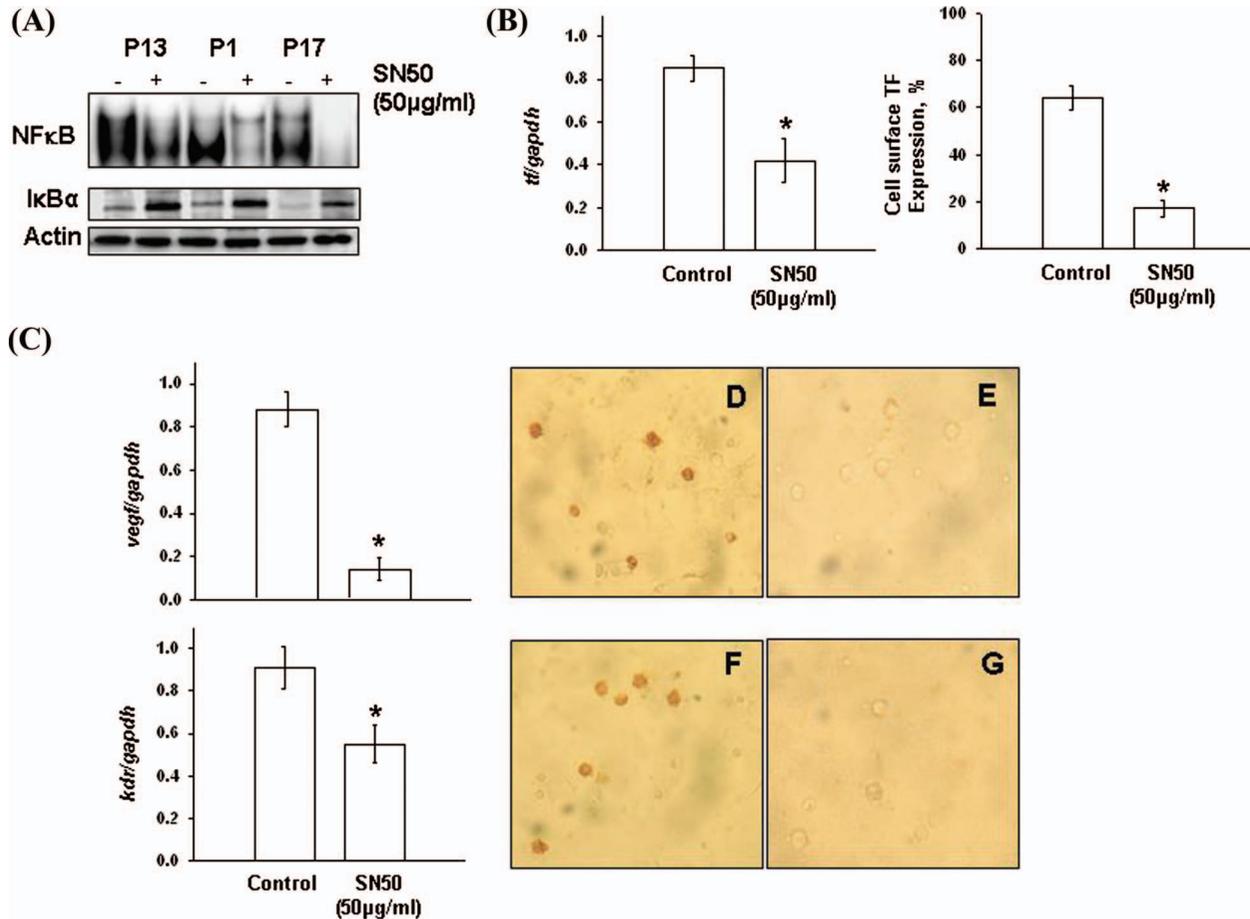


Figure 8. NFκB blockade effects in the constitutive expression of TF and VEGF. SN50 was administered to cells at a dose of 50 μg/ml per 5×10^5 cells during 3 h. Cells were then harvested and processed to evaluate *tf*, *vegf*, and *kdr* mRNA gene expression and TF, VEGF, and KDR protein synthesis, as well as NFκB activation as described in Material and Methods. (A) NFκB binding activity in blasts from AML patients after SN50 treatment. Panels are representative of three AML patients. (B) Relative *tf* mRNA gene expression levels and cell surface TF presence in SN50-treated blast cells and controls. Significant differences at $p < 0.05$ versus control cells. (C) Relative *vegf* and *kdr* mRNA gene expression levels. The bar graphs show mean levels ± SEM of *vegf* and *kdr* mRNA of 10 treated AML samples. (D–G) Representative photographs from AML blasts immunostained with anti-VEGF or anti-KDR antibodies, followed by peroxidase-conjugated avidin and DAB staining. (D,E) Representative photographs from AML blasts immunostained with anti-VEGF, before and after treatment with SN50, respectively; (F,G) Representative from AML blasts immunostained with anti-KDR, before and after treatment with SN50, respectively; original magnification, × 120.

leukemias, as a result of genetic and epigenetic aberrations involving both receptor-associated and cytoplasmic tyrosine kinases, as well as inhibitory phosphatases. In addition, its pharmacological blockade by selective small-molecule inhibitors has been shown to profoundly impair leukemic cell proliferation and clonogenic growth. Our in vitro studies further suggest a direct involvement of MEK/ERK activation on both TF and VEGF expression in AML cells.

These results also support recent studies, which have demonstrated that some transcription factors regulating *tf* can also induce *vegf* transcription [11]. VEGF is regulated in human cancer cell lines by the ERK signaling pathway and, at the same time, activation of the MAP kinase pathway is required to

a large degree for TF activation by the VEGF protein [27]. Thus, the MAPK seems to be a common pathway that independently regulates VEGF and TF expression and, at the same time, is involved in the control of TF or VEGF expression induced by each other.

We also showed that among the investigated leukemia cases, NFκB was frequently constitutively activated, and that this activation correlated with the increased expression of the *tf* mRNA. Many studies have shown that increased TF expression can be explained on the basis of the induction of different transactivating transcription factors, such as members of the nuclear factor kappa B family [28,29]. NFκB has also been shown to regulate the expression of proangiogenic molecules, such as IL-8 [30]. In

this study, we set out to determine whether the activation of NF κ B correlated with the degree of neoplastic angiogenesis. By using an inhibitor of NF κ B nuclear translocation, we have demonstrated that NF κ B signaling blockade significantly repressed the constitutive expression of both TF and VEGF found in AML cells. Thus, our data support the role that NF κ B may play in angiogenesis, an essential feature of progressive tumor growth.

Moreover, a recent study has described the involvement of the Ras-induced Raf/MEK/ERK pathway in activating NF κ B DNA binding and transactivation [19]. Thus, the simultaneous activation of the ERK pathway found in the leukemia samples could help to explain this concomitant activation. Moreover, our *in vitro* studies showing the inhibition of the NF κ B binding activity after MEK/ERK blockade further support this hypothesis. Furthermore, recent data suggest that in a variety of tumors, several NF κ B target genes encode secreted growth factors that induce NF κ B activation [31]. Therefore, autocrine loops might be also relevant in the constitutive activation of NF κ B in cancer.

Clinical studies showed a high incidence of fatal outcomes among patients in whom a coordinated increase in the expression and activation of the above mentioned proteins was observed. Both the TF and VEGF proteins, at remarkably high levels and simultaneously synthesized in blast AML cells, might determine an increase in tumor angiogenesis, thus linking both proteins to pathophysiological processes. Furthermore, the constitutive activation status of intracellular pathways such as MEK/ERK MAP kinase or NF κ B, which are also linked to the activation of many other oncogenic proteins, might contribute to the adverse development of the disease. Activation of these pathways might be triggered by autocrine production of growth factors or by a positive synergistic relationship between the leukemic and endothelial cells, through the paracrine production by each of mitogenic growth factors.

Taken together, our study strongly suggests that there is a coordinated deregulation of cellular receptors, proangiogenic factors and intracellular pathways in the same leukemia sample, which might be related to clinical features. These altered signals may be critical to predict a poor outcome; therefore, their study could provide information needed to tailor therapy for individual patients with acute leukemia.

Acknowledgements

This work was supported by grants from the Fondo de Investigación Sanitaria (PI041291 and PI050910), the Junta de Andalucía (24/2005 and 60/2005) of Spain, and FIJC-06/ESP.

