Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma

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Bone marrow angiogenesis is an attractive target for the treatment of multiple myeloma (MM). Here, we investigate the antiangiogenic power of lenalidomide, a molecule structurally similar to but less toxic than thalidomide. Lenalidomide impacts angiogenesis in vivo and in vitro, and selectively blocks migration of bone marrow endothelial cells of patients with active disease (MMECs). It downregulates key angiogenic genes and VEGF/VEGFR2-mediated downstream signaling pathways involved in MMECs migration, and the NF-κB pathway. Proteomics analysis shows that lenalidomide-treated MMECs specifically modulate the expression levels of angiogenesis-related molecules governing MMECs migration, cell-shape and cytoskeletal remodeling, energy metabolism and protein clearance. Altogether, these data imply the intrinsic complexity of the signaling pathways modulated by lenalidomide at the cellular and molecular level. Data also suggest that lenalidomide, by targeting angiogenesis, may exert an indirect anti-MM effect. The identified genes and proteins offer candidate targets in MMECs for the development of targeted antiangiogenic therapies.
Abstract

Purpose: To determine the in vivo and in vitro antiangiogenic power of lenalidomide, a ‘lead-compound’ of IMiDs® immunomodulatory drugs in bone-marrow (BM) endothelial cells (ECs) of patients with multiple myeloma (MM) in active phase (MMECs).

Experimental Design: The antiangiogenic effect in vivo was studied using the chorioallantoic membrane (CAM) assay. Functional studies in vitro (angiogenesis, “wound” healing and chemotaxis, cell viability, adhesion, and apoptosis) were performed in both primary MMECs and ECs of patients with monoclonal gammopathies of undetermined significance (MGECs) or healthy human umbilical vein endothelial cells (HUVECs). Real-time RT-PCR, Western blotting and differential proteomic analysis were used to correlate morphological and biological ECs features to the lenalidomide effects at gene and protein level.

Results: Lenalidomide exerted a relevant antiangiogenic effect in vivo at 1.75 μM, a dose reached in interstitial fluids of patients daily treated with 25 mg. In vitro, lenalidomide inhibited angiogenesis and migration of MMECs, but not of MGECs or control HUVECs; and had no effect on MMECs viability, apoptosis, or fibronectin- and vitronectin-mediated adhesion. Lenalidomide-treated MMECs showed changes in VEGF/VEGFR2-signaling pathway and on several proteins controlling ECs motility, cytoskeleton remodeling, and energy metabolism pathways.

Conclusions: This study provides information on the molecular mechanisms associated with the antimigratory and antiangiogenic effects of lenalidomide in primary MMECs, thus giving new avenues for effective endothelium-targeted therapies in MM.
Introduction

Lenalidomide (CC-5013, Revlimid®; Celgene Corp., NJ) is an oral immunomodulatory drug (IMiD®) with demonstrated efficacy and tolerability in patients with multiple myeloma (MM) (1,2). It is an analogue of thalidomide, but has greater potency and a better toxicity profile (3). We have previously shown that thalidomide is antiangiogenic by downregulating key genes involved in autocrine and paracrine angiogenic loops of MM patients (4).

Earlier studies have demonstrated that in the bone marrow (BM) microenvironment TNF-α increases cell-cell adhesion by inducing NF-κB-dependent upregulation of adhesion molecules on both MM and stromal cells. Although TNF-α only modestly triggers the MM plasma cell proliferation, the subsequent activation of NF-κB stimulates IL-6 - another key survival signal - in stromal cells, and both factors are targeted by IMiDs® (5). Lenalidomide shows in vivo antitumor properties due to stimulation of patients’ T-cells and IL-2/IFN-γ production, and inhibition of TNF-α (6). In MM cells, it upregulates apoptotic genes thus blocking growth (3,7). It also inhibits MM cell adhesion to stromal cells via downregulation of ICAM-1 and VCAM-1. In peripheral blood mononuclear cells, it reduces the expression of angiogenic factors, such as VEGF and bFGF (3), TNF-α and IL-6 (8). However, its precise mechanisms of action and molecular targets in a well-defined subpopulation of BM stromal cells, such as MM endothelial cells (MMECs), are still elusive.

Methods

Reagents

Lenalidomide (Celgene Corp.) was dissolved in DMSO (Sigma-Aldrich, Milwaukee, WI) as a stock solution of 10 mM, and stepwise diluted in culture medium before use: the 1.75 μM dose is the interstitial fluid concentration following the therapeutic oral dose of 25 mg/day for an adult patient weighing 70 kg. Recombinant human VEGF_{165} and TNF-α were purchased from Sigma-Aldrich; heat-inactivated FBS, DMEM and RPMI 1640, antibiotic/antimycotic,
glutamine, trypsin/EDTA, and PBS without Ca\(^{2+}\) and Mg\(^{2+}\) were from Euroclone (Pero, Milan, Italy).

**Patients and endothelial cell (EC) cultures**

Patients fulfilling the International Myeloma Working Group diagnostic criteria (9) for active MM (n=44) and monoclonal gammopathy of undetermined significance (MGUS) (n=31) were studied. MM patients were at diagnosis with symptomatic disease and an increase in M-component level in the 3 months before analysis (n=28), or were in relapse (n=12), or leukemic phase (n=4). They were 24 male and 20 female, aged 44-75 (median 61.5) years, staged as IIA (n=6), IIB (n=8), IIIA (n=23), and IIIB (n=7), and were not under treatment with lenalidomide. MGUS patients were 18 male and 13 female and aged 43-78 (median 65.7) years. The study was approved by the Ethics Committee of the University of Bari Medical School, and all patients provided their informed consent in accordance with the Declaration of Helsinki.

BM primary ECs from MM (MMECs) and MGUS (MGECs) patients were obtained by centrifugation on Ficoll gradient of heparinized BM aspirates followed by incubation with magnetic microbeads coated with Ulex europaeus agglutinin-1 lectin (10). Control (healthy) human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA), and cultured in endothelial growth media-2 (Lonza, Walkersville, MD) with 10% FBS.

Plasma cell conditioned media (CM) were prepared as previously described (10).

**Chorioallantoic membrane (CAM) assay**

Fertilized White Leghorn chicken eggs were incubated at 37°C at constant humidity (11). On day 3, the shell was opened and 2-3 mL of albumen removed to detach the CAM. On day 8, the CAMs were implanted with 1 mm\(^3\) sterilized gelatin sponges (Gelfoam Upjohn\(^{®}\), Kalamazoo, MI) loaded with serum-free medium (SFM) alone (negative control) or CM of MM plasma cells.
alone (positive control) or with 1.75 μM lenalidomide. The angiogenic response was evaluated on day 12 as the number of vessels converging toward the sponge at 50X, and photographed in ovo (Olympus stereomicroscope, Rozzano, Italy).

**Functional studies**

**Cell viability and apoptosis assays.** Cell viability was determined by MTT assay (12): cells (5 × 10^3/100 μL/well) were plated in triplicate in 96-well plates in serum-free DMEM (negative control) or complete DMEM alone (positive control) or with the lenalidomide doses for 72 hours, and treated with MTT for the last 4 hours. The absorbance was measured at 570 nm with 655 nm as a reference wavelength. Crystal violet assay was performed as previously described (10). For apoptosis, 5.0 × 10^5 cells were washed with ice-cold PBS without Ca^{2+} and Mg^{2+}, incubated with 7-AAD and PE annexin V (BD Biosciences™, San Jose, CA) and analyzed by flow cytometry (FACSCantoII, Becton Dickinson, San Jose, CA).

**Chemotaxis.** This was performed in triplicate using the Boyden microchamber technique (13) toward SFM alone (negative control), or admixed with the CM of MM plasma cells alone (positive control) or with the lenalidomide doses. Cells were counted on 400X oil-immersion field/membrane.

**“Wound” healing.** ECs were grown to confluence on fibronectin-coated (10 μg/mL) 6 cm^2 dishes. A “wound” was made by scraping the cell monolayer with a P200 pipette tip. Cells were exposed for 24 hours to SFM alone (negative control) or admixed with the CM of MM plasma cells alone (positive control) or with the lenalidomide doses, fixed, and quantified for motility by counting the cells migrating into the total wound area of each 10X field using an EVOS digital inverted microscope (Euroclone). At least three different fields were randomly chosen across the wound length (12).
**Adhesion assays.** ECs were plated (2 × 10^3 cells/well) in triplicate in 96-well fibronectin-coated (10 µg/mL), vitronectin-coated (10 µg/mL), and uncoated plates in SFM alone (positive control) or with the lenalidomide doses, then fixed at 30 minutes with 2.5% glutaraldehyde, stained with crystal violet-methanol, and counted as in the cell viability assay, reading absorbance at 595 nm (12).

**Angiogenesis assay.** ECs were plated (1 × 10^5 cells/well) in duplicate in Matrigel®-coated (Becton Dickinson) 24-well plates in SFM alone (positive control) or with the lenalidomide doses. After 18 hours, the skeletonization of the mesh, acquired with the EVOS microscope, was followed by measurement of its topological parameters (“mesh areas”, “length”, and “branching points”) with a computed image analysis (14).

**Western Blotting**

Total ECs protein lysates were subjected to immunoblot with primary and secondary antibodies to: VEGFR-2, phospho-Erk-1/2, Erk-1/2, VE-cadherin, Src, phospho-NF-κB p65, NF-κB p65, CXCL12/SDF-1, CCL2/MCP-1, myosin light chains (MLCs) and phospho-MLCs (Cell Signaling Technology, Danvers, MA), phospho-VEGFR-2 (Y1054) and IEX-1 peptide (Abcam, Cambridge, UK), phospho-VE-cadherin and phospho-Src (Invitrogen Corp., Carlsbad, CA), BNIP3 (GeneTex Inc., Irvine, CA), VEGF, bFGF, and SEPW1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), β-actin (Sigma-Aldrich), and mouse and rabbit horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules CA). Immunoreactive bands were visualized by enhanced chemiluminescence (LiteA/ blot extend substrate, Euroclone) and the Gel Logic 1500 Imaging System (Eastman Kodak Co., Rochester, NY), quantified with the Kodak Molecular Imaging Software, and expressed as arbitrary optical density (OD).
MMEC proteomics

Two-dimensional gel electrophoresis (2-DE), imaging, and quantification. Total lysates of MMECs and MGECs were prepared as previously described (12). The 2-DE analysis was performed in duplicate using isoelectric focusing (IEF) on an IPG-phor system (GE Healthcare Bio-Sciences, Piscataway, NJ) with precast gel strips pH 3-10, followed by SDS-polyacrylamide gel (SDS-PAGE) (Hoefer SE 600 Ruby, GE Healthcare Bio-Sciences), digitized by an ImageScanner (Amersham Biosciences) and analyzed by the ImageMaster TM 2D Platinum v.5.0 software (GE healthcare Bio-Sciences) for spot detection and gels matching, as previously described (15). Spots exhibiting an intensity difference between untreated and lenalidomide-treated samples with a P value of .05 by the two-tailed Student’s t-test for equal or unequal variance (depending on the calculated variance of spots), were considered to be differentially expressed.

In-gel trypsin digestion and analysis by mass spectrometry of selected protein spots. A preparative gel was obtained by loading whole MMECs lysates. Selected differential protein spots were excised out the gel, destained with 15 mM K3Fe(CN)6, and 50 mM Na2S2O3, washed with sequential incubation in 25 mM NH4HCO3, in 50% ACN and dried with 100% ACN. The gel proteins were subjected to cystein reduction and alkylation with 10 mM DTT in 100 mM ambic and 55 mM iodacetamide in 100 mM ambic, respectively, and subsequently reswollen with 10 ng/µL trypsin in 50 mM ambic (10 µg/mL) and digested overnight at 37°C. Two µL of 1% TFA were added to stop the enzymatic reaction. Peptides were purified and concentrated by solid phase extraction (SPE) in ZipTip C18 pipette tips (Millipore) and spotted directly onto a MALDI ground still target (Bruker Daltonics Bremen, Germany) upon elution with CHCA matrix (5 mg/mL in 50% ACN, 0.1% TFA). MALDI-MS and MALDI-MS/MS were performed on the UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). MS data were acquired in positive reflectron mode. All acquisitions were performed in a mass range of 700-3500 Thomson (m/z) with voltages of 25 and 21.7 kV for the first and second ion extraction.
stages, 9 kV for the lens, 26.3 and 13.8 kV for reflector 1 and 2, respectively. Quadratic external calibration of TOF was performed on monoisotopic mass of bradykinin (clip 1-7) [M+H]+, angiotensin II [M+H]+, angiotensin I [M+H]+, substance P [M+H]+, bombesin [M+H]+, ACTH (clip 1-17) [M+H]+, ACTH (clip 18-39) [M+H]+, and somatostatin [M+H]+. MS were analyzed by the Bruker Flex Analysis 3.0 software. MALDI-TOF MS/MS analysis was performed in LIFT mode. Precursor ions were selected manually. MS/MS spectra were acquired with 4,000-8,000 (maximum/maximum) laser shots using the instrument calibration file. Peptide mass fingerprint obtained from MS analysis were used for protein identification in the Swiss-Prot database (SwissProt 2010_04.fasta; 516 081 sequences; 181 677 051 residues) using the peptide search routine MASCOT 2.3 algorithm (http://www.matrixscience.com) restricted to Homo sapiens taxonomy with carbamidomethyl of cysteines as fixed modification, oxidation of methionines as variable modification. All peptide mass values were considered mono-isotopic and mass tolerance was set at 50 ppm. MASCOT scores greater than 56 were considered significant ($P \leq .05$). A database search for MS/MS data was performed using the same setting of MS analysis, with a fragment tolerance of 0.4 Da. The main cellular location, function and biological relationships of identified proteins were searched and determined on PubMed with EndNote software v.5.0.

**Real-Time reverse transcriptase (RT) PCR (RT-PCR)**

Total ECs RNA was isolated using the RNeasy® Mini kit (Qiagen, Hilden, Germany), and reverse transcribed into total complementary DNA (cDNA) with the iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using the “StepOne™ real-time RT-PCR system” (Applied Biosystems, Foster City, CA). The reaction mixture contained SYBR® Green PCR master mix (Applied Biosystems), cDNA and the following specific (forward/reverse) primers (Invitrogen): VEGF (5’-AAGGAGGAGGGCAGAATCAT-3’/5’-CCAGGCCCTCGTCATTG-3’), bFGF (5’-CCGGCACCGCCGAGTTGAC-3’/5’-CACATTTAGAAGCCAGTAATCT-3’),
CCL2 (5'-CAACGAGAATGGGTTCCAGGAT-3'/5'-TCTTCCGGAGTTTGGTTTG-3'),
CXCL12 (5'-TCAATTGCTCTCCAGATAATGT-3'/5'-CACGCTGCATAGGAATTGG-3'),
COL4A1 (5'-CGTAACTAAACACCCCTGCTTCAT-3'/5'-CACTATTGAAGCTTTATCGCTGGTCTT-3'),
CRYAB (5'-AGGTGCATGGAAAACATGAAGA-3'/5'-GGAACTCCCTGGAGATGAAACC-3'),
Notch1 (5'-CATGGTACCAATCATGAATCTTTGTT-3'/5'-CTGGAGGGACCAAGAATTGTATAA-3'),
BNIP3 (5'-ATATTCCCCCAAGGAGTTCCCT-3'/5'-ACGCTCGTGGTTCCTCATTGCT-3'),
IER3 (5'-CCGCACTCCCCAAAAAGA-3'/5'-GCTCTCGCGCACCAGGTA-3'),
SEPW1 (5'-CGGCCGCCTGGACAT-3'/5'-AATCAACTTCCGGCTACCA-3'), and control GAPDH (5'-GAAGGTGAAGGTCGGAGT-3'/5'-CATGGGTGGAATCATATTGGAA-3'). Reaction mixture and amplification conditions were performed according to the manufacturer’s instructions. Each RNA was tested in triplicate and the threshold cycle values were normalized to GAPDH and averaged ± SD. The relative gene expression (fold change) between untreated and lenalidomide-treated ECs was calculated with the $2^{-\Delta\Delta CT}$ method (16).

Immunofluorescence microscopy

MMECs plated on chamber slides (5 × 10³ cells/chamber) (LabTek, Nalge Nunc International, Naperville, IL) were treated with VEGF (10 ng/mL) alone or with 1.75 μM lenalidomide, fixed (4% paraformaldehyde), permeabilized (0.2% Triton X-100), blocked (3% BSA/PBS 1X), then stained with a murine mAb to p120 catenin (p120ctn, pp120 Src substrate, BD Transduction Laboratories™, Lexington, KY) and fluorescein (FITC)-conjugate goat anti-mouse IgG (Sigma-Aldrich). Images were obtained using an Axioplan-2 microscope/63X oil-immersion objective (Carl Zeiss, Jena, Germany).

Results

Lenalidomide suppresses in vivo and in vitro MM-related angiogenesis
To investigate whether lenalidomide could directly inhibit the MM-related angiogenesis we first examined the *in vivo* CAM assay. CAMs implanted with a gelatin sponge soaked with the CM of MM patients’ plasma cells was surrounded by more newly-formed capillaries converging radially towards the sponge in a “spoked-wheel” pattern (mean number of vessels 24 ± 7, Figure 1A, middle panel) than with SFM (11 ± 4, left panel). When lenalidomide 1.75 μM was added, a significant inhibition in MM-induced angiogenesis was seen (5 ± 2, *P* < .01, right panel).

Next, we examined whether lenalidomide could impact MMECs *in vitro* angiogenesis using the Matrigel assay. After 18 hours of incubation, MMECs spread throughout the Matrigel surface and aligned to form branching and anastomosing thick tubes with multicentric junctions, which gave rise to a closely knit network of capillary-like structures (Figure 1B, left panel). Lenalidomide dose-dependently inhibited this network, which progressively became a poorly organized plexus with few and no anastomosed tubes with scarce junctions. The 0.25 and 0.50 μM concentrations significantly lowered the mesh areas (~34% and ~48%, respectively), the vessel length (~45% and ~58%), and the vessel branching points (~48% and ~55%, *P* < .05, Figure 1B,C). The MM therapeutic dose of 1.75 μM almost completely inhibited these angiogenic topological parameters (mesh area ~67%, vessel length ~76%, branching point ~80%, *P* < .001, Figure 1B,C). The 2.5 μM fully inhibited MMEC angiogenesis: cells were either isolated or aggregated in small clumps without junctions (mesh area ~90%, vessel length and branching points ~95%, Figure 1B,C). Similar, though fainter, effects were observed in the ECs of MGUS patients (MGECs, Supplementary Figure S1A) while there were no significant effects in HUVECs (data not shown).

**Lenalidomide affects MMECs functions involved in angiogenesis**

The MMECs migratory activity was significantly inhibited in a dose-dependent manner, as evidenced both in the “wound” healing assay (Figure 2A) and the chemotaxis assay (Figure 2B). MMECs migration was stimulated by CM of MM plasma cells, and reduced in the “wound” by
56% and 75% at 0.5 and 1.75 μM, respectively (number of migrated cells in the “wound” area: 42 ± 3 and 24 ± 2; P < .03 and P < .01); in the Boyden microchamber, 0.5 and 1.75 μM inhibited by 27% (P < .05) and 55% (P < .01). A not significant effect on MGECs chemotaxis (Supplementary Figure S1B), and no effect on HUVECs were observed (data not shown).

Of note, independently of the dose (0.1-10 μM), lenalidomide did not induce cell death of MMECs (nor MGECs nor HUVECs) as assessed by both MTT and crystal violet assays, and did not significantly interfere with their capacity to attach to and spread on fibronectin and vitronectin surfaces as well as on uncoated dishes. Similar results were obtained with MGECs (Supplementary Figure S1C), and HUVECs (data not shown). Apoptosis was also not induced (Figure 2C).

**Lenalidomide downregulates key genes involved in the MMECs angiogenic profile**

To check possible targets of the lenalidomide antiangiogenic activity, we investigated whether it could modulate the expression profile of genes that are primarily involved in MM angiogenesis (VEGF and bFGF), in MMECs migration (CCL2 and CXCL12) and in several critical biological functions closely related to angiogenesis (BNIP3, IER3, SEPW1, COL4A1, CRYAB and Notch1) (17). A significant downregulation was observed only in active MMECs at the 1.75 μM therapeutic dose (Figure 3). Compared to untreated MMECs, VEGF was 40% less expressed on average (P < .03); bFGF 50% (P < .001); CCL2 and CXCL12 ~ 45% (P < .001); BNIP3 40% (P < .03); IER3 and SEPW1 ~ 35% (P < .03); while COL4A1, CRYAB and Notch1 were not affected (Figure 3). Immunoblotting analysis confirmed the relative protein inhibition (Figure 3). In contrast, lenalidomide produced no or negligible gene/protein regulation in MGECs (Supplementary Figure S1D) or HUVECs (data not shown).

**Lenalidomide abolishes MMECs migration and angiogenesis by affecting VEGF/VEGFR2-mediated downstream signaling pathways**
Lenalidomide was shown to exert an inhibitory effect on MMECs angiogenesis by targeting chemotaxis. We have previously found that MMECs functions, including chemotaxis, are mediated by a VEGF/VEGFR2 autocrine loop (12,18). To assess whether lenalidomide could affect VEGF_{165}-triggered MMECs angiogenesis, serum-starved MMECs were exposed to VEGF_{165} alone or with added lenalidomide. The VEGF_{165}-triggered VEGFR2 tyrosine phosphorylation lowered significantly and dose dependently with the drug (−25% and −48% with 0.5 and 1.75 μM; \(P < .03\) and \(P < .01\), respectively, Figure 4A).

Since the activation of the mitogen-activated protein kinases Erk-1/2 is closely involved in the migration and tubular-like formation of ECs (19,20), we investigated whether lenalidomide could inhibit Erk-1/2 in MMECs, and found that it sizably affected, in a dose-dependent fashion, the VEGF_{165}-induced phosphorylation of Erk-1/2 (−34% and −80% at 0.5 and 1.75 μM; \(P < .003\) and \(< .001\), respectively; Figure 4A). In addition, Erk-1/2 influences the cell motility machinery by phosphoactivating myosin light chain kinase (MLCK) activity. This leads to phosphoactivation of myosin light chains (MLCs) which promotes cytoskeletal myosin-actin contraction needed for cell movement (21). Here we found that lenalidomide also markedly reduces the VEGF_{165}-induced phosphorylation of MLCs in MMECs (−60% at 1.75 μM, \(P < .01\), Supplementary Figure S2).

It is well documented that VEGFR2 is the principal receptor that mediates the mitogenic and chemotactic effects of VEGF on ECs (22), and that the VEGF/VEGFR2 loop stimulates the tyrosine phosphorylation of VE-cadherin, which is critical for the sprouting of capillary networks during angiogenesis and the metastatic spread of tumor cells (23,24). Thus, we tested whether lenalidomide could impact VEGF_{165}-mediated VE-cadherin phosphorylation in MMECs. The VEGF_{165} treatment significantly increased tyrosine phosphorylation of VE-cadherin (on Y^{658}), which was effectively diminished by 0.5 and 1.75 μM lenalidomide (−30% and −66% respectively; \(P < .01\); Figure 4B). This phosphorylation is mediated by Src tyrosine kinase activity and the activation of the VE-cadherin-Src complex is increased in angiogenic
tissues or VEGF-induced ECs, being a necessary step for the cell switch from the quiescent to the angiogenic phenotype (24,25). We found that lenalidomide consistently reduced VEGF$_{165}$-induced Src phosphorylation (on Y$^{418}$) in a dose-dependent manner (−20% and −53% at 0.5 and 1.75 μM, respectively; $P < .03$ and < .01; Figure 4B). Overall, data suggest that lenalidomide inhibition of VEGF$_{165}$-triggered angiogenesis in MMECs is mediated by disruption of both Src kinase and subsequent VE-cadherin tyrosine phosphorylation.

Since p120ctn is a VE-cadherin-binding protein in the adherent junctions, and affects cell-cell contacts and motility (26), we wondered whether it was modified by lenalidomide. Immunofluorescence studies showed that in VEGF$_{165}$-stimulated confluent MMECs, the p120ctn-specific fluorescence is diffusely distributed through the cytoplasm, probably because p120ctn cytosolic localization mediates the VEGF-triggered MMECs migration. In contrast, 1.75 μM lenalidomide elicited a massive sequestration of p120ctn at the cell-cell junctions (Supplementary Figure S3), which may contribute to the inhibition of the MMECs motility.

Furthermore, since NF-κB regulates critical genes associated with inflammation and cancer metastases (27), we investigated whether lenalidomide could also modulate the NF-κB-mediated pathway. The NF-κB transcriptional activation occurs via heterodimerization of its subunits p50 and p65. Lenalidomide inhibited, in a dose-dependent manner, the TNF-α-triggered phosphorylation of the p65 subunit (−65% and −86% at 0.5 and 1.75 μM, respectively; $P < .003$ and < .001; Figure 4C), without affecting basal p65. However, lenalidomide exerts comparable effects, albeit not significant, in MGECs, but not in HUVECs (Supplementary Figure S4).

**Lenalidomide-induced changes in the MMECs proteome**

To further dissect the inhibitory effects of lenalidomide at the molecular level, we pursued comparative proteomics analysis of MMECs of two patients (one at diagnosis and one at relapse) cultured without (Figure 5A) or with (Figure 5B) lenalidomide 1.75 μM for 72 hours,
and the same analysis was in parallel performed for MGECs, as control BM-derived endothelial cells (Figure 5C and 5D). At least two 2-DE gels were run per sample, followed by computer-assisted spot matching and silver staining images to enable localization of variation spots. Over 500 protein spots were visualized per gel, 10 proteins that were differentially expressed by the treatment (2-fold change) in MMECs were identified by peptide sequencing based on tandem mass spectrometry (MS/MS) and database searching (Figure 6 and Supplementary Table S1). Among these proteins, five were down-regulated: the cell membrane-embedded LBP/p40(RSSA), cytoplasmatic vimentin and keratin2C1, mitochondrial DLDH and glutamate dehydrogenase (DHE3), while five were upregulated: cytoplasmatic septin2 (SEPT2), γ-actin (ACTG), PNPH, the glycolytic enzyme ENOA, and PSB4. As shown in Figure 6, all the identified proteins were not significantly affected by lenalidomide treatment in MGECs. Interestingly, basal expression levels of vimentin, keratin2C1, mitochondrial DLDH, glutamate dehydrogenase (DHE3) and ENOA were increased in untreated MMECs vs. their normal counterpart (Figure 6, panels A of MMECs and MGECs).

In accordance with the above-mentioned anti-migratory and antiangiogenic effects of lenalidomide, most of the differently expressed MMECs proteins in response to lenalidomide are angiogenesis-related agents controlling cell shape and invasiveness (LBP/p40) (28), cytoskeletal dynamic remodeling (vimentin, keratin2C1, septin-2, γ-actin) (29-32), and coordinated events at the interface between cytoskeleton and membrane biology (polymerization of F-actin microfilaments and transport of organelle or ribosomal subunits during protein biogenesis). Alterations of enzymatic activities (DLDH, DHE3, PNPH and ENOA) (33, 34) are seemingly specific indicators of changes in energy metabolism pathways and oxidative stress responses, even in the absence of a significant apoptosis induction in lenalidomide-treated MMECs. Upregulated levels of PSB4 (35) could be part of a compensatory response to changes in cellular bioenergetics due to altered protein clearance and turnover.
Discussion

The clinical benefit of lenalidomide in treating myelodysplastic syndromes, MM, chronic lymphocytic leukemia, and non-Hodgkin’s lymphoma has drawn heightened attention to its mechanism of action, since specific targets of IMiDs® compounds remain undefined. In MM plasma cells lenalidomide inhibits cell growth and promotes apoptosis, and blocks their adhesion to stromal cells in the BM milieu (3,7). In stromal cells, lenalidomide reduces the expression of angiogenic factors and of several additional factors that support the plasma cell growth. In addition, lenalidomide stimulates T-cells and natural killer cells (6). IMiDs®, including lenalidomide, also have antiangiogenic properties that are independent of their immunomodulatory effects (36). We have already demonstrated that thalidomide exerts a direct antiangiogenic activity on MMECs due to downregulation of key genes mandatory for autocrine and paracrine loops of neovascularization, even if at ~10× higher doses than those of lenalidomide used here (4). However, while the IMiDs share many of thalidomide’s biological properties, the relative potency and range of these effects vary substantially from each other and from thalidomide. Thus, each molecule cannot be assumed to have the same overall biological effects or therapeutic properties as thalidomide or other IMiDs. In fact, as shown here, lenalidomide is antiangiogenic in vivo (CAM assay) and in vitro, hence overlapping thalidomide (37,38). However, lenalidomide halts only MMECs chemotaxis but not proliferation, while thalidomide halts both ECs functions (38), including proliferation in MMECs (10). Tentatively, we suggest that the drugs’ differential effects may derive from distinct mechanisms of action at the molecular level: thalidomide potently inhibits ECs functions, possibly through preventing Erk-1/2 nuclear translocation and/or inhibition of Akt/PKB phophorylation, thus halting mainly VEGF-mediated survival/proliferation (38), while lenalidomide inhibits markedly myosin light chains (MLCs) phosphorylation in the cytoplasm (Supplementary Figure S2) thus acting much more on cell migration. These observations further underscore striking pharmacodynamic differences between lenalidomide and thalidomide.
Accordingly in this study, we first pointed out the strong potential of lenalidomide in inhibiting in vivo angiogenesis induced by the CM of MM plasma cells (CAM assay) at 1.75 μM, which corresponds to the interstitial fluid level in patients treated with 25 mg/day/os. Then, we examined the activity of lenalidomide in targeting functions associated with in vitro angiogenesis, and found that it impairs both MMECs angiogenesis, and their migration activity, bringing on the maximum inhibition at the same dose effective for antiangiogenic activity in vivo. Of note, the angiogenic and chemotactic cell functions were significantly hampered only in MMECs derived from patients with active disease, while the drug exerted only negligible or no effect on ECs from non-active MM patients or MGECs, or on HUVECs. Previous studies (39) showed that lenalidomide inhibits VEGF, bFGF, and TNF-α-mediated migration of HUVECs only at doses 10-100 × higher than those used here and it inhibits angiogenesis in human umbilical artery explants, with approximately 50% inhibition at 1 μM (40). It is possible that the pro-angiogenic factors produced by the umbilical artery are similar to those factors present in the MMECs culture, or are sufficient to reveal the anti-angiogenic activity of lenalidomide.

While lenalidomide produces in vitro growth arrest and apoptosis of MM plasma cells and of a variety of solid tumor cell types (3), the drug does not affect viability and apoptosis of MMECs (nor MGECs nor HUVECs), thus indicating that the MMECs antiangiogenic effect does not correlates with those cell functions. Also, lenalidomide does not interfere with the MMECs fibronectin- and vitronectin-mediated adhesion. Data indicate that lenalidomide may inhibit MM angiogenesis due to an anti-migratory rather than cytotoxic or apoptogenic mechanisms on MMECs.

Accordingly, we investigated the molecular mechanisms involved in the inhibition of MM angiogenesis by lenalidomide. The drug induces inhibition of genes directly involved in the angiogenic cascade, such as VEGF and bFGF, and of the CCL2 and CXCL12 chemokines that are relevant for migration and homing of MMECs (41,42). Lenalidomide displayed a selective effect on other angiogenesis-related genes shown to be upregulated in active MMECs (17):
inhibition of BNIP3, IER3 and SEPW1, but no effect on COL4A1, CRYAB and Notch1. BNIP3 is overexpressed in many malignancies types due to hypoxia (17,43). Since hypoxia favors the malignant progression, the BNIP3 overexpression indicates high metastatic ability and aggressive phenotype, and implies a poor prognosis. Perhaps the downregulation of BNIP3 in active MMECs by lenalidomide can be explained as a way to overtake the hypoxia-mediated invasive potential, hence affecting their overangiogenic phenotype. IER3 is a member of the “stress-associated early response gene” family involved in a wide range of cell functions under stress conditions and in oncogenesis (44). Tentatively, the IER3 downregulation by lenalidomide in active MMECs may account for an effective way to block their overangiogenic phenotype, hence the MM plasma cell spreading and invasion. SEPW1 has antioxidant function that protects cells from oxidative stress (45). Since SEPW1-mediated homeostasis in MMECs may be crucial for their life and survival, the lenalidomide-induced SEPW1 downregulation may represent a way to overcome protection of the cells from oxidative stress, thus halting MM angiogenesis. Among the genes not regulated by lenalidomide, CRYAB is antiapoptotic (46) while Notch1 drives proliferation of primary MM cells (47). Given their roles in proliferation and apoptosis, they are unlikely to be affected by lenalidomide which, indeed, has no effects on these MMECs functions as shown here. Although COL4A1 is a major component of the vascular basement membrane, involved in the migration (‘sprouting’) of MM microvessels (10), the lenalidomide-mediated downregulation of other major genes governing the sprouting, such as VEGF, bFGF, CCL2, CXCL12, BNIP3, IER3, and SEPW1 can overcome the role of COL4A1.

We found that MMECs angiogenesis is sustained by the VEGF/VEGFR2 autocrine loop (18). VEGF strongly activates, via VEGFR2, the MAP kinase Erk-1/2, which is the last signaling mediator to the nucleus involved in cell proliferation and chemotaxis (19,20). As a consequence, Erk-1/2 specific inhibitors reduce endothelial tubulogenesis in vitro (20). The block by lenalidomide in active MMECs of the VEGF_{165}-induced phosphorylation of both
VEGFR2 and Erk-1/2 further confirms its antiangiogenic power in the MM BM milieu in which VEGF is highly represented (10).

VEGF increases tyrosine phosphorylation of the adherens junction components, such as VE-cadherin, β-catenin, plakoglobin, p120 catenin and the cell-cell adhesion molecule PECAM-1 in cultured ECs (23), and the VEGF-mediated VE-cadherin phosphorylation causes the loosening of cell-cell contacts in established vessels to allow ECs migration and vessel sprouting during angiogenesis (24). Src kinase is enriched at cell-cell contacts and implicated in regulating the cadherins and catenins phosphorylation; and the VEGF-induced-VE-cadherin tyrosine phosphorylation (on Y

Overall, these data indicate that lenalidomide impacts on MMECs migration in active MM, thereby inhibiting angiogenesis. This inhibition is associated with, and partly mediated by, modifications of intracellular signals triggered by the VEGF-regulated VE-cadherin.

Furthermore, we performed immunolocalization studies on p120ctn, which binds the cytoplasmic tail of cadherin and localizes into adherens junctions, thus playing a key role in cell-cell adhesion and motility (26). In epithelial cells, p120ctn shows a cytoplasmic diffuse distribution in sparse cultures that is indicative of the cell migratory state; while in confluent cells, the formation of cadherin-mediated cell-cell contacts is accompanied by sequestering p120ctn to the junction regions, thus abolishing its effects on cell migration (26). Our immunolocalization results suggest that, in active MMECs, lenalidomide strongly overcomes VEGF-mediated localization of p120ctn in the cytoplasm, by sequestering the protein at the cell-cell junctions. Perhaps the VEGF-triggered cytoplasmic distribution of p120ctn could impair VE-cadherin-p120ctn binding, thus making ECs free to migrate, while lenalidomide-induced...
sequestration at cell-cell contacts may stabilize the p120ctn-VE-cadherin interaction, thus blocking the MMECs motility.

NF-κB regulates several genes involved in angiogenesis, including the chemokine genes governing ECs migration (49). It stimulates angiogenesis by inducing IL-8 and VEGF expression (50). By demonstrating here that lenalidomide inhibits the activity of the p65 subunit of NF-κB in active MMECs, we suggest its role in inhibiting cell migration and angiogenesis in MM.

We further extended these findings, through a comparative proteomics analysis of the lenalidomide-treated MMECs vs untreated cells. The functional characterization of the identified proteins strengthens the inhibitory effects of lenalidomide on MMECs motility and angiogenic potential associated to changes in cytoarchitecture and mitochondrial energy metabolism. Some of the identified proteins (ie, LBP/p40, vimentin, keratin2C1, septin2, and γ-actin) are closely correlated with tumor growth, invasion and metastasis (28-31). How MM signaling networks could affect turnover and function of such proteins in MMECs remains a major challenge for future investigations. However, all these proteins could represent attractive biomarkers for monitoring resistance to antiangiogenic and cytoskeleton-disrupting drugs (32) in lenalidomide-treated MM patients.

In conclusion, our findings emphasize the intracellular mechanisms whereby lenalidomide exerts its antiangiogenic potential in active MM patients, and indicate that this drug may be of improved therapeutic value and could enhance responses to chemotherapy and radiotherapy in MM.
References


6. Marriott JB, Clarke IA, Dredge K, Muller G, Stirling D, Dalgleish AG. Thalidomide and its analogues have distinct and opposing effects on TNF-α and TNFR2 during co-stimulation of both CD4+ and CD8+ T cells. Clin Exp Immunol 2002;130:75-84.


FIGURE LEGENDS

Figure 1. Lenalidomide inhibits angiogenesis in CAM and Matrigel. (A) CAMs were incubated with gelatin sponges loaded with serum free medium (left panel), and with conditioned medium of MM plasma cells either alone (middle) or supplemented with 1.75 μM lenalidomide (right). Note the inhibition of MM angiogenesis by the drug. Images were acquired with a stereomicroscope. Original magnification: X50. (B) Lenalidomide inhibits MMECs angiogenesis in the Matrigel in a dose-dependent manner. MMECs arranged to form a closely knit capillary-like plexus (left panel), while the tube formation was gradually blocked with increasing lenalidomide doses with a full inhibition at 2.5 μM (right panel). A representative patient is shown. Images were acquired with a digital inverted microscope. Original magnification: X80. (C) Skeletonization of the mesh was followed by measurements of its topological parameters: mesh area, vessel length and branching points. Data are presented as mean ± SD of percent inhibition. Significance assessed by the Wilcoxon signed-rank test. CAM = chorioallantoic membrane; Len = lenalidomide; MM = multiple myeloma; MMECs = Endothelial cells from patients with multiple myeloma; SFM = serum free medium.

Figure 2. Lenalidomide affects MMECs functions associated with angiogenesis. Lenalidomide inhibits MMECs migration in a dose-dependent manner, using the ‘wound’ healing (A) and the Boyden microchamber (B) assays. Images were acquired with a digital inverted microscope. Original magnification: X200. MMEC chemotaxis columns/bars: means of migrated MMECs counted in five to eight X400 fields per patient in 27 patients ± SD. (C) Lenalidomide increasing doses do not affect MMECs viability and adhesion nor induce cell apoptosis. MMEC viability columns/bars: means ± SD in duplicate experiments per patient in 44 patients. MMEC adhesion symbols/bars: means ± SD in duplicate experiments per patient in 32 patients. The MMEC apoptosis is from one representative patient out of 28.
Significance assessed by the Wilcoxon signed-rank test. Len = lenalidomide; MM = multiple myeloma; MMECs = Endothelial cells from patients with multiple myeloma; SFM = serum free medium.

**Figure 3. Lenalidomide downregulates key genes and proteins of MM angiogenesis.**
Expression levels of genes (Real-Time RT-PCR) and proteins (Western blotting) in MMECs untreated and treated with lenalidomide. Data are expressed as mean relative expression ± SD of each gene/GAPDH mRNA levels of three independent experiments for each out of 20 patients. Gene expression fold change in untreated MMECs was arbitrarily set as 1 ± SD. Significance was assessed by the Wilcoxon signed-rank test. Len = lenalidomide; MM = multiple myeloma; MMECs = Endothelial cells from patients with multiple myeloma; OD = optical density.

**Figure 4. Lenalidomide impacts the VEGF_{165}/VEGFR2 downstream signaling in MMECs.** Serum-starved MMECs from 16 patients were pretreated with the lenalidomide doses for 6 hours, then stimulated with 10 ng/mL VEGF_{165} for 10 minutes or with TNF-α for 20 minutes. Whole cell lysates were prepared and probed with the indicated antibodies. The band intensity assessed with the Kodak Molecular Imaging Software as OD units is given as mean ± SD. Bands refer to a representative patient. Significance of changes was assessed by the Wilcoxon signed-rank test. MMECs = Endothelial cells from patients with multiple myeloma; OD = optical density.

**Figure 5. Lenalidomide-induced changes in the proteomic profiles of MMECs.** 2-DE gels of whole protein lysates from MMECs and MGECs incubated (A,C) without or (B,D) with 1.75 μM lenalidomide for 72 hours, respectively. Over computer-assisted matching of about 500 protein spots visualized (silver staining images of two gels/sample), 10 proteins were found to be differentially expressed in lenalidomide-treated MMECs (2-fold change). Specifically, the squares and circles in gels indicate downregulated or upregulated proteins,
respectively, upon lenalidomide treatment. 2-DE = 2-dimensional gel electrophoresis; MMECs = Endothelial cells from patients with multiple myeloma; MGECs = endothelial cells from patients with MGUS; MGUS = monoclonal gammopathy of undetermined significance.

**Figure 6. Representative areas of 2-DE gel maps.** Enlarged panels of 2-DE spot patterns (Figure 5) show differentially expressed proteins identified by MS (1-10) from (A) untreated and (B) lenalidomide-treated MMECs and MGECs. 2-DE = 2-dimensional gel electrophoresis; MMECs = Endothelial cells from patients with multiple myeloma; MGECs = endothelial cells from patients with MGUS; MGUS = monoclonal gammopathy of undetermined significance.
Abbreviations

7-AAD = 7-Amino-Actinomycin D
ACN = acetonitrile
ACTH = adrenocorticotropic hormone
bFGF = basic fibroblast growth factor
BNIP3 = BCL2/adenovirus E1B 19-KDa interacting protein 3
BSA = bovine serum albumin
CCL2 = chemokines (C-C motif) ligand 2
CHAPS = [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate]
CHCA = α-cyano-4-hydroxycinnamic acid
COL4A = collagen type IV alpha
CRYAB = alpha-B-Crystallin
CXCL12 = chemokines (C-X-C motif) ligand 12
DLDH = dihydrolipoyl dehydrogenase
DMEM = Dulbecco’s Modified Eagle’s Medium
DMSO = dimethyl sulfoxide
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
ENOA = enolase-alpha
Erk = extracellular signal regulated kinase
FBS = fetal bovine serum
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
ICAM-1 = inter-cellular adhesion molecule 1
IER3 = immediate early response 3
IEX-1 = Immediate early gene X-1
IFN-γ = interferon gamma
IL = interleukin
IPG = immobilized pH gradient
LBP = laminin-binding precursor
MALDI = matrix-assisted laser desorption/ionization
MCP-1 = monocyte chemotactic protein-1
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PE = annexin V = phycoerythrin annexin V
PECAM-1 = platelet endothelial cell adhesion molecule-1
PMSF = phenylmethylsulfonyl fluoride
PNPH = purine nucleoside phosphorylase
PSB 4 = proteasome subunit beta 4
RPMI = Roswell Park Memorial Institute
RSSA = ribosomal protein SA
SD = standard deviation
SDF-1 = stromal cell-derived factor 1
SDS = sodium dodecyl sulfate
SEPW1 = selenoprotein W1
TFA = trifluoroacetic acid
TNF-α = tumor necrosis factor-alpha
tris = tris(hydroxymethyl)aminomethane
VCAM-1 = vascular cell adhesion molecule-1
VE-cadherin = vascular endothelial cadherin
VEGF = vascular endothelial growth factor
VEGFR = