

Amifostine exerts anti-angiogenic activity and suppresses vascular endothelial growth factor secreted by hemopoietic stem/progenitor cells

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ABSTRACT

Objective: To assess, in vitro, the effect of Amifostine (AMF, WR-2721) on angiogenesis and levels of vascular endothelial growth factor (VEGF) secreted from hemopoietic stem/progenitor cell populations.

Methods: We conducted the study in the research laboratories of the Hashemite University, Jordan between September 2003 and January 2005 where we took samples were from Myelodysplastic syndrome (MDS) patients and healthy donors attending Al-Hussein Cancer Center and We determined the proliferation of human umbilical vein endothelial cells (HUVECs) in cultures supplemented with media conditioned with AMF-treated and AMF-untreated pure hemopoietic cells [CD34+ cells, and erythroid, myeloid and megakaryocytic progenitors]. Furthermore, in the same conditioned media, we evaluated levels of elaborated VEGF by a sensitive enzyme linked immunosorbent assay.

Results: Biologically, media conditioned with AMF-treated cells reduced proliferation of HUVECs compared to media conditioned with untreated control cells ($p < 0.05$). In cultures of AMF-untreated cells, elaboration of VEGF was higher ($p < 0.05$) in media conditioned with cells from MDS patients compared to healthy donors. A 30 minutes pre-exposure of cells to AMF (500 mM) suppressed levels of VEGF secreted within 24 hours in 63 of 89 evaluated cultures. The percentage of reduction of VEGF in AMF-sensitive cultures was comparable in cultures of MDS cells (18%, 2-37%; median, range) and normal cells (12%, 2-45%).

Conclusion: The results showed that AMF exerts an anti-angiogenic activity and suppresses the secretion of VEGF in hemopoietic stem/progenitor cells obtained from both healthy individuals and patients with MDS.

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Angiogenesis is the formation of new capillaries from the existing vasculature. We know that the progression (growth, dissemination, and metastasis) of solid tumors is dependent on angiogenesis.¹⁻³ The outcome of this active process depends upon the balance between several positive and negative regulatory molecules.¹⁻⁴ Among these molecules, vascular endothelial growth factor (VEGF) is reported with apparent angiogenic activity.^{5,6} Studies of angiogenesis in hematological

malignancies, in particular, in patients with myeloid leukemias and Myelodysplastic syndrome (MDS) revealed a positive correlation between disease progression and angiogenesis as assessed by estimating bone marrow (BM) microvessel density and the expression profile of angiogenic factors especially VEGF.⁷⁻¹¹ The increased vascularity reported in myeloid malignancies,⁷ the prognostic importance of angiogenic factors in leukemias and MDS,⁹ and the detection of receptor of angiogenic

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factors in leukemic cells,^{9,11} suggest that angiogenic factors may have a direct effect on marrow vascularity, growth of leukemic cells and disease progression. Based on these findings, several anti-angiogenic agents are now available for treating leukemia and MDS.¹² Amifostine (AMF) is a phosphorylated Amino-Thiol that exerts a broad-spectrum cytoprotection against side effects of chemotherapy and radiotherapy.^{13,14} The pharmacological benefit of AMF ensues from the fact that it selectively protects normal tissues, leaving the anti-tumor effects of chemotherapeutic agents and ionizing radiation either unchanged or enhanced.¹⁴ Although, reports suggest that AMF acts by lowering the apoptotic threshold, preclinical data indicate that it may inhibit the formation of spontaneous metastasis.¹⁵ In the hematopoietic system, studies found that pre-exposure to AMF stimulates *in vitro* colony formation and improves survival of BM progenitors in cultures from normal individuals and some patients with MDS.^{16,17} To date, it remains unanswered whether AMF affects the expression of angiogenic factors in hemopoietic stem cells (HSC)/progenitors. In the present study, we evaluate, *in vitro*, the effect of AMF on secretion of VEGF by immature hemopoietic cells. We quantified the VEGF in media conditioned with highly pure CD34+ cells and a lineage-specific precursor cells. Moreover, we examined the effect of conditioned media on proliferation of endothelial cells in cultures of human umbilical vein endothelial cells (HUVECs).