References

1. Lee JT, McCubrey JA. The Raf/MEK/ERK signal transduction cascade as target for chemotherapeutic intervention in leukemia. *Leukemia* 2002;16:486–507.
2. Burnett AK. Introduction: modern management of acute myeloid leukemia. *Semin Hematol* 2001;38:1–2.
3. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetic on outcome in AML: analysis of 1612 patients entered into the MRC AML 10 trial. *Blood* 1998;92:2322–2333.
4. Winton EF, Langston AA. Update in acute leukemia 2003: a risk adapted approach to acute myeloblastic leukemia in adults. *Semin Oncol* 2004;2:80–86.
5. Tallman S, Kwaan HC. Intravascular clotting activation and bleeding in patients with hematologic malignancies. *Rev Clin Exp Hematol* 2004;8(1):E1.
6. Lopez-Pedreria Ch, Jardí M, Malagón MM, Inglés-Esteve J, Dorado G, Torres A, et al. Tissue factor (TF) and urokinase plasminogen activator receptor (uPAR) and bleeding complications in leukemic patients. *Thromb Haemost* 1997;77:62–70.
7. Velasco F, López-Pedreria Ch, Borrell M, Fontcuberta J, Borell M, Torres A. Elevated levels of tissue factor pathway inhibitor in acute non-lymphoblastic leukemia patients with disseminated intravascular coagulation. *Blood Coagul Fibrinol* 1997;8:70–72.
8. Nadir Y, Katz T, Sarig G, Hoffman R, Oliven A, Rowe JM, et al. Hemostatic balance on the surface of leukemic cells: the role of tissue factor and urokinase plasminogen activator receptor. *Haematologica* 2005;90(11):1549–1556.
9. Rak J, Milsom C, May L, Klement P, Yu J. Tissue factor in cancer and angiogenesis: the molecular link between genetic tumor progression, tumor neovascularization, and cancer coagulopathy. *Semin Thromb Hemost* 2006;32:54–70.
10. Versteeg HH, Spek CA, Peppelenbosch MP, Richel DJ. Tissue factor and cancer metastasis: the role of intracellular and extracellular signaling pathways. *Mol Med* 2004;10:6–11.
11. Chen J, Bierhaus A, Schiekofer S, Andrassy M, Chen B, Stern DM, et al. Tissue factor: a receptor involved in the control of cellular properties, including angiogenesis. *Thromb Haemost* 2001;86:334–345.
12. Sampson MT, Kakkar AK. Coagulation proteases and human cancer. *Biochem Soc Trans* 2002;30:201–207.
13. Belting M, Dorrel MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, et al. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 2004;10:502–509.
14. Ruf W, Mueller BM. Tissue factor signaling. *Thromb Haemost* 1999;82:175–182.
15. Towatari M, Lida H, Tanimoto M, Iwata H, Hamaguchi M, Saito H. Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia* 1997;11:479–484.
16. Versteeg HH, Pappelenbosch MP, Spek CA. The pleiotropic effects of tissue factor: a possible role for factor VIIa-induced intracellular signaling? *Thromb Haemost* 2001;86:1353–1359.
17. Bellamy WT. Expression of vascular endothelial growth factor and its receptors in multiple myeloma and other hematopoietic malignancies. *Semin Oncol* 2001;28:551–559.
18. Padro T, Bieker R, Ruiz S, Steins M, Retzlaff S, Burger H, et al. Overexpression of vascular endothelial growth factor (VEGF) and its cellular receptor KDR (VEGFR-2) in the bone marrow of patients with acute myeloid leukemia. *Leukemia* 2002;16(7):1302–1310.

19. Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth? *J Invest Dermatol Symp Proc* 2000;5:24–33.
20. Birkenkamp KU, Geugien M, Schepers H, Westra J, Lemmink HH, Vellenga E. Constitutive NF- κ B DNA-binding activity in AMLs is frequently mediated by Ras/PI3K/PKB-dependent pathway. *Leukemia* 2004;18:103–112.
21. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991;19:2499.
22. Schreiber E, Mathias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with mini-extracts, prepared from a small number of cells. *Nucleic Acids Res* 1989;17:6419.
23. Zwacka RM, Zhang Y, Zhou W, Halldorson J, Engelhardt JF. Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor kappaB independently of IkappaB degradation. *Hepatology* 1998;28:1022–1030.
24. Ghannadan M, Wimazal F, Simonitsch I, Sperr WR, Mayerhofer M, Sillaber C, et al., Immunohistochemical detection of VEGF in the bone marrow of patients with acute myeloid leukemia. Correlation between VEGF expression and the FAB category. *Am J Clin* 2003;119(5):663–671.
25. Hussong JW, Rodgers GM, Shami PJ. Evidence of increased angiogenesis in patients with acute myeloid leukemia. *Blood* 2000;95:309–313.
26. Dias S, Shmelkov SV, Lam G, Rafii S. VEGF(165) promotes survival of leukemic cells by Hsp-90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* 2002;99:2532–2540.
27. Mechtcheriakova D, Schabbauer G, Lucerna M, Clauss M, Martin R, Binder BR, et al. Specificity, diversity and convergence in VEGF and TNF α signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *FASEB J* 2001;15:230–242.
28. Hall AJ, Vos HL, Bertina RM. Lipopolysaccharide induction of tissue factor in THP-1 involves Jun protein phosphorylation and nuclear factor kappa B nuclear translocation. *J Biol Chem* 1999;274:376–383.
29. Oeth P, Parry GC, Mackman N. Regulation of the tissue factor gene in human monocytic cells. Role of AP-1, NFkappa B/Rel, and Sp1 proteins in uninduced and lipopolysaccharide-induced expression. *Arterioscler Thromb Vasc Biol* 1997;17:365–374.
30. Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ. Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. *Cancer Res* 2000;60:5334–5339.
31. Lu T, Sathe SS, Swiatkowski SM, Hampole CV, Stark GR. Secretion of cytokines and growth factors as a general cause of constitutive NFkappaB activation in cancer. *Oncogene* 2004;23:2138–2145.

Copyright of Leukemia & Lymphoma is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.