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BRIEF COMMUNICATION

Erythropoietin is involved in the angiogenic potential of bone marrow macrophages in multiple myeloma

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Abstract Erythropoietin (Epo) is the crucial cytokine regulator of red blood cell production, and recombinant human erythropoietin (rHuEpo) is widely used in clinical practice for the treatment of anemia, primarily in kidney disease and in cancer. Increasing evidence suggests several biological roles for Epo and its receptor, Epo-R, unrelated to erythropoiesis, including angiogenesis. Epo-R has been found expressed in various non-haematopoietic cells and tissues, and in cancer cells. Here, we detected the expression of Epo-R in bone marrow-derived macrophages (BMMAs) from multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) patients and assessed whether Epo/Epo-R axis plays a role in MM macrophage-mediated angiogenesis. We found that Epo-R is over-expressed in BMMAs from MM patients with active disease compared to MGUS patients. The treatment of BMMAs with rHuEpo significantly increased the expression and secretion of key pro-angiogenic mediators, such as vascular endothelial growth factor, hepatocyte growth factor and monocyte chemotactic protein (MCP-1/CCL-2), through activation of JAK2/STAT5 and PI3 K/Akt pathways. In addition, the conditioned media harvested from rHuEpo-treated BMMAs enhanced bone marrow-derived endothelial cell migration and capillary morphogenesis in vitro, and induced angiogenesis in the chorioallantoic membrane of chick embryos in vivo. Furthermore, we found an increase in the circulating levels of several pro-angiogenic cytokines in serum of MM patients with anemia under treatment with Epo. Our findings highlight the direct effect of rHuEpo on macrophage-mediated production of pro-angiogenic factors, suggesting that Epo/Epo-R pathway may be involved in the regulation of angiogenic response occurring in MM.

Keywords Angiogenesis · Erythropoietin · Macrophages · Multiple myeloma · Tumor growth

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Introduction

Vacca and colleagues demonstrated for the first time that bone marrow microvascular density was significantly increased in multiple myeloma (MM) compared to monoclonal gammopathy of undetermined significance (MGUS) and moreover in active versus non-active myeloma [1]. These findings suggest that active MM may represent the 'vascular phase' of plasma cell tumors, and non-active MM and MGUS their 'prevascular phase' [2]. Myeloma plasma cells induce angiogenesis through the secretion of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), matrix metalloproteinase-2 and -9 (MMP-2

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and MMP-9), and by induction of host inflammatory cell infiltration [3]. Moreover, interactions between plasma cells, hematopoietic stem cells, bone marrow stromal cells (BMSCs), and endothelial cells are intimately involved in all biological stages of intramedullary growth [4]. As concerns the role of bone marrow macrophages (BMMAs) in MM, when they were exposed to VEGF and FGF-2 they transformed into cells similar to paired MM endothelial cells, and generate capillary-like networks mimicking those of MM endothelial cells [5].

Erythropoietin (Epo) is a pleiotropic cytokine that exerts diverse biological effects and angiogenesis is one of its extra-hematopoietic functions [6]. Moreover, Epo modulates effects on macrophage number and function, and this effect was associated with the expression of Epo receptor (Epo-R) in BMMAs [7].

This study was designed to determine whether MM BMMAs express Epo-R and whether they are affected to express their angiogenic potential directly by Epo, examining the effects of Epo on macrophage phenotype and functions, under both in vitro and in vivo experimental conditions.

Materials and methods

Reagents

Recombinant Human Epo (rHuEpo, Eprex®, epoietin alfa) was provided by Janssen-Cilag (Saunderton, UK). Recombinant $VEGF_{165}$, LY294002 and wortmannin were purchased from Sigma-Aldrich (St Louis, MO), FGF-2 from Peprotech Inc. (Rocky Hill, NJ), and recombinant soluble human Epo-R (rHuEpo sR) from R&D Systems Inc. (Minneapolis, MN). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, antibiotic/antimycotic, trypsin/EDTA, heat-inactivated fetal bovine serum (FBS), and PBS without $Ca2^{+}$ and Mg^{2+} were from Euroclone (Pero, Italy).

Patients and cell culture

Fifty-six patients fulfilling the International Myeloma Working Group diagnostic criteria for active MM (n = 38) and MGUS (n = 18) were studied. MM patients were aged 45–77 years and D&S staged as IIA (n = 5), IIB (n = 6), IIIA (n = 18) and IIIB (n = 9). MGUS patients were aged 42–75 years. The study was approved by the local ethics Committee of the University of Bari Medical School, and all patients gave their informed consent in accordance with the Declaration of Helsinki. Bone marrow-derived primary endothelial cells (BMECs) and macrophages (BMMAs) from MM and MGUS patients were obtained as previously described [5, 8–10]. Serum samples were collected from 38 patients with MM. Peripheral blood was processed immediately after venipuncture by centrifugation at 1,500×*g* for 10 min and serum stored at – 80 °C.

Real-time reverse transcription-polymerase chain reaction (Real-Time RT-PCR)

Real-Time RT-PCR reactions were carried out as previously described [10], using the "StepOne Real-Time RT-PCR system" (Applied Biosystems, Foster City, CA), primers (Invitrogen Corp., Carlsbad, CA, Suppl. Table 1) and SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. The relative gene expression (fold change) was calculated with the $2^{-\Delta\Delta CT}$ method [11], using GAPDH as endogenous control.

Western blotting

Total protein lysates (30 μg) from BMMAs were separated on 4–12 % NuPAGE® gels (Invitrogen Corp.), electrotransferred to a polyvinylidene difluoride membrane (PerkinElmer Life Science Inc., Boston, MA) and immunoblotted with anti-Epo-R (M20, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-pAkt$^{(Ser473)}$, Akt, pJAK2$^{(Tyr1007/1008)}$, JAK2, pSTAT5$^{(Tyr694)}$, and STAT5 (Cell Signaling Technology Inc., Danvers, MA), and anti-β-actin antibodies (Sigma-Aldrich). Then, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence ("LiteAblot extend" substrate, Euroclone) and the Gel Logic 1,500 Imaging System (Eastman Kodak Co., Rochester, NY), quantified with the Kodak Molecular Imaging Software, and expressed as arbitrary optical density units (OD).

Epo-R small interfering RNA transient transfection (siRNA)

BMMAs (4×10^5) were transiently transfected for 72 h with 50 nM of small interfering RNAs (siRNAs) specific for human Epo-R and negative control scramble siRNAs (SMART-pool; Dharmacon RNA Technologies, Lafayette, CO) or with the transfection reagent alone (Lipofectamine, RNAiMAX siRNA transfection reagent, Invitrogen Corp).

Preparation of BMMAs CM and cytokine measurement

Conditioned Media (CM) from BMMAs were prepared by seeding 2.5×10^5 cells/ml in serum free medium (SFM), in SFM supplemented with rHuEpo alone or in presence of rHuEpo sR, or LY294002, or wortmannin, were collected after 24 h of culture, and centrifuged at 1,500 rpm at 4 °C

for 10 min to remove cell debris. Cytokines were measured by using the "Fluorokine® Multianalyte Profiling (MAP) Kits" for the Luminex® Platform (R&D Systems and Luminex Corporation, Austin, TX), allowing simultaneous quantification of the following cytokines in single samples: VEGF, HGF, FGF-2, monocyte chemotactic protein (MCP)-1/CCL-2, interleukin(IL-6), IL-8, IL-1α, IL-1β, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage-CSF (GM-CSF), tumor necrosis factor alpha (TNF-α). VEGF, HGF, FGF-2, MCP-1/CCL-2, IL-6, IL-8, G-CSF, GM-CSF and TNF-α circulating levels were also detected in the serum of MM patients treated or not treated with rHuEpo.

Chemotaxis assay

BMECs were seeded into the upper compartment of a Boyden micro chamber on a polycarbonate membrane (Neuro Probe, Inc., Warwickshire, UK) pre-coated with 10 μg/ml fibronectin (Sigma-Aldrich), towards: (i) SFM alone; (ii) SFM supplemented with 1.5 % FBS and 10 ng/ml VEGF and FGF-2; (iii) CM of BMMAs untreated and treated with 30 and 60 U/ml of rHuEpo; (iv) CM of BMMAs treated with rHuEpo/rHuEpo sR (30 U/ml and 9 μg/ml, respectively), in the lower compartment. After 6 h at 37 °C, migrated BMECs were fixed, stained (SNABB-DIFF Kit, Labex AB, Helsingborg, Sweden) and counted in five randomly chosen fields/well under a digital inverted light microscope EVOS (Euroclone) (×400).

Scratch wound assay

BMECs (4×10^4 cells/well) were grown to confluence on fibronectin-coated (10 μg/ml) 24-well plates in complete DMEM. A scratch wound was made by scraping the cell monolayer in the middle with a sterile pipette tip. DMEM was removed and cells were exposed for 24 h to SFM alone or in complete DMEM or in CM of BMMAs untreated or treated as in chemotaxis assay. Wound closure was monitored under an EVOS inverted microscope. After 24 h, cells were fixed and stained (SNABB-DIFF Kit). Cell migration was determined by counting the BMECs that had moved in the "wound", and indicated as the percentage of relative wound closure compared with control (10 % FBS).

In vitro morphogenesis assay on Matrigel®

BMECs were plated (4×10^4 cells/well) in duplicated in reduced growth factors Matrigel®-coated (Becton–Dickinson, San Jose, CA) 48-well plates in SFM alone or with CM of BMMAs as in chemotaxis and wound assays. After 16 h, tridimensional organization was observed under an EVOS inverted microscope, followed by measurement of vessel areas and length in three separate microscopic fields at ×200 [10].

In vivo chick embryo chorioallantoic membrane (CAM) angiogenesis assay

Fertilized chicken eggs were incubated at 37 °C at constant humidity. On day 8, sterilized gelatin sponges adsorbed with SFM alone or supplemented with VEGF (200 ng/embryo), or with CM of BMMAs alone or pretreated for 24 h with 30 U/ml rHuEpo, were implanted on the top of the CAM, as previously described [12]. CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope. Blood vessels entering the implants or the sponges within the focal plane of the CAM were counted by two observers in a double blind fashion at 50× magnification.

Results

Bone-marrow-derived macrophages express Epo-R

Epo-R mRNA was expressed by primary BMMAs of both MGUS and MM patients (Fig. 1a). However, Epo-R was significantly over-expressed in BMMAs isolated from MM patients with active disease (∼60 %). Immunoblotting performed using an anti-human Epo-R antibody, identified a band of 59 kDa in MM and MGUS BMMAs lysates, and in K562 cells, used as positive control. Epo-R protein level was significantly higher in active MM compared to MGUS patients (∼45 %) (Fig. 1b).

RHuEpo up-regulates production of pro-angiogenic mediators by BMMAs

We performed a multiplex ELISA assay to simultaneously measure several cytokines including VEGF, HGF, FGF-2, MCP-1/CCL-2, IL-6, IL-8, IL-1α, IL-1β, CSF, GM-CSF and TNF-α. Primary BMMAs from MM patients were treated with 10–30–60 U/ml of rHuEpo in SFM for 24 h and, then, the CM were harvested. The assay revealed that rHuEpo increased the production of VEGF (1.15, 1.30 and 1.50-fold), HGF (1.34, 1.70 and 3.40-fold), and MCP-1/CCL-2 (1.40, 1.90 and 2.10-fold) at 10–30–60 U/ml into the CM of BMMAs as compared to controls (Fig. 2a). On the contrary, rHuEpo did not affect the secretion of FGF-2, IL-6, IL-8, IL-1α, IL-1β, CSF, GM-CSF, and TNF-α (data not shown).