Methods. Patients, samples, and the generation of erythroid, myeloid and megakaryocytic progenitors from CD34+ cells. Eight samples were obtained from consenting healthy allogeneic donors of HSC (6 BM and 2 granulocyte-colony stimulating factor-mobilized peripheral blood stem cells [PBSCs] samples). Another 15 BM samples were obtained from MDS patients with French American British (FAB) types: refractory anemia (RA) (6 patients), RA with ringed sideroblasts (RARS) (2 patients), RA with excess blasts (RAEB) (3 patients), RAEB in transformation (RAEB-t) (2 patients), and chronic myelomonocytic leukemia, (CMML) (2 patients).¹⁸ Patients and healthy donors were of closely matched age and gender. From low-density (<1.077 g/ml) mononuclear cells, highly pure CD34+ cell populations were isolated by 2 cycles of positive selection using the magnetic cell sorting system (CD34 isolation kit; Miltenyi Biotech; Auburn, CA).¹⁹ The CD34+ cells were grown at 10⁴/ml in serum-free medium (Iscove's modified Dullbecco's medium [IMDM]; Life Technologies; Rockville, MD) supplemented with 20% BIT 9500 (detoxified charcoal filtered bovine serum albumin, iron-saturated transferrin,

and insulin; Stem Cell Technologies), 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. Growth of burst forming unit-erythroid (BFU-E) was stimulated with recombinant human (rh) erythropoietin (2U/ml), rh stem cell factor (20 ng/ml) and rh interleukin-6 (10 ng/ml). Colony forming unit-granulocyte monocyte (CFU-GM) growth was stimulated with rh interleukin-3 (IL-3, 10 ng/ml) plus rh granulocyte-macrophages colony stimulating factor (10 ng/ml). Colony forming unit megakaryocyte (CFU-Meg) growth was stimulated with rh-IL3 (10 ng/ml) plus rh thrombopoietin (50 ng/ml). All cytokines are products of Peprotech, Rocky Hill, NJ and were kindly provided by Dr. Frank Ruscelli, Basic research laboratories, NCI-Frederick, NIH, USA. Cultures were incubated at 37°C, in a fully humidified atmosphere with 5% CO₂. After 5-7 days, the generation of different blood cell precursors from CD34+ cells was assessed by fluorescence-activated cell sorter (FACS) analysis as previously described.¹⁹ The IgG1 isotype control mouse monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC) were used along with FITC-conjugated anti-human specific mAbs to CD34, CD41, and CD33 (Becton Dickinson; San Diego, CA), and glycophorin A (GPA) (Immunotech; Marseille, France).

Cell treatment with AMF, collection of conditioned media and quantification of VEGF secreted by hemopoietic cells. Aliquots of CD34+ cells, BFU-E-, CFU-GM-, and CFU-Meg-derived cells were treated with AMF (500 mM, WR-2721, Schering Plough, kindly provided by Dr. Xenophon Yataganas, Laikon General Hospital, Athens, Greece) or with an equal volume of its solvent (sterile water) for 30 minutes at 37°C, and washed 3 times with 5% BIT 9500 in IMDM. Then, both AMF-pretreated and control cells were re cultured in serum-free conditions at equal densities (1x 10⁶ cells/ml). After 24 hours, the cells were harvested, centrifuged and supernatants representing conditioned media were collected. Conditioned media was either used freshly or stored at -20°C for less than one month. Collected conditioned media were analyzed for VEGF by Quantikine human immunoassay from R and D according to the manufacturer's instructions. The sandwich ELISA assay allowed a quantitative analysis of VEGF with sensitivity greater than 5 pg/ml. Proliferation of HUVECs. The HUVECs were isolated as previously described.¹ In brief; the HUVECs were harvested with 0.1% collagenase (Worthington Biomedical Corp) and grown in complete growth medium [199 (M199) containing 30% fetal calf serum, basic-fibroblast growth factor (FGF) (10 ng/ml), heparin (10 U/ml) and hydrocortisone (1 mg/ml)]. When confluent, endothelial cells were harvested by trypsin digestion with 0.5% trypsin-EDTA. Endothelial cells were identified by their typical cobblestone morphology and the

presence of von Willebrand factor by indirect immunofluorescence assay (data not shown). To test the influence of conditioned media on the growth of endothelial cells, isolated endothelial cells were seeded 24 hours before the experiment in 96 wells coated with fibronectin at 2.5×10^3 cells/ml. The next day, wells were washed 3 times with pre-warmed M199, and grown in 100 μ l of: a) HUVEC growth medium, b) serum-free M199 medium, c) serum-free M199 media conditioned with AMF-treated cells and, d) M199 media conditioned with water-treated cells. Media was replaced by fresh ones every 2 days. At day 4, proliferation of HUVECs was evaluated by colorimetric method using WST-1 reagent (Chemicon International Inc., Temecula, CA) according to the manufacturer's instructions. Absorbance readings were converted to percentage of proliferation considering 100% proliferation in wells containing HUVEC growth media. Cultures in each testing were in triplicates (3 wells) and values obtained are the mean of the 3 readings.

Statistical analysis. To study the effect of AMF, results are expressed as a mean value \pm SD and the paired Student t-test value was used to determine the statistical significance of differences between means. Medians were compared, and Kruskal-Wallis analysis was used, to compare the values between normal individuals and MDS patients.

Results. It was possible to isolate highly pure, and cell populations from BM aspirates ($94 \pm 2\%$) and mobilized PBSCs collections ($96 \pm 3\%$). Following stimulation of CD34+ cells to give lineage-specific cells, using FACS analysis indi-

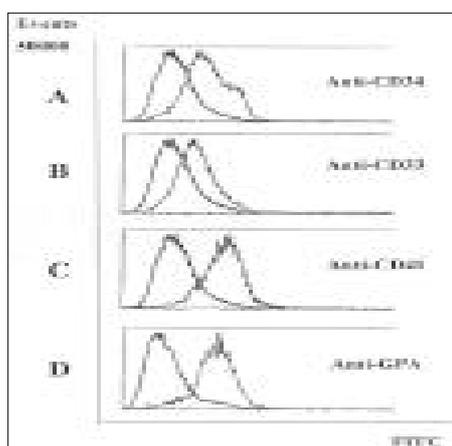


Figure 1 - Representative fluorescence-activated cell sorter analysis results of a) CD34+ cells, b) colony forming unit-granulocyte monocyte, c) colony forming unit-megakaryocyte, and d) burst forming unit-erythroid derived cells. Cells were stained with lineage specific fluorescein isothiocyanate (FITC) labeled monoclonal antibodies as indicated. GPA - glycophorin A

cated that approximately $98 \pm 2\%$ of BFU-E-derived cells are GPA+, $90 \pm 4\%$ of CFU-GM-derived cells are CD33+, and $91 \pm 7\%$ of CFU-Meg-derived cells are CD41+ (Figure 1). In very few cases, when purity did not exceed 85%, cells were not used further to prepare conditioned media. To determine whether cell treatment with AMF would affect the biology of endothelial cells, proliferation of HUVECs was evaluated in serum free media conditioned with AMF-treated and control cells. Stimulated proliferation of HUVECs was observed in serum free media conditioned with control cells including CD34+ cells, myeloid, erythroid and megakaryocytic precursors compared to serum free media (Figure 2). This stimulation of proliferation was significantly reduced when HUVECs were grown in media conditioned with the same but AMF-treated cells ($p < 0.05$, versus control cells). To compare levels of VEGF secreted by normal versus MDS cells, conditioned media collected from AMF-untreated cultures were evaluated, not in separate experiments, by sandwich ELISA. We found a significant increase ($p < 0.05$) in VEGF secretion by CD34+ cells, CFU-GM-, and CFU-Meg-, but not BFU-E-derived cells from patients with MDS ($n=15$) compared to healthy donors ($n=8$) (Figure 3). The distribution of VEGF levels elaborated in media

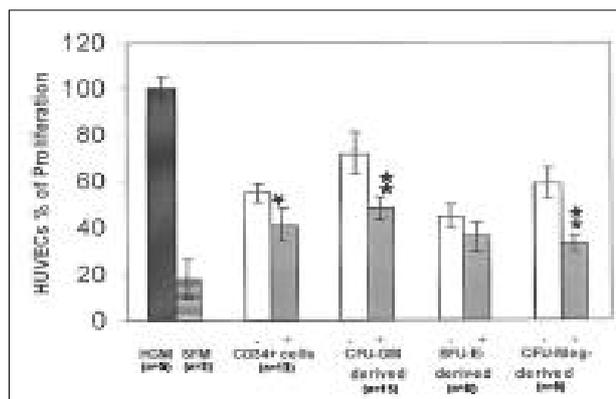


Figure 2 - Effect of media conditioned with Amifostine-treated hemopoietic cells on proliferation of human umbilical vein endothelial cells (HUVECs). Media conditioned with different pure cell populations untreated and pre-treated with Amifostine as described in methods. Human umbilical vein endothelial cells were cultured for 4 days in HUVEC growth media (HGM), serum free media (SFM), and media conditioned with untreated control cells (-) or AMF-treated cells (+). Percentage of HUVECs proliferation was determined by comparing optical density (OD) readings. The ODs obtained from wells treated with HGM were representing optimal 100% proliferation. * $p < 0.05$ and ** $p < 0.01$ (compare effect of media conditioned with Amifostine-treated cells versus control cells). CFU-GM - colony forming unit-granulocyte monocyte, BFU-E - burst forming unit-erythroid, CFU-Meg - colony forming unit-megakaryocyte.

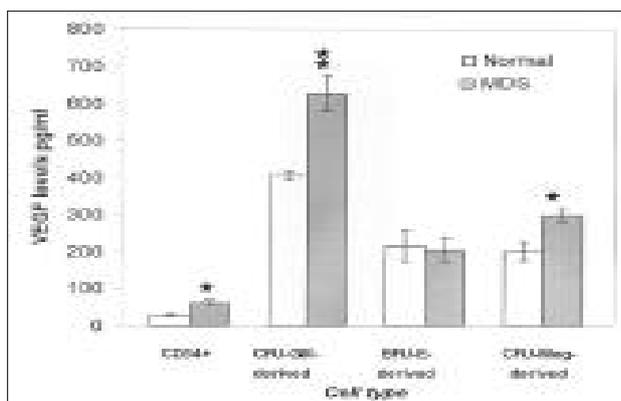


Figure 3 - Levels of vascular endothelial growth factor (VEGF, pg/ml) secreted by hemopoietic cells from normal individuals and myelodysplastic syndrome (MDS) patients as determined by ELISA in media conditioned with normal hematopoietic cells (n=8) and cells from patients with MDS (n=13-15). Values expressed as median + 1 standard error (SEM). P-values are calculated when results of MDS cells were compared with those of normal cells using Kruskal-Wallis test. * $p < 0.05$ and ** $p < 0.05$. CFU-GM - colony forming unit-granulocyte monocyte, BFU-E - burst forming unit-erythroid, CFU-Meg - colony forming unit- megakaryocyte.

