VEGF INDUCES MCL-1 UPREGULATION AND PROTECTS MULTIPLE MYELOMA CELLS AGAINST APOPTOSIS


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Abstract

Interleukin-6 (IL-6) triggers multiple myeloma (MM) cell proliferation and protects against apoptosis by upregulating Myeloid-cell-leukemia 1 (Mcl-1). Vascular endothelial growth factor (VEGF) induces modest proliferation of MM cells and induces IL-6 secretion in a paracrine loop involving MM cells and bone marrow stromal cells. Using murine embryonic fibroblast cell line as a model (Mcl-1\textsuperscript{wt/wt} and Mcl-1\textsuperscript{Δ/Δ} MEFs), we here demonstrate that deletion of Mcl-1 reduces fetal bovine serum (FBS), VEGF, and IL-6 induced-proliferation. We also show that VEGF upregulates Mcl-1 expression in a time and dose manner in 3 human MM cell lines and MM patient cells. Importantly, we demonstrate that the pan-VEGF inhibitor, GW654652, inhibits VEGF induced-upregulation of Mcl-1, and as with Mcl-1 siRNA is associated with decreased proliferation and induction of apoptosis. Finally, we show that VEGF protects MM patient cells against FBS starvation-induced apoptosis. Our studies therefore demonstrate that VEGF-induced MM cell proliferation and survival is mediated via Mcl-1, providing the preclinical framework for novel therapeutics targeting both Mcl-1 and/or VEGF to improve patient outcome in MM.

Key words: multiple myeloma; vascular endothelial growth factor (VEGF); Mcl-1; apoptosis.
Multiple myeloma (MM) is a clonal B-cell malignancy characterized by the accumulation of malignant plasma cells within the bone marrow (BM). Binding of MM cells to bone marrow stromal cells (BMSC) promotes tumor cell growth, survival, and drug resistance by both MM cell-BMSC contact and triggering of cytokines secretion. Among these cytokines, interleukin-6 (IL-6) produced by BMSC plays a major role on both proliferation and survival of tumor cells. In turn, MM cells secrete vascular endothelial growth factor (VEGF), which further promotes production of IL-6 in BMSC, as well as migration and proliferation of the tumor cells. Thus VEGF is both an autocrine growth factor and trigger of IL-6-mediated paracrine MM cell growth. Recent reports have highlighted the major role of VEGF in MM pathogenesis, demonstrating that VEGF also increases micro-vessel density in the BM. Moreover, VEGF increases bone-resorption by osteoclasts and inhibits maturation of dendritic cells. Taken together, these reports have promoted pre-clinical MM studies which confirm the promise of VEGF targeting therapies. Although the impact of VEGF on MM cell proliferation and migration is well documented, its role in conferring protection against apoptosis remains unknown. As reported in other hematological malignancies such as leukemia, VEGF upregulates Bcl-2 and thus protects leukemia cells against chemotherapy. Furthermore, among the Bcl-2 family members induced by VEGF, Katoh et al have demonstrated that Mcl-1 was critical to protect leukemia cell lines against etoposide-induced-apoptosis.
Myeloid-cell-leukemia 1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family which is distinguished from other Bcl-2 family members, like Bcl-2 or Bcl-xL, by its short half-life and ability to protect cells against a large variety of cytotoxic stimuli. Moreover, Mcl-1 downregulation is a critical and pivotal checkpoint controlling mitochondrial apoptotic events, such as cytochrome c release and caspase activation. Recently, Opferman et al demonstrated that Mcl-1 is also required for development and maintenance of B and T lymphocytes. In MM, IL-6 activates the JAK/Stat-3 pathway leading to the upregulation of Mcl-1 expression. Using oligonucleotide antisense technique (ASO), Zhang et al and Derenne et al demonstrated that specific inhibition of Mcl-1, but not of Bcl-2 or Bcl-xL, induces apoptosis of MM cells. Conversely, the anti-apoptotic effect of IL-6 is mediated through Mcl-1 upregulation. However, Zhang et al demonstrated that IL-6 failed to upregulate Mcl-1 expression in almost two third of MM cell lines and primary MM cells, despite triggering phosphorylation of STAT-3. Thus, the mechanisms whereby cytokines regulate Mcl-1 may involve distinct signaling pathways. Mcl-1, besides triggering anti-apoptotic effects, is also involved in cell cycle progression and pivotal in regulating cell homeostasis.

In the present report, we investigated whether VEGF, as IL-6, can regulate Mcl-1 expression and thereby influence survival and proliferation of MM cells. Using Mcl-1^{wt/wt} and Mcl-1^{A/null} murine embryonic fibroblast cells lines (MEFs) as a model to investigate the role of Mcl-1, we first demonstrate that Mcl-1 is involved in both IL-6 and VEGF-induced cell proliferation. Second, we show that VEGF upregulates Mcl-1 expression in human MM cell lines (HMCLs) and MM patient cells; and conversely, confirm that specific downregulation of Mcl-1 expression by siRNA inhibits proliferation and induces apoptosis. Furthermore, we demonstrate that VEGF protects MM patient cells against fetal bovine serum (FBS) starvation
induced-apoptosis. Taken together, these data confirm the pivotal role of VEGF in survival of MM cells, thereby providing the preclinical rationale for targeting Mcl-1 and VEGF in novel therapeutics to improve patient outcome in MM.

Materials and methods

Cells and cell culture
The human MM cell lines (HMCLs) MM1s, MM1r and U266, as well as patient MM cells were maintained in RPMI 1640 medium with 2 mM L-glutamine (Mediatech, Cellgro, AK, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 10 µg streptomycin (Mediatech). Murine embryonic fibroblast cell line Mcl-1<sup>wt/wt</sup> and Mcl-1<sup>Δnull</sup> MEFs were kindly provided by J Opferman (Howard Hughes Institute, Dana-Farber Cancer Institute, Boston, MA).<sup>18</sup> MEFs were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 10 µg streptomycin and 2 mM L-glutamine, 2 mercapto-ethanol (Sigma) and MEM non-essential amino-acid (Gibco, Grand Island, NY).

Isolation of patient’s tumor cells
Patient’s BM samples were harvested after informed consent. Mononuclear cells were obtained after Ficoll-Paque centrifugation (Pharmacia Biotech Uppsala, Sweden), and MM
patient cells (96 % CD38+ CD45RA−) were separated by antibody-mediated selection using RosetteSep (Stemcell Technologies, Vancouver, BC, Canada) as previously described. 26

**Reagent**

Indazolylpyrimidine GW654652 (Glaxosmithkline) is a tyrosine kinase inhibitor that inhibits all 3 VEGF receptors. 12

**Stimulation of cells**

Cell lines were starved overnight in their respective culture medium supplemented with 0.25% or 0.5% FBS for MEFs and HMCLs, respectively. Cells were stimulated with FBS and mouse recombinant VEGF (m-rVEGF) or IL-6 (m-rIL-6) for MEFs and human recombinant VEGF or IL-6 for HMCLs. All cytokines were obtained from R&D Systems, Mineapolis, MN. Duration of stimulation and doses of cytokines are indicated for each experiments.

**DNA synthesis and cell proliferation assay**

Cell growth was assessed by addition of 0.5µCi of 3H-thymidine per well during the last 8 hours of each experiment. Cells were harvested onto glass-fiber filtermates using an automatic cell harvester (Tomec Harvester 96 Mach III, Hamden, CT, USA), and radioactivity counted using the Wallac Trilux Betaplate scintillation counter (Turku, Finland). Each condition was performed in quadruplicate.

**Cell viability assays**

Cell viability was assessed by 3-(4,5 dimethylthiazol-2-y)-2,5 diphenyltetrazolium bromide (MTT; Chemicon International, Temecula, Ca, USA) assay, according to manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). Cells were seeded in
96-well plates. Cell viability was evaluated as previously described.\textsuperscript{27,28} Cell survival was estimated as a percentage of the value of untreated control.

**Flow cytometry and cell cycle analysis**

For cell cycle analysis, DNA was stained with propidium iodide. Briefly, 1x10\textsuperscript{6} cells were washed with 1xPBS, re-suspended in 70% ethanol, and then incubated for 30 min on ice. After incubation, cells were washed twice with 1x PBS and re-suspended in the presence of RNase for 20 min at 37\textdegree{}C. After two washes in 1x PBS, cells were re-suspended in propidium iodide on ice for 20 min for cytometric analysis. Apoptotic cells were detected as a subdiploid peak, as described by Zamai et al.\textsuperscript{29} Flow cytometry was analysed using Cytomics RXP program (Beckman Coulter).

Apoptotic MM patient cells were assayed with double staining using CD38 and Apo2.7 mAbs coupled to FITC and PE, respectively (Immunotech, Marseille, France). After staining, MM and non-MM patient cells were gated according to their CD38 expression (CD38\textsuperscript{++} and CD38\textsuperscript{+/-} for MM cells and non-MM cells, respectively), and apoptotic cells were assessed by Apo 2.7 expression, as previously described.\textsuperscript{30} Thus, the percentage of apoptotic cells in each cell subset was separately measured.

**Cell lysis and Western-blot**

Cells were washed 2 times with 1xPBS and suspended in lysis buffer (10mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% triton X-100, 1 mM sodium vanadate, 1 mM phenylmethyl sulfonyl fluoride and 2 mg/ml aprotinin). After 40 min on ice, lysates were cleared by centrifugation at 13 000g/min for 30 min at 4\textdegree{}C and were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, prior to electrophoretic transfer onto Hybond C super membrane (Amersham, Arlington Heights, IL). The blots were probed
overnight with either Mcl-1, Bcl-2, Actin, Erk-1/2 (Santa-Cruz Biotechnology; Santa-Cruz, Ca), Bax, Bad, Bcl-xl, XIAP, survivin or cIAP antisera (Cell Signaling Technology, Beverly, MA) prior to incubation with secondary antibodies and exposure to enhanced chemoluminescence substrate.

**Transfection of Mcl-1 siRNA**

MM.1s cells were transiently transfected with indicated amounts of Mcl-1 duplex (5’-UAA CAC CAG TAC GGA CGG G dTdT; dTdT AUU GUG GUC AUG CCU GCC C – 5’ targeting 5’ – TAA CAC CAG TAC GGA CGG C – 3’) or non-specific control duplexes (pool of 4) using the Cell line Nucleofector Kit V Solution (Amaxa Biosystems, Cologne, Germany), as previously described. Following transfection, MM.1s cells were subjected to western blot analysis, MTT assays, and H[dT] uptake assays.
Results

Mcl-1 contributes to FBS induced-proliferation and mediates both IL-6 and VEGF induced-proliferation in MEFs.

Recently, Opferman et al have generated a Mcl-1null allele and Mcl-1Δnull MEFs that failed to express Mcl-1 protein; using this model system, they demonstrated that Mcl-1 is required for the development of B and T lymphocytes, since deletion of Mcl-1 lead to a profound reduction of these hematopoïetic cells. In the present study, Mcl-1wt/wt and Mcl-1Δnull MEF cell lines were used as a model to study Mcl-1 involvement in proliferation. To study the consequences of Mcl-1 deletion on growth factor and cytokine-mediated proliferation, Mcl-1wt/wt and Mcl-1Δnull MEFs were starved overnight in DMEM with 0.25 % FBS, followed by culture in the absence or presence of various doses of FBS. As in Figure 1a, low dose FBS (1.25%) triggered MEF proliferation similarly in these two cell lines. However, at higher concentration of FBS the number of proliferating cells was significantly lower in Mcl-1Δnull compared to Mcl-1wt/wt MEFs. In addition, we compared the cell cycle distribution of Mcl-1Δnull and Mcl-1wt/wt MEFs (Figure 1b). Interestingly, the percentage of cells in S phase was significantly lower in Mcl-1Δnull MEFs versus Mcl-1wt/wt MEFs, 21% and 30% respectively, demonstrating that lack of Mcl-1 reduces DNA synthesis in MEFs. Next, we compared the response of these cell lines to IL-6 and VEGF. Mcl-1wt/wt and Mcl-1Δnull MEFs were starved in DMEM with 0.25 % FBS overnight, followed by culture for 2 days in the absence or presence of 25 ng/ml of m-rIL-6 or m-rVEGF for two days (Figure 1c). Both m-rIL-6 and m-rVEGF significantly increased proliferation of Mcl-1wt/wt MEFs cells (increase of 45% and
20% compared to un-stimulated cells, respectively). In contrast, Mcl-1\(^{Δnull}\) MEFs did not respond to m-rVEGF, and proliferation induced by m-rIL-6 was only modest (10% increase). Taken together, these experiments demonstrate that deletion of Mcl-1 reduces FBS- and inhibits VEGF-induced proliferation.

**VEGF upregulates Mcl-1 expression in HMCLs**

Since both VEGF and IL-6 promote MM cell proliferation, and IL-6 upregulates Mcl-1, we next investigated whether VEGF could also upregulate Mcl-1 expression. MM1s, MM1r and U266 cells were starved overnight in RPMI 0.5% FBS, followed by culture in the absence or presence of either 50 ng/ml of IL-6 or VEGF. After 6h stimulation, cells were lysed and Mcl-1 expression determined by Western-blot analysis (Figure 2). IL-6 and VEGF, to a lesser extent, up-regulated Mcl-1, but not Bcl-2, expression in these 3 HMCLs. MM1s cells were the most, and MM1r cells were the least sensitive. Thus, we conducted further experiments on HMCLs in MM1s cells. Time and dose-dependent Mcl-1 upregulation by VEGF was observed in MM1s cells (Figure 3a and b): Mcl-1 expression was slightly upregulated at 1 ng/ml and peaked at 5 ng/ml VEGF. Moreover, time course experiments show that VEGF triggered upregulation of Mcl-1 is transient, peaking at 6h and returning to baseline after 24h (Figure 3b).

The effects of VEGF on other Bcl-2 family members were similarly investigated. No modulation of Bcl-2, Bax, Bad or Bcl-x\(_L\) protein expression was observed, suggesting that Mcl-1, among bcl-2 family members, is specifically targeted by VEGF. Other anti-apoptotic proteins, such as survivin and cIAP, were also upregulated whereas XIAP expression was unchanged. Interestingly, both Mcl-1 and survivin protein expression were significantly downregulated after overnight starvation, while expression of the other anti-apoptotic proteins
remained stable. This suggests that starvation, as a pro-apoptotic stimulus, acts mainly through downregulation of Mcl-1 and/or survivin (Figure 3b).

To confirm the specific link between VEGF stimulation and Mcl-1 upregulation, VEGF-R was inhibited using GW654652, a pan-VEGF-R inhibitor. After overnight starvation, MM1s cells were cultured for 1h in the absence or presence of various doses of GW654652, followed by culture without or with 50 ng/ml of VEGF for 6h. As shown in Figure 3c, upregulation of Mcl-1 triggered by VEGF was blocked by GW654652 in a dose-dependent manner, thereby confirming the link between VEGF stimulation and Mcl-1 upregulation.

Inhibition of Mcl-1 by Mcl-1 siRNA induces apoptosis and inhibits proliferation of MM1s cells

To investigate the consequences of specific inhibition of Mcl-1 on both proliferation and apoptosis, we performed Mcl-1 siRNA transfection in MM1s cells. Mcl-1 protein expression (using western-blot analysis), proliferation (using 3H-thymidine incorporation), and cell viability (using MTT assay) were determined 24 and 48h after transfection. Mcl-1 siRNA transfection down regulated Mcl-1 expression in a dose-dependant manner (Figure 4a); without any modification of Bcl-2 or Bcl-2 family member expression (data not shown). Mcl-1 downregulation also inhibited 3H-thymidine incorporation (42 % and 61% decrease at 24 and 48 h, respectively) (Figure 4b). Similarly, cell viability after Mcl-1 down-regulation was 75 % and 60% relative to control cultures at 24 and 48h, respectively (Figure 5a).

To confirm that cytotoxicity was due to apoptosis, cell cycle analysis was performed. After 24 and 48 h, the percentage of apoptotic cells (subG1 peak) was 22 % and 41 % in Mcl-1 siRNA treated cells, versus 15 % and 15 %, respectively, in control cultures.
Taken together, these experiments demonstrate that specifically targeting Mcl-1 inhibits proliferation and induces apoptosis in MM1s HMCLs. Thus, downregulation of Mcl-1 is sufficient by itself to inhibit proliferation and induce apoptosis in MM cells.

**VEGF overcomes inhibition of DNA synthesis induced by FBS starvation in MM1s cells**

We next investigated whether VEGF could overcome FBS starvation in MM1s cells. MM1s cells were starved in RPMI 0.5% FBS overnight, followed by culture with or without various doses of VEGF. DNA synthesis was evaluated by $^3$H-thymidine incorporation (Figure 6). Only 12% of starved cells incorporated $^3$H-thymidine in the absence of VEGF. In contrast, 33% and 49% of cells still incorporated $^3$H-thymidine in presence of 10 and 25 ng/ml of VEGF, respectively. Higher concentrations of VEGF did not increase the percentage of proliferating cells (data not shown). These results demonstrate that VEGF overcomes, at least in part, FBS starvation, thereby maintaining MM cell viability and proliferation.

**VEGF upregulates Mcl-1 expression and protects patient MM cells against FBS starvation induced-apoptosis.**

We then investigated the effects of VEGF in two patient MM cells. After selection, MM cells (patient A) were starved overnight in RPMI 0.5% FBS, followed by culture in the absence or presence of 50 ng/ml of IL-6 or VEGF for 6 h. Mcl-1 expression was determined by Western-blot analysis (Figure 7a). As in HMCLs, both IL-6 and VEGF induced Mcl-1 upregulation. In contrast to HMCLs, Mcl-1 upregulation triggered by VEGF was stronger than by IL-6; like HMCLs, Bcl-2 expression remained unchanged.

BM mononuclear cells from another patient (patient B), were cultured in RPMI 10% FBS or starved with RPMI 2% FBS, with or without 25ng/ml of VEGF for 2 days. Cells were then stained with both CD38-FITC and Apo 2.7- PE. Apoptosis was assessed by flow cytometry:
in MM cells as CD38 bright cells (CD38++); and in non-MM cells as CD38 low/negative cells (CD38+/−) (Figure 7b). Eighteen percent CD38++ MM cells were present in the BM sample. Gated analysis showed that a total of 15% of MM cells in the control group (cultured in RPMI supplemented with 10% FBS) versus 93% in the FBS-starved group without VEGF and 48% in the FBS starved group with VEGF were apoptotic (Apo2.7+). Gated analysis on non-MM mononuclear cells showed that VEGF also reduced FBS starvation induced-apoptosis from 21% to 15%. Thus, these results demonstrate that VEGF protects MM patient cells against FBS starvation induced-apoptosis.

**Discussion**

VEGF induces angiogenesis, vasculogenesis, vasodilatation, and increases vascular permeability.33 Because of its ability to promote the growth of tumor vascular environment, VEGF is a major growth factor mediating tumor progression.34 In MM, VEGF increases micro vessel density (MVD), reflecting bone marrow angiogenesis. Thus, MVD has been reported to increase during disease progression from monoclonal gammapathy of undetermined significance (MGUS) to active MM.5,35,36 In addition, a high BM MVD is an adverse prognosis factor.37 Taken together, these reports demonstrate that VEGF, by its effects on the BM microenvironment of MM cells, is a major factor in MM progression. Furthermore, by inducing IL-6 in the BM milieu, VEGF is involved in both autocrine or paracrine MM cells growth. In addition, VEGF also directly targets MM cells. Indeed, Bellamy et al and Podar et al reported expression of VEGFR-1 on MM cells and primary patient MM cells; Kumar et al demonstrated that VEGFR-2 was also expressed on these cells.6,38,39 Importantly, VEGF triggers signaling cascades in MM cells including ERK pathway mediating cell growth and PI3-Kinase protein kinase-C dependent cascade mediating
migration. To date, however, the impact of VEGF protecting against MM cell apoptosis remains unclear. In the present study, we demonstrate that VEGF upregulates expression of anti-apoptotic proteins, including Mcl-1, survivin and cIAP. Furthermore, we demonstrate that FBS starvation induced-apoptosis in MM cells is partially blocked by VEGF confirming that VEGF is a potent anti-apoptotic cytokine in MM.

Up-regulation of Mcl-1 triggered by VEGF, also reported in chronic lymphocytic leukemia cells, may account, at least in part, for the VEGF anti-apoptotic effect. Among the Bcl-2 family members, Mcl-1 is a key anti-apoptotic protein in the intrinsic pathway of apoptosis. Indeed, Mcl-1 interacts with Bim, a pro-apoptotic BH3-only-protein of the Bcl-2 family, thereby protecting against its pro-apoptotic effect. Furthermore, Nijhawan et al demonstrated that downregulation of Mcl-1 is a required and early event in UV radiation induced-apoptosis. Thus, Mcl-1 downregulation is a pivotal and early checkpoint for some pro-apoptotic stimuli. As reported by Derenne et al, we confirm in the present study that Mcl-1 downregulation by Mcl-1 siRNA induces apoptosis of MM cells, confirming that Mcl-1 is required for MM cell survival.

In MM, the main cytokine reported to upregulate Mcl-1 is IL-6. Relative to IL-6, Mcl-1 induced upregulation triggered by VEGF was weaker in HMCLs, but stronger in patient MM cells, suggesting that signaling pathway(s) triggered by VEGF mediating upregulation of Mcl-1 may differ from those induced by IL-6. Similarly to Podar et al, we found that VEGF did not activate the JAK/STAT-3 pathway, the main signaling pathway whereby IL-6 upregulates Mcl-1. Furthermore, because Mcl-1 induced-upregulation was stronger by VEGF than by IL-6 in patient MM cells, VEGF may be a more potent in vivo stimulus to up-regulate Mcl-1.
than IL-6. Ongoing studies of additional patient MM cell samples are delineating the respective roles of VEGF versus IL-6 in Mcl-1 regulation *in vivo*.

Like other proteins including p53, the E2F family proteins and survivin, Mcl-1 is involved not only in apoptosis but also in cell cycle regulation. Indeed, Mcl-1 is a cell cycle regulator through its interaction with proliferating cell nuclear antigen (PCNA), a cell cycle-regulatory protein essential for G1 to S phase transition. Fujise et al demonstrated that Mcl-1 interacts functionally and physically with PCNA through a specific amino acid motif. Interestingly, among the Bcl-2 family proteins only Mcl-1 has this motif and interacts with PCNA. In this report, the authors suggested that Mcl-1 is up regulated by DNA damage, thereby slowing cell cycle progression by binding PCNA. Thus Mcl-1 would slow cell cycle and act as an anti-apoptotic protein, ensuring cell survival until DNA repair is completed. By its dual function on apoptosis and cell cycle regulation, Mcl-1 would prevent replication of altered DNAs. In contrast, our study demonstrates that FBS triggered-proliferation is significantly reduced in Mcl-1 \(^{\Delta\text{null}}\) MEFs compared to Mcl-1 \(^{\text{wt/wt}}\) MEFs. Importantly, we also demonstrate a decreased percentage of S phase cells in Mcl-1 \(^{\Delta\text{null}}\) MEFs, suggesting that Mcl-1 is required for cell cycle progression. Furthermore, we demonstrate that VEGF fails to promote cell growth and, that IL-6 induced-proliferation is significantly reduced in deleted MEFs. Moreover, Mcl-1 siRNA inhibits HMCL proliferation. Taken together, our results demonstrate that Mcl-1 promotes IL-6 and VEGF-triggered cell growth and cell cycle progression.

We believe that Fujise’s reports and our present study highlight the complexity of the role of Mcl-1 in cell cycle regulation. As reported by Craig et al, Mcl-1 interacts with a large panel of proteins involved in cell cycle or/and apoptosis, including other Bcl-2 family members, and thus creates a “coordinated network” influencing viability, proliferation, and differentiation.
Because of its rapid and inducible expression, short-term effects, ability to interact with other proteins, and rapid turnover, Mcl-1 has the “parfait profil” of a protein responsive to a large spectrum of stimuli influencing differentiation, proliferation, and survival.

In conclusion, we show in the present report that VEGF protects patient MM cells against FBS starvation induced-apoptosis and that VEGF, like IL-6, is not only a growth, but also an anti-apoptotic factor. Indeed, VEGF up-regulates Mcl-1 expression in MM cells, thereby mediating, at least in part, its anti-apoptotic capacity. As further confirmation of its potent anti-apoptotic role, we also demonstrate that VEGF upregulates survivin and cIAP anti-apoptotic proteins. Moreover, we show that Mcl-1 mediates proliferation and cell cycle progression, and is required for IL-6 or VEGF-induced MM cell proliferation. Taken together, these results provide the preclinical framework for targeting VEGF and Mcl-1 in novel MM therapeutics.
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LEGENDS

Figure 1: Deletion of Mcl-1 in MEFs modifies FBS induced-proliferation.

A/ Deletion of Mcl-1 in MEFs reduces FBS induced-proliferation. Mcl-1<sup>wt/wt</sup> and Mcl-1<sup>Δ/Δ</sup> MEFs were cultured in 96 well plates overnight in DMEM with 0.25% FBS. They were then cultured without or with the indicated percentage of FBS for 48 h; proliferation was determined by ³H-thymidine uptake during the last 8 h. Data represents mean +/- SD for quadruplicate samples. One representative experiment of 3 is shown. CPM: count per minutes.

B/ Lack of Mcl-1 expression reduces the percentage of MEFs in S phase. Cell cycle analysis was performed on Mcl-1<sup>wt/wt</sup> and Mcl-1<sup>Δ/Δ</sup> MEFs using PI incorporation. The percentage (+/- SD) of cells in each cell cycle phase is presented.

C/ Mcl-1 is required for m-rIL-6 or m-rVEGF induced-proliferation. Mcl-1<sup>wt/wt</sup> and Mcl-1<sup>Δ/Δ</sup> MEFs were starved overnight in DMEM with 0.25% FBS and, followed by culture with or without m-rIL-6 or m-rVEGF (25ng/ml) for 48 h; cell growth assessed as described in Materials and Methods. Proliferation index is defined as the number of CPM in the stimulated cell divided by the CPM in the control unstimulated cells. Data represents mean +/- SD of three different experiments.

Figure 2: VEGF triggers up-regulation of Mcl-1 in HMCLs.

HMCLs (MM1s, MM1r and U266) were starved overnight in RPMI 1640 with 0.5% FBS and then cultured in the absence on presence of VEGF (50 ng/ml) or IL-6 (50 ng/ml) for 6 h. Cell
lysates (30 µg in each lane) were analysed by Western-blot analysis with Mcl-1, Bcl-2 and Actin or Erk-1/2 antisera. Actin and Erk-1/2 were used as loading controls. One representative experiment of three is shown.

Figure 3: VEGF triggers time and dose-dependent protein expression in MM1s cells, which is specifically inhibited by GW654652.

A/ VEGF-triggers dose dependent up-regulation of Mcl-1 expression in MM1s cells. MM1s cells were starved overnight in RPMI 1640 supplemented with 0.5% FBS, followed by culture in the presence or absence of the indicated doses of VEGF for 6 h. Cells lysates (30 µg) were investigated by Western-blot analysis with indicated antisera. Actin was used as a loading control.

B/ VEGF triggers time-dependent modulation of protein expression in MM1s cells. MM1s cells were cultured overnight in RPMI 1640 supplemented with 0.5% FBS, followed by culture with VEGF (50 ng/ml) for 6 h. Cells lysates (30 µg) were studied by Western-blot analysis with indicated antisera. Actin served as a loading control.

C/ VEGF-triggered Mcl-1 upregulation is inhibited by GW654652. After overnight starvation followed by addition of the indicated doses of GW654652 (1h), cells were cultured in presence or absence of 50 ng/ml VEGF (6h). Cells lysates (30 µg) were analysed by Western-blot analysis. One representative experiment out of three is shown.

Figure 4: Treatment with Mcl-1 siRNA decreases MM cell proliferation.

A/ Down-regulation of Mcl-1 expression in a dose-dependent manner by Mcl-1 siRNA. MM1s cells were transfected with indicated doses of Mcl-1 siRNA (and scrambled siRNA, 5µg). Mcl-1 expression was determined by Western-blot analysis at 24h (left panel) and 48h (right
panel). Whole cell lysates of MM1s cells served as an additional control. Because no change in Bcl-2 expression was observed, Bcl-2 is used as a loading control.

B/ Transfection of Mcl-1 siRNA inhibits MM1s proliferation. After transfection with Mcl-1 siRNA or scrambled siRNA (5µg), 2x10^4 MM1s cells per well were cultured for 24 and 48h. Proliferation assays were performed as described in Materials and Methods. Non-transfected cells served as an additional control. Results shown are the percentage of ^3^H-thymidine incorporation compared to control. One representative experiment out of three is shown.

**Figure 5: Downregulation of Mcl-1 by Mcl-1 siRNA induces apoptosis in MM cells.**

A/ Mcl-1 down-regulation by Mcl-1 siRNA induces cytotoxicity. 2x10^4 MM1s cells per well were transfected with Mcl-1 siRNA or scrambled siRNA (5µg), followed by culture for 24 and 48h in 96 well plates. MM1s cell survival was determined at 24 and 48h by MTT assay. Results shown are percentage of viability compared to control.

B/ Mcl-1 down-regulation by Mcl-1 siRNA induces apoptosis. Cell cycle analysis was performed at 24 and 48h (upper panel and lower panel, respectively) following transfection with Mcl-1 siRNA or scrambled siRNA (5µg) (right panel and left panel, respectively). A sub-G1 peak represents apoptotic cells. One representative experiment of three is shown.

**Figure 6: FBS-starvation-induced cell death is partially rescued by VEGF.**

MM1s cells were starved overnight in RPMI 1640 supplemented with 0.5% FBS in 96 well plates, followed by culture for 48h with the indicated doses of VEGF (in RPMI 1640 with 0.5% FBS), in RPMI 1640 supplemented with 10% FBS, or in RPMI 1640 supplemented with 0.5% FBS. Proliferation was measured by ^3^H-thymidine incorporation. Results shown are compared to control cells cultured in RPMI 1640 supplemented with 10% FBS.
Figure 7: VEGF up-regulates Mcl-1 and protects patient MM cells from FBS starvation-induced apoptosis.

A/ VEGF upregulates Mcl-1 expression in patient MM cells. Patient MM cells were starved overnight in RPMI 1640 with 0.5% FBS, followed by culture for 6h in the absence or presence of either IL-6 (50 ng/ml) or VEGF (50ng/ml). Cells lysates (30 µg per lane) were analysed by Western-blot analysis using indicated antisera. Actin was used as a loading control.

B/ FBS starvation induced-apoptosis is inhibited by VEGF.

Patient BM mononuclear cells were cultured 48h in RPMI 1640 with 10% or 2% FBS, in the presence or absence of VEGF (25 ng/ml). The subset of MM cells (CD38++ cells) (left panel) and non-MM cells (CD38 +/- cells) (right panel) were evaluated for apoptosis using Apo2.7 staining. The percentage of apoptotic MM cells or non-MM cells cultured in control FBS 10% (upper lane) in FBS 2% (middle lane), and in FBS 2% supplemented with VEGF 25 ng/ml (bottom lane) is indicated.
Figures

Figure 1

Graph showing the relationship between % FBS in medium and CPMs/10^6 FB with data points for MEFs Mcl-1 Δw/w and MEFs Mcl-1 Δe/e.
Figure 1

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Figure 1.

![Graph showing proliferation index](image-url)
Figure 2

<table>
<thead>
<tr>
<th>Control</th>
<th>H-6</th>
<th>VEGF</th>
<th>Control</th>
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<th>VEGF</th>
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<tbody>
<tr>
<td>Mcl-1</td>
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<tr>
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<tr>
<td>Erk1</td>
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29
Figure 3

<table>
<thead>
<tr>
<th>a</th>
<th>VEGF (ng/mL)</th>
<th>h</th>
<th>Hours</th>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>5</td>
<td>50</td>
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<tr>
<td>Mcl-1</td>
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<td>+</td>
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</tr>
<tr>
<td>Bcl-2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
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<tr>
<td>c</td>
<td>GW (µg/mL)</td>
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<td>XIAP</td>
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<tr>
<td>0</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>VEGF (50 ng/mL)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5

(a) Cell viability (% of control)

Control Scrambled siRNA Mcl-1 siRNA (5 μg)

grey 1 grey 2

(b) 24 h

Control Mcl-1 siRNA

5b-GL 15%

48 h

5b-GL 15%

5b-GL 41%
Figure 6
Figure 7

a

patient A

<table>
<thead>
<tr>
<th></th>
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<th>VEGF</th>
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<td>Bcl-2</td>
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<tr>
<td>Actin</td>
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</tr>
</tbody>
</table>
Figure 7

Patient B

CD 38

SSC

MM cells (CD38+*)

Mononuclear cells (CD38*)

FBS 10%

15 %

7.0 %

FBS 2%

93 %

21 %

FBS 2% + VEGF 25 ng/ml

48 %

15 %

Apo 2.7+
VEGF induces MCL-1 upregulation and protects multiple myeloma cells against apoptosis

Steven Le Gouill, Klaus Podar, Martine Amiot, Teru Hideshima, Dharminder Chauhan, Kenji Itshitsuka, Shaji Kumar, Noopur Raje, Paul G Richardson, Jean-Luc Harousseau and Kenneth C Anderson

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