Over-expression of VEGF, HGF and MCP-1/CCL-2 was confirmed at mRNA level by means of Real-Time RT-PCR analysis. We evaluated the time-course of VEGF, HGF and MCP-1/CCL-2 changes in their mRNA levels after

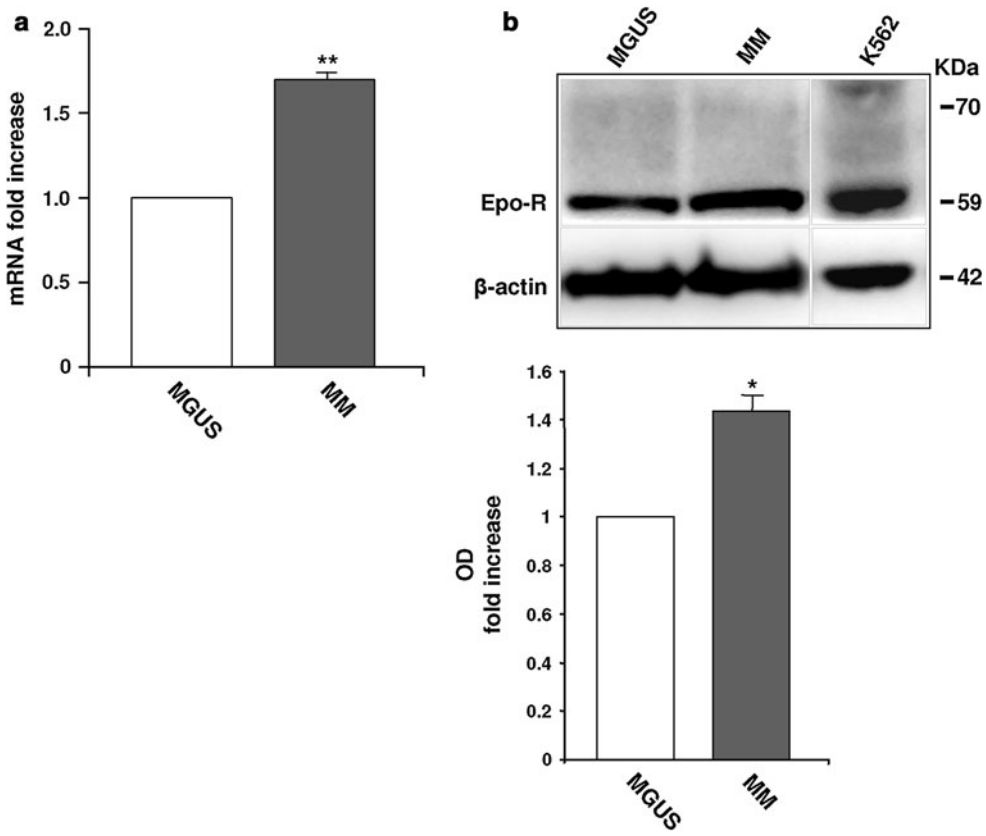

Fig. 1 Epo-R expression in bone-marrow derived macrophages (BMMAs) from MGUS and MM patients. **a** Real-Time reverse transcription-PCR (normalized to GAPDH) as mean ± SD of 18 MM and 9 MGUS patients. **b** Western blot of representative MM and MGUS patients, and K562 control lysate. Fold increase of optical density (OD) in MM (*grey bar*) versus MGUS (*empty bar*) BMMAs as mean ± SD of 17 MM and 8 MGUS patients. Significances * $P < 0.03$ and ** $P < 0.003$ by Wilcoxon signed-rank test

BMMAs stimulation with 30 U/ml rHuEpo. Expression of VEGF increased markedly after 6–12 h of rHuEpo stimulation by 30 % and over 100 %, respectively, in a dose-dependent way, with its mRNA level which remained still high 24 and 48 h post-treatment (74 %). Also HGF expression increased significantly after 6–12 h of stimulation (38 and 47 %, respectively); however its mRNA level returned to almost control level after 24 and 48 h. By contrast, MCP-1/CCL-2 mRNA expression raised in 12 h of treatment (25 %) and returned to almost control level in 24 and 48 h (Fig. 2b).

Stimulation of Epo-R elicits activation of JAK2/STAT5 signaling pathway and regulates VEGF, HGF and MCP-1/CCL-2 secretion by BMMAs via PI3K/Akt signaling pathway

Epo interacts with its receptor and regulates cell functions through activation of many signaling pathways including STAT5, MAPK and PI3K/Akt [13]. To ascertain which signaling pathways are involved in BMMAs activation by rHuEpo, we focused our study on Epo's signal transduction components: JAK2, STAT5, Akt and ERK1/2. Thus, we investigated the phophorylation of Epo-R-associated JAK2, STAT5, Akt and ERK1/2. Time-course experiments demonstrated that rHuEpo stimulation elicits an increment in the phosphorylation of JAK2 after 5 min of exposure (+59 %), whereas activation of STAT5 was detected at 30 and 60 min after treatment (+51, +48 %, respectively) (Fig. 3a).

A significant increase in the phosphorylation of Akt, which markedly peaked after 30 min following rHuEpo exposure (+56 %) and returned to basal level after 60 min, was also observed. In addition, Akt activation was significantly hampered by PI3K specific inhibitors LY294002 and wortmaninn (−47 and −62 %, respectively) (Fig. 3b). By contrast, ERK1/2 phosphorylation was induced at 30 min and persisted until 60 min following rHuEpo stimulation (data not shown). However, rHuEpo produced no or negligible not statistically significant effects on JAK2/STAT5 and PI3K/Akt pathway in BMMAs from MGUS patients (Supplemental Figure 1). Interestingly, small interfering RNA-mediated knockdown of Epo-R expression, by more than 80 %, after 72 h of transfection, did not result in any increase in the phosphorylation of JAK2, STAT5 and Akt following rHuEpo stimulation (Fig. 3c).

Furthermore, to evaluate whether activation of JAK2/PI3K/Akt signaling cascade affected VEGF, HGF and MCP-1/CCL-2 secretion, we stimulated BMMAs with 30 U/ml of rHuEpo in presence of LY294002 and wortmannin. LY294002 inhibited rHuEpo-mediated VEGF, HGF and MCP-1/CCL-2 secretion of about 31, 53, and 49 %, respectively, amount almost comparable to that

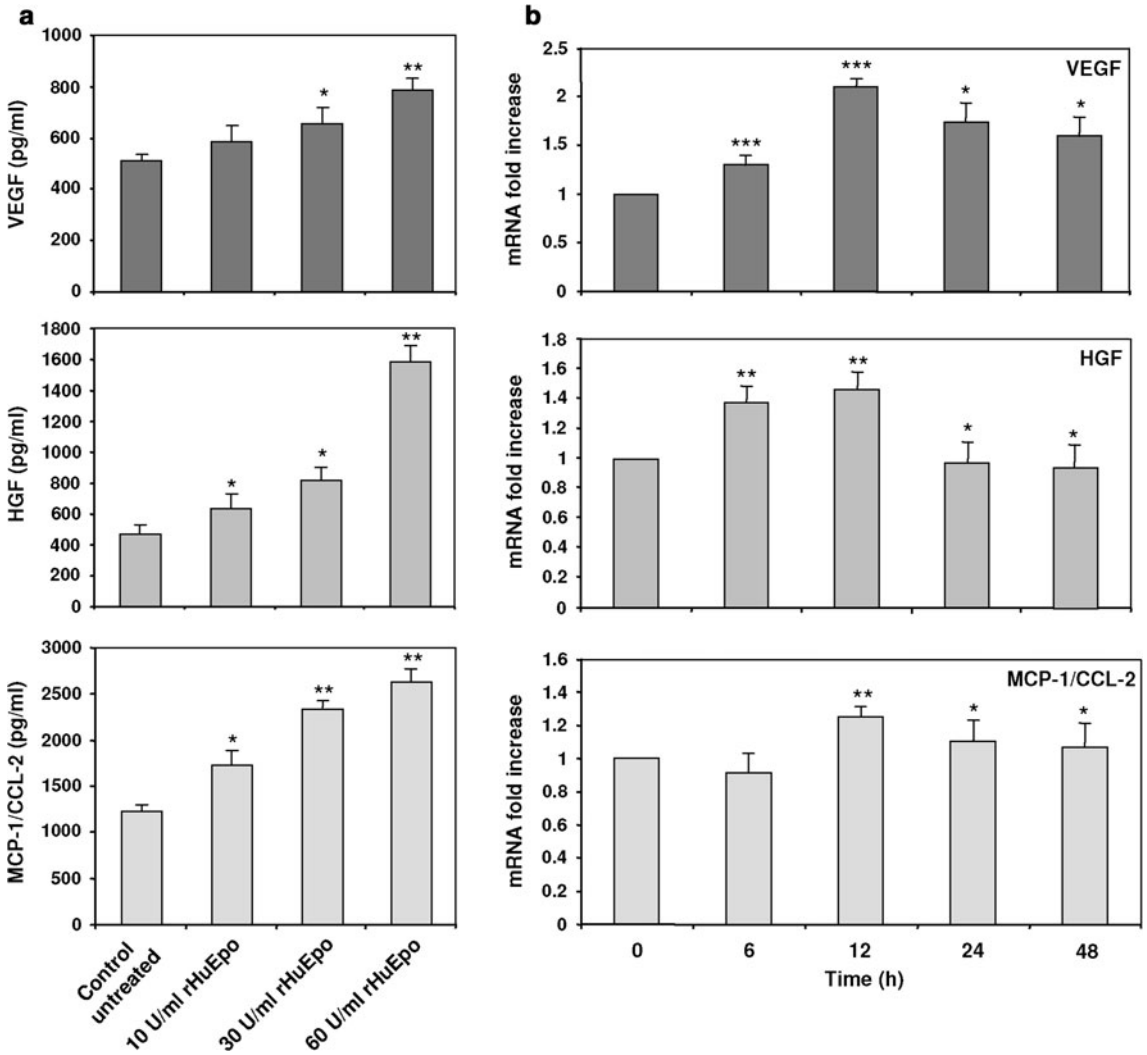

Fig. 2 Pro-angiogenic mediators production in conditioned media of BMMAs-treated with rHuEpo. **a** Secreted VEGF, HGF, and MCP-1/CCL-2 levels quantified by multiplex ELISA in the conditioned media (CM) of BMMAs untreated and treated with 10, 30 and 60 U/ml rHuEpo for 24 h. Histograms are expressed in pg/ml and are the mean ± SD of 28 MM patients. **b** Changes in VEGF, HGF, and MCP-1/CCL-2 mRNA expression at different times (0, 6, 12, 24 and 48 h) in response to 30 U/ml rHuEpo treatment, determined by Real-Time reverse transcription-PCR. Values are expressed as mean relative expression ± SD of each gene/GAPDH mRNA level of 24 MM patients. Significances * $P < 0.05$, ** $P < 0.03$ and *** $P < 0.01$ by Wilcoxon signed-rank test

released by untreated BMMAs (Fig. 3d); analogously wortmannin treatment significantly reduced VEGF, HGF and MCP-1/CCL-2 production of about 34, 38, and 51 %, respectively. These data suggest that activation of JAK2/PI3K/Akt signaling pathway in response to Epo/Epo-R axis stimulation is sufficient to stimulate BMMAs to release pro-angiogenic mediators.

CM from rHuEpo-treated BMMAs enhances BMECs motility in vitro

We performed a chemotaxis assay using a modified Boyden chamber. SFM alone, SFM supplemented with 1.5 % FBS and 10 ng/ml of both VEGF and FGF-2, used as negative and positive controls, respectively, untreated, rHuEpo-treated BMMAs CM and CM of BMMAs treated with rHuEpo in the presence of 9 μg/ml of a rHuEpo sR, which binds with high affinity and is a potent antagonist of Epo, were loaded in the lower compartment of the chamber. Serum-starved BMECs were added to the upper compartment.

CM of untreated BMMAs induced a migratory response per se (2.7-fold-increase) as compared to SFM (Fig. 4a). The CM from rHuEpo-treated BMMAs significantly enhanced BMECs migration of about 3.7 and 4.3-fold at 30 and 60 U/ml, respectively, as compared to control SFM. RHuEpo treatment at 60 U/ml induced a migratory response almost comparable to chemotaxis induced by VEGF and FGF-2 (4.7-fold-increase vs. control). Furthermore, CM from BMMAs treated with 30 U/ml of rHuEpo

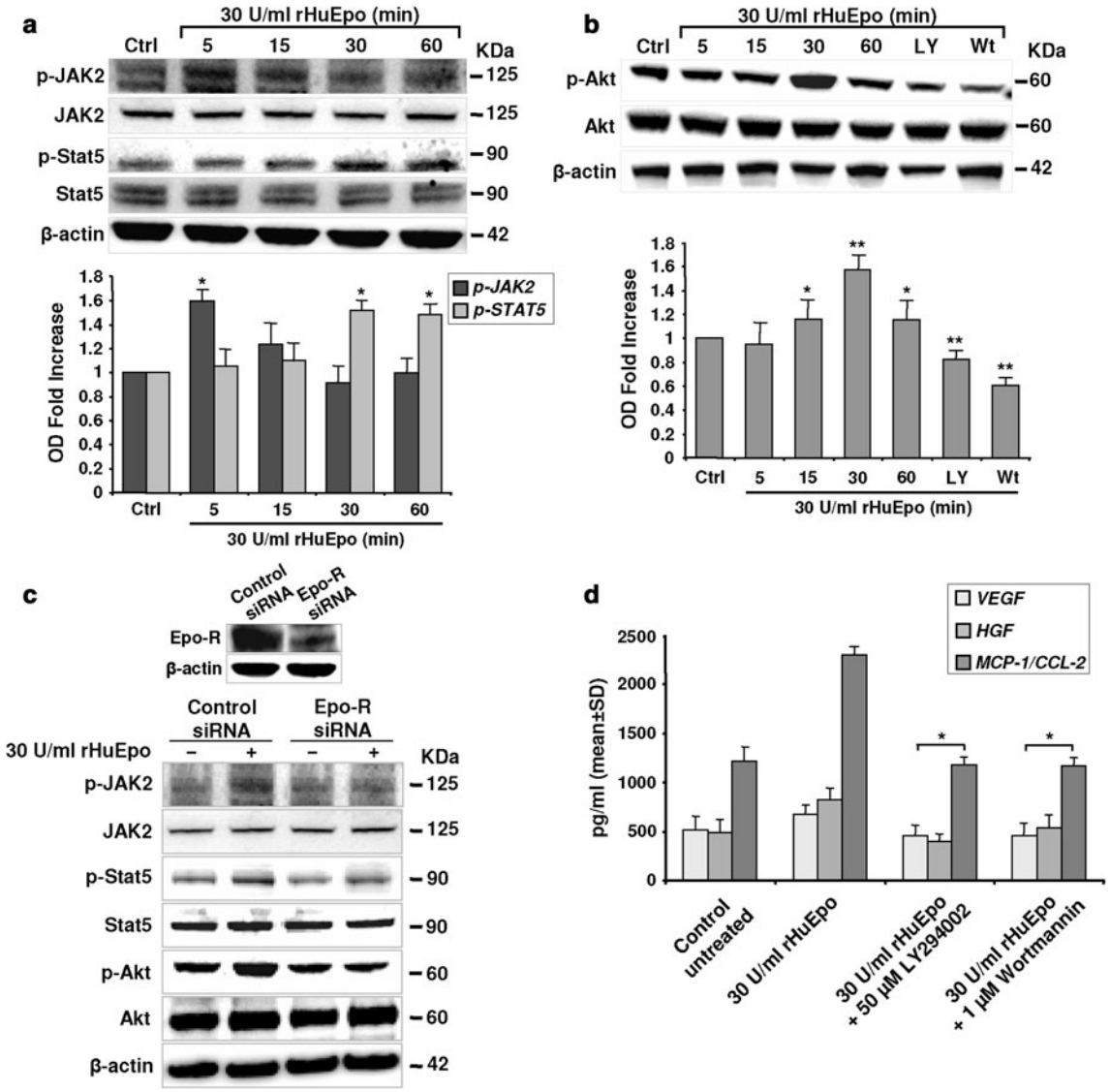

Fig. 3 RHuEpo stimulates JAK2/STAT5/Akt phosphorylation and mediates secretion of VEGF, HGF and MCP-1/CCL-2 from BMMAs through PI3K/Akt pathway. **a**, **b** Representative immunoblots of rHuEpo-treated MM-BMMAs. Fold increase of optical density (OD) of phosphorylated JAK2, STAT5 and Akt expressed as mean ± SD of 18 MM patients. **c** Representative immunoblot of siRNA-mediated Epo-R knockdown and its effect on JAK2, STAT5 and Akt phosphorylation. **d** Secreted VEGF, HGF, and MCP-1/CCL-2 levels quantified by multiplex ELISA in the conditioned media (CM) of BMMAs untreated, or treated with 30 U/ml rHuEpo alone or supplemented with 50 μM LY294002 or 1 μM wortmannin for 24 h. Histograms are expressed in pg/ml and are the mean ± SD of 16 MM patients. Significances * $P < 0.05$ and ** $P < 0.03$ by Wilcoxon signed-rank test. LY = LY294002; Wt = wortmannin

in the presence of rHuEPo sR, significantly inhibited rHuEpo-dependent BMECs migration (~1.8-fold).

We have studied also the effect of Epo on BMECs motility by a scratch wound healing assay (Fig. 4b). CM from untreated BMMAs was able, per se, to stimulate a migratory effect on BMECs compared to SFM (~36 % of wound closure). In the presence of rHuEpo-treated BMMAs CM we observed an increased in BMECs motility, demonstrated by a 57 and 80 % of wound closure, at 30 and 60 U/ml, respectively, which was considerably hampered by the antagonist rhEpo sR (30 % of wound closure).

CM from rHuEpo-treated BMMAs stimulates angiogenesis in vitro and in vivo

We performed a capillary morphogenesis assay on Matrigel® substrate in vitro. After 16 h of incubation, BMECs spreaded and formed capillary-like structures. Incubation of BMECs with the CM of BMMAs increased the number and the lengths of capillary tubes compared to BMECs in SFM (+34 and +38 %, of relative vessel area and of relative length, respectively) which were significantly augmented in the presence of 30 U/ml (+79 and +72 %,

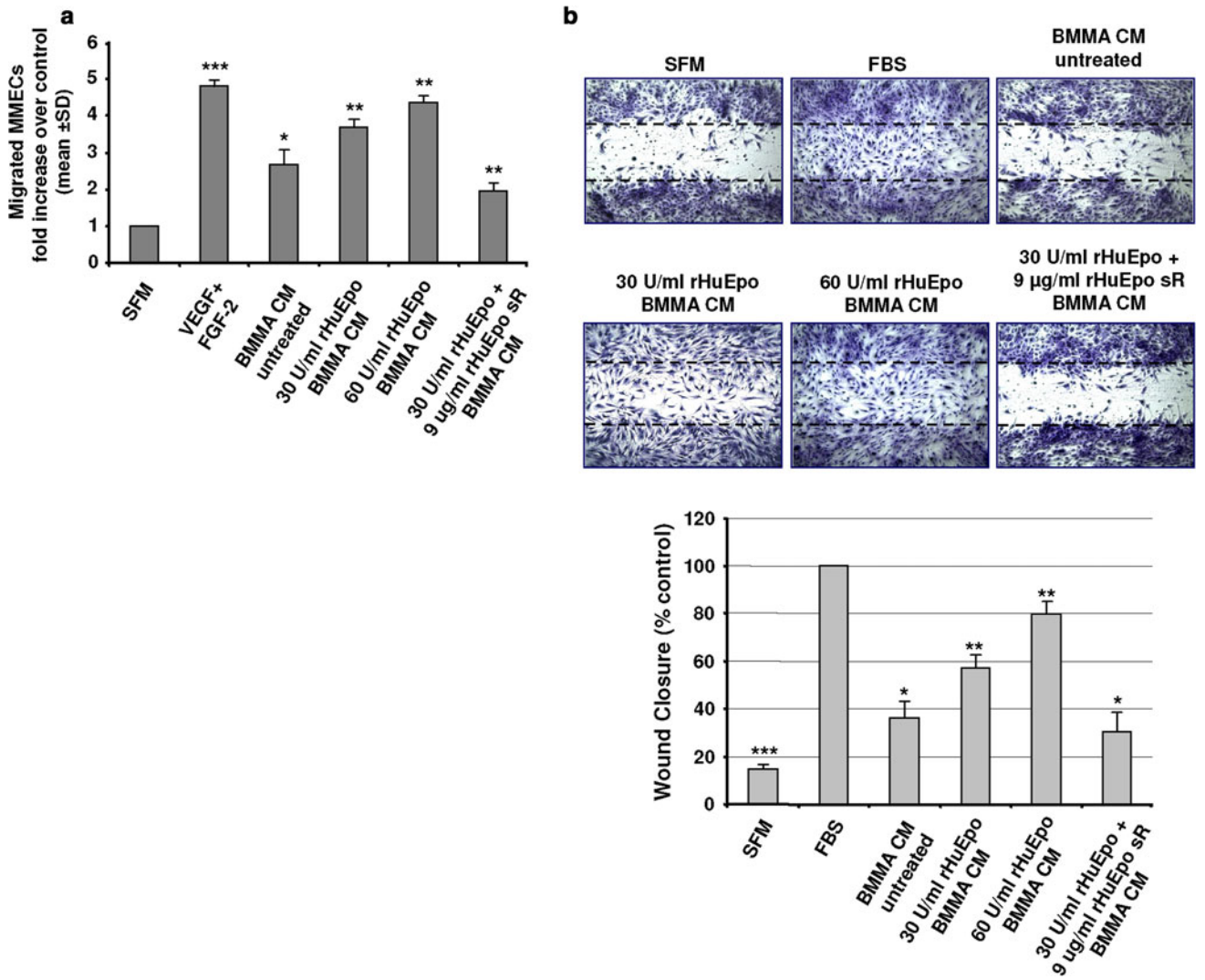

Fig. 4 RHuEpo-treated BMMAs affect BMECs motility in vitro. Conditioned media (CM) harvested from BMMAs treated with rHuEpo promote BMECs migration. **a** Boyden micro chamber assay. Data in *bar graphs* represent fold increase of migrated BMECs over SFM, used as control (mean ± SD) counted in five 400 × fields/membrane. **b** Scratch wound healing assay. Representative images of the wound closure after 24 h from the scratch are shown. *Graph* represents the relative closure of the cell-free area compared with control at the same time point. Migration of control cells in complete medium (10 % FBS) was arbitrarily set to 100 %. Data are mean ± SD of 12 MM patients. Significances * $P < 0.05$, ** $P < 0.03$ and *** $P < 0.01$ by Wilcoxon signed-rank test. Original magnification ×80 by EVOS digital inverted microscope. *SFM* serum free medium, *FBS* fetal bovine serum

respectively) and 60 U/ml of rHuEpo (+96 and +94 %, respectively). Treatment with the antagonist rhEpo sR suppressed the formation of the vessel-like networks induced by 30 U/ml rHuEpo (−34 and −28 %, respectively) (Fig. 5a).

We have previously demonstrated that rHuEpo alone exerts a strong angiogenic activity in the CAM assay [14]. In this study, we have compared in the same assay the angiogenic activity of CM of BMMAs untreated and treated with 30 U/ml rHuEpo. After 4 days of treatment, macroscopic observation of the CAMs showed that CM of BMMAs induced an angiogenic response, characterized by newly formed capillaries spreading radially towards the sponge significantly higher (mean number of vessels around the implant = 22 ± 4) as compared to SFM alone (mean number of vessels = 8 ± 2). The angiogenic response was significantly enhanced in the presence of CM of BMMA-treated with 30 U/ml rHuEpo (mean number of vessels = 30 ± 4), and was comparable to that exerted by VEGF (mean number of vessels = 28 ± 3) (Fig. 5b).

Epo enhances the circulating levels of angiogenic cytokines in patients with MM

Sera of 38 MM patients, including 16 patients with anemia who had received adjuvant Epo therapy, were investigated

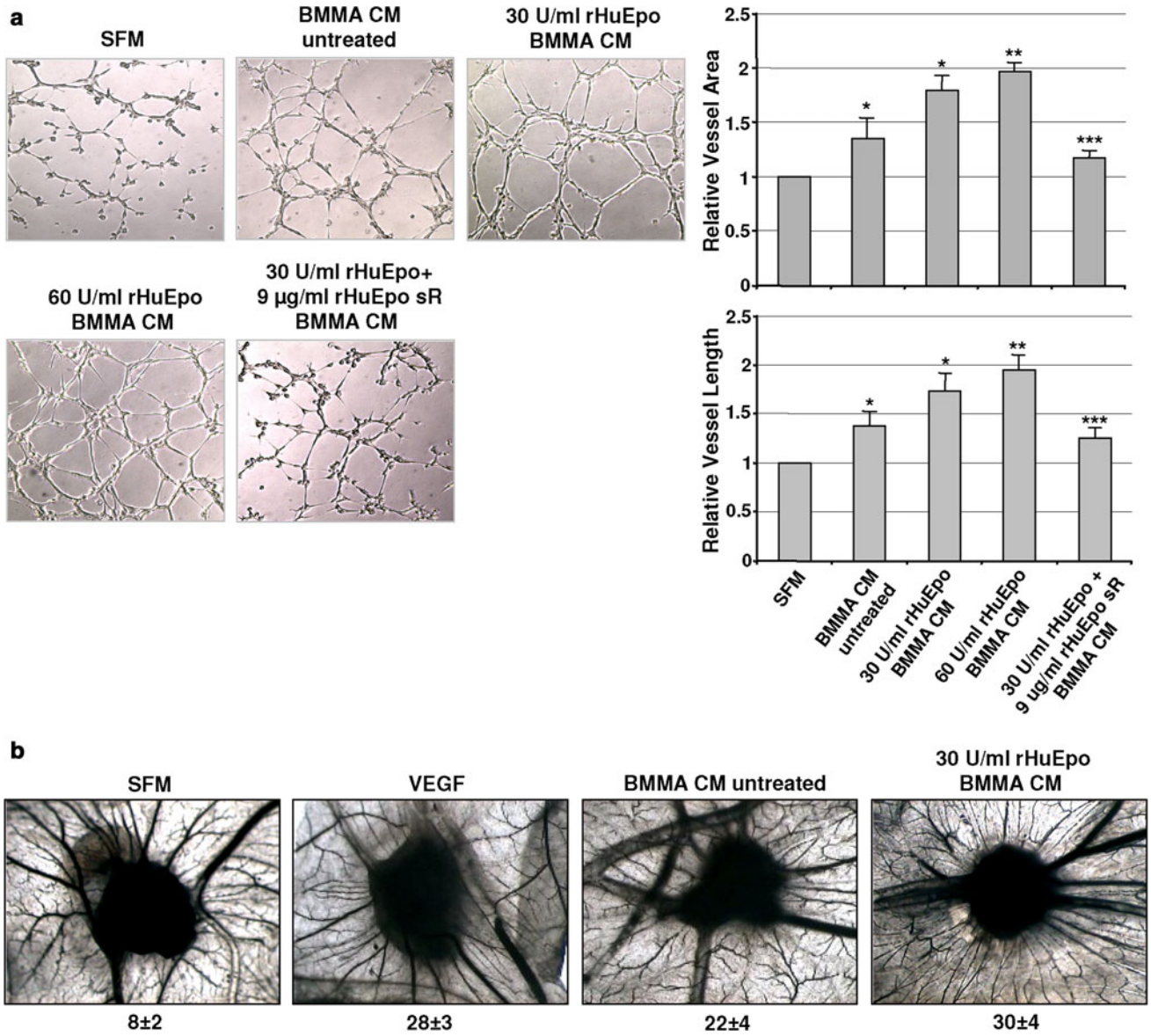

Fig. 5 RHuEpo-treated BMMAs conditioned media enhance angiogenesis in vitro and in vivo. **a** Morphogenesis in vitro on Matrigel® substrate. Images are representative of one patient out of 15 total MM patients. Data are presented as relative vessel area and length (mean ± SD), by the EVOS "Micron" image software. Original magnification ×200. Significances * $P < 0.05$, ** $P < 0.03$ and *** $P < 0.01$ by Wilcoxon signed-rank test. **b** Macroscopic pictures of gelatin sponges soaked with SFM alone or supplemented with VEGF or with the CM of BMMAs untreated and treated with 30 U/ml rHuEpo, implanted on the chick embryo chorioallantoic membrane (CAM). Note numerous allantoic vessels developing radially towards the implants with the exception of the sponge adsorbed with SFM alone. Original magnification ×50. Vessel counts are indicated. Significance $P < 0.001$ for BMMA CM untreated versus SFM alone and 30 U/ml rHuEpo-treated BMMA CM versus untreated. *SFM* serum free medium, *CM* conditioned media

for the simultaneous detection of VEGF, HGF, FGF-2, MCP-1/CCL-2, IL-6, IL-8, G-CSF, GM-CSF and TNF-α circulating levels. Epo strongly enhanced serum levels of VEGF, MCP-1/CCL-2, IL-8, GM-CSF and TNF-α by 49, 57, 68, 56, and 48 %, respectively, compared to their levels in untreated patients. HGF, FGF-2, and IL-6 concentrations were slightly affected (22, 32, and 26 %, respectively), whereas G-CSF was poorly affected (13 %) (Fig. 6).

Discussion

In this study, we have provided evidence for a novel role for Epo. We have demonstrated for the first time that: (1) MM BMMAs express EpoR; (2) rHuEpo markedly up-regulates production of pro-angiogenic mediators by BMMAs, including VEGF, HGF, and MCP-1/CCL-2. The capacity of Epo to modulate the release of other angiogenic factors express the capability of Epo to indirectly control

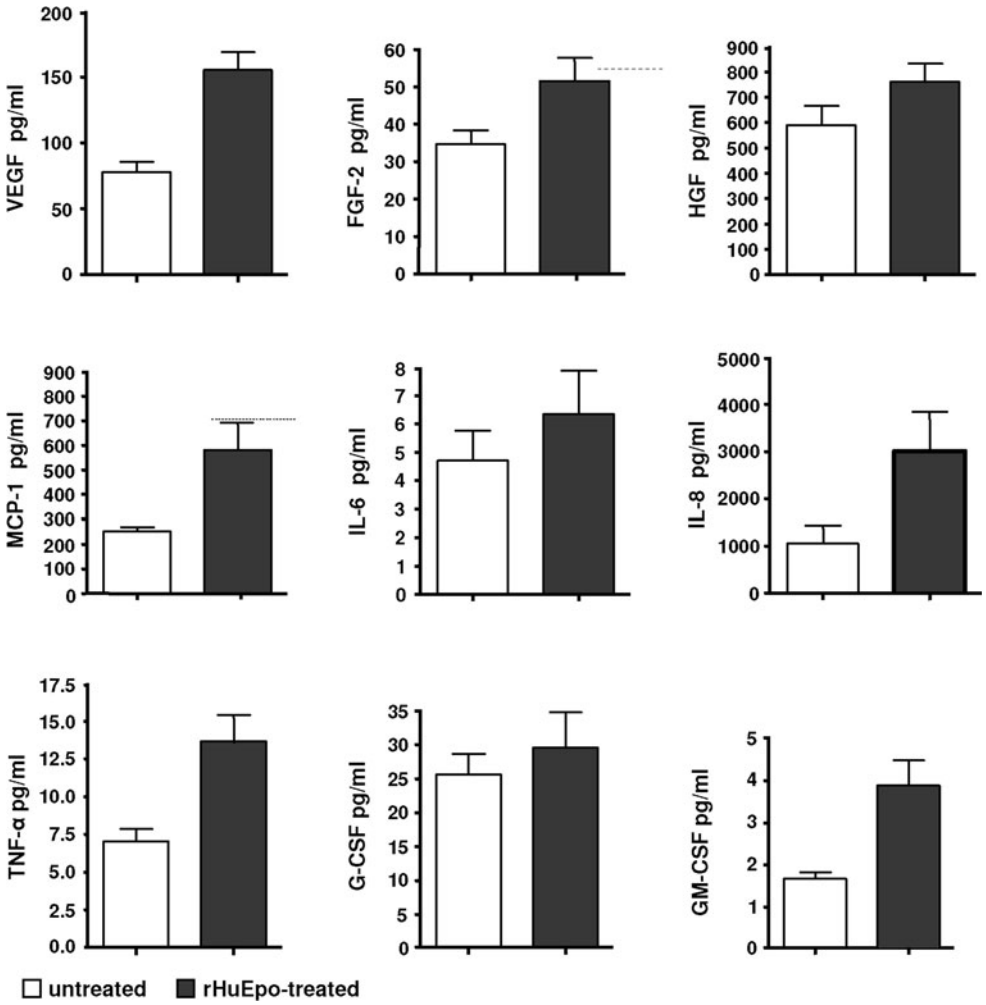

Fig. 6 Erythropoietin therapy enhances serum circulating levels of angiogenic cytokines in MM patients. Circulating levels of VEGF, HGF, FGF-2, MCP-1/CCL-2, IL-6, IL-8, G-CSF, GM-CSF and TNF-α quantified by multiplex ELISA in the sera of 16 erythropoietin-treated MM patients compared to 22 untreated ones. Significance $P < 0.0001$

tumor angiogenesis. In particular, Epo and VEGF directly target vascular endothelial cells through EpoR and VEGFR signaling pathways to induce tumor angiogenesis [6]; (3) rHuEpo regulates VEGF, HGF and MCP-1/CCL-2 secretion by BMMAs via PI3 K/Akt signaling pathway; (4) CM from rHuEpo-treated BMMAs enhances BMECs motility in vitro, and these effects were inhibited by a rHuEpo sR; (5) CM from rHuEpo-treated BMMAs stimulates angiogenesis in vitro, by using a capillary morphogenesis assay on Matrigel® substrate, and in vivo, by using the CAM assay and the angiogenic response is higher as compared to that induced by CM of BMMAs alone and is comparable to that of VEGF.

Epo favors tumor progression through effects on angiogenesis through an autocrine/paracrine loops [6]. Tumor cells release increasing amount of VEGF and placental growth factor in response to Epo [15]. In a Lewis lung carcinoma xenograft model in mice subcutaneous administration of Epo promotes tumor angiogenesis [16]. On the contrary, exogenous Epo decreased both the host- and tumor-derived VEGF expression, suggesting that the effect of Epo is independent of VEGF production [17]. Injection of Epo with tumor cells in vivo stimulated tumor neovascularization and growth, while injection of Epo antagonist proteins with tumor cells inhibited angiogenesis and tumor growth [18]. In a mouse tumor model PDGF-BB induced Epo expression by targeting perivascular cells expressing PDGFR-β and Epo stimulated tumor angiogenesis in a paracrine way [19]. Moreover, a correlation between Epo/Epo-R expression, angiogenesis, and tumor progression has been established in several human tumors [20–25].

Epo may exhibit also modulatory effects on macrophages number and function in vivo and in vitro, enhancing their pro-inflammatory phenotype [7]. Transgenic tg6 mice overexpressing human Epo have increased populations of liver and duodenal mucosa macrophages [26, 27], and liver macrophages in tg6 mice also had enhanced erytrophagocytotic activity [27]. Expression of Epo-R was shown in wound healing macrophages, and Epo enhanced the activity of this cell population [28].

Overall, our findings and literature data suggest that Epo may be involved in the regulation of angiogenic response occurring in MM through a direct effect on tumor macrophages. Our suggestion is confirmed by data showing that

Epo administration to patients with MM and myelodysplastic syndrome induced bone marrow angiogenesis and further malignant transformation in plasma cell leukemia and acute monoblastic leukemia, respectively [29, 30]. Moreover, Epo treatment of patients with MM as well as murine models of myeloma was associated with improved immunological functions [31–35].

The results of our study might also have important clinical implications. RHuEpo and Epo-stimulating agents (ESA) are used to treat or prevent anemia in patients receiving chemotherapy for various cancers, including MM. However, it has been demonstrated that they negatively affected patient survival [36, 37]. Among the possible causes of this negative effect of Epo there is its pro-angiogenic activity. In this study we have shown that Epo treatment enhances the circulating levels of angiogenic cytokines in patients with MM as compared to untreated ones. However, the role of neovascularization in tumor progression during ESA therapy remains to be determined, because it could serve to either increase tumor growth or enhance the efficacy of chemotherapy and radiotherapy. It is also important to note that ESA treatment of non-cancer related disease, including end-stage renal disease, could stimulate dormant tumor growth by mechanisms of promoting angiogenesis and tumor growth [38].

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Conflict of interest None.

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