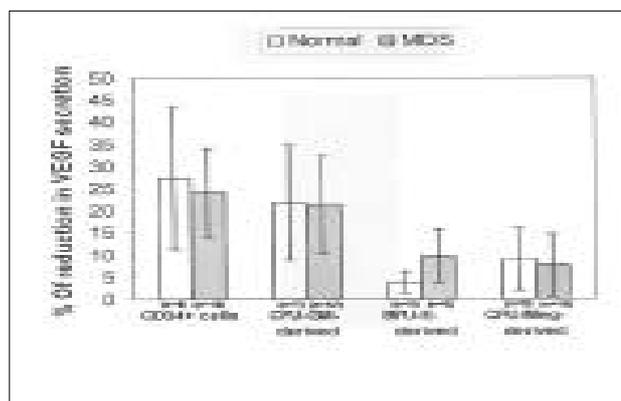


Figure 4 - Effect of AMF on secretion of vascular endothelial growth factor (VEGF) by hemopoietic cells. Media conditioned with various AMF-treated and AMF-untreated cells were collected as described in the methods, and analyzed by ELISA for VEGF elaboration. Percent reduction in VEGF contents resulted from AMF treatment was calculated and expressed as mean values of n number of cultures + 1 SD. MDS - myelodysplastic syndrome AMF - Amifostine, CFU-GM - colony forming unit-granulocyte monocyte, BFU-E - burst forming unit-erythroid, CFU-Meg - colony forming unit-megakaryocyte.

Table 1 - Distribution of levels of vascular endothelial growth factor (VEGF) secreted from hemopoietic cells obtained from patients with low and high risk myelodysplastic syndrome (MDS) patients compared to those secreted by cells from healthy individuals.

Cell type	Healthy individuals percentile value		MDS patients*			
	25th	75th	Low risk		High risk	
			<25th	>75th	<25th	>75th
CD34+	19.25	35.0	1/8	5/8	0/7	7/7
Colony forming unit-granulocyte monocyte-derived	391.25	436.5	1/8	6/8	0/7	6/7
Burst forming unit-erythroid-derived	116.75	341.75	2/8	0/8	0/7	1/7
Colony forming unit- megakaryocyte-derived	168.0	210.0	0/6	6/6	0/7	6/7

*results expressed as number MDS cultures/total that gave levels of VEGF below 25th percentile or above 75th value determined in cultures of same type of normal cells.

conditioned with cells from low risk (RA and RARS) and high risk (RAEB, RAEB-t and CMML) MDS patients were compared with those from normal cells (Table 1). The majority (17 of 22 and 19 of 21) of cultures of CD34+ cells, CFU-GM-, and CFU-Meg-derived cells (from low risk and high risk patients) gave VEGF levels above the corresponding 75th percentile value obtained from normal cell cultures. In an attempt to explain how AMF reduced growth of HUVECs, levels of VEGF in media conditioned with AMF-treated and AMF-untreated hemopoietic cells were compared. Pure CD34+ cells, myeloid, erythroid and megakaryocytic precursors were pretreated with the

predetermined optimal dose (the lowest dose with maximal effect) of AMF (500 mM). After 24 hours of culture in serum free conditions, media conditioned with the same number of AMF-treated and control cells were compared for VEGF contents. Pretreatment with AMF reduced levels of VEGF in 63 of 89 cultures (23/31 and 40/58 cultures of normal and MDS cells) and remained unchanged in the rest of cultures (26/99). The percentages of reduction of VEGF in the AMF-sensitive cultures (n=63) were comparable, 12%, 2-45%, and 18.5%, 2%-37% (median, range) in cultures of normal and MDS cells (Figure 4). The number of MDS patients studied from each FAB

subgroup was statistically low to compare the effects of AMF on VEGF secretion by cells from different MDS subgroups.

DISCUSSION. Vascular endothelial growth factor plays a significant role in endothelial cell activation during early angiogenesis and maintains the differentiation state of blood vessels.^{1,6,20} Moreover, it regulates hemopoietic stem cell development, extracellular matrix remodeling, and the production of inflammatory cytokines.²⁰⁻²² It is a critical factor contributing to neoplastic angiogenesis and overexpressed in myeloid leukemias and MDS.⁷⁻¹¹ Recent drugs with anti-apoptotic activity such as amifostine and anti-angiogenic activity including VEGF receptor kinase inhibitors, thalidomide and recombinant VEGF neutralizing antibody are in clinical trials in patients with MDS.^{12,23} However, mechanisms of action of these modalities of treatment and their use in combination to improve treatment outcome are not clear. Previous studies have shown that AMF inhibits apoptosis of hemopoietic progenitors while it induces apoptosis in myelodysplastic cell lines and increases angiostatin production, indicating a possible action through angiogenesis.^{15,24,25} Recently, Giannopoulou et al²⁶ showed, in the chicken embryo chorio-allantoic membrane, a reduction of m-RNA levels of VEGF190 and VEGF165 isoforms by AMF.²⁶ In the present pre-clinical study, we evaluated the effect of AMF on secretion of VEGF by hemopoietic stem/progenitor cells.

We isolated purified CD34+ cells, myeloblasts, erythroblasts, and megakaryoblasts employing advanced cell isolation and culture techniques. We used sensitive ELISA to detect picograms of VEGF in media conditioned with these cells. We confirmed the previous findings of Majka et al,²⁷ that normal hemopoietic progenitor/precursor cells secrete significant amounts of VEGF, which were highest, in our study, in media conditioned with myeloid cells. The production of VEGF by hemopoietic cells and the expression of VEGF receptors demonstrated in macrophages, megakaryocytes and primitive HSCs,^{22,28-30} suggests that secreted VEGF would have direct impact on the development of hemopoietic cells. In accord, it was found that the VEGF promotes myeloid and suppresses erythroid progenitor cells development.³¹ Compared to cultures of cells from healthy donors, we found increased secretion of VEGF from MDS cells in most media conditioned with CD34+ cells, myeloblasts and megakaryoblasts, but not erythroblasts. Different studies demonstrated an increased cellular expression and plasma level of VEGF in patients with MDS.^{10,13,32} Aguayo et al,³³ showed the highest increase in VEGF in plasma samples from CMML patients compared to other FAB types of MDS.³³ Employing immunocytochemical staining of BM cells, Bellamy et al,³⁴

showed increased staining intensity of VEGF in myelomonocytic cells from MDS patients and detected no signals in erythroblasts and plasma cells.³⁴ Collectively, these data suggest that VEGF is not upregulated in erythroid precursors from MDS patients, and thus VEGF has no suppressive effect on erythropoiesis during disease development.

It is evident in our findings that in vitro pretreatment of different hemopoietic cells with a clinically applicable dose of AMF is associated with a significant reduction in VEGF secretion. This reduction in VEGF secretion was comparable in media conditioned with cells from healthy donors and MDS patients. Interestingly, we found cells belonged to all hemopoietic lineages to be responsive to AMF, by showing a decrease in VEGF secretion. However, the response of CD34+ cells and CFU-GM-derived cells was more than that of BFU-E- and CFU-Meg-derived cells. In contrast to our findings in hemopoietic cells, Grdina et al,³⁵ reported no effect of AMF on levels of adhesion molecules, cytokines and growth factors including VEGF secreted by human endothelial and glioma cells. Biologically, we examined the effect of media conditioned with AMF-treated and control cells on proliferation of HUVECs. Media conditioned with control cells stimulated HUVECs proliferation confirming that different hemopoietic cells secrete proteins, which work together to positively regulate the growth of endothelial cells.²⁷ We found here that treatment of HUVECs with media conditioned with AMF-treated hemopoietic cells exhibits a suppressive effect on the proliferation of HUVECs. This suppression is partially attributed by the direct effect of AMF on the production of VEGF, or other angiogenesis related molecules not evaluated in our study. Although, we found that media conditioned with CD34+ cells contained far lower levels of VEGF than those found in media conditioned with CFU-GM cells (**Figure 4**), media conditioned with CD34+ and CFU-GM cells stimulate comparable proliferation of HUVECs (**Figure 2**). The fact that CD34+ cells secrete other angiogenic factors like HGF, which were undetectable in media conditioned with CFU-GM, may explain this inconsistency.²⁷

In summary, we showed that AMF suppresses, in vitro, the secretion of VEGF by different hemopoietic cells and induces anti-angiogenic activity in HUVECs cultures. This suggests that patients with MDS, and other myeloid malignancies may benefit from the anti-angiogenic effect of AMF in addition to its effect on cell survival and apoptosis.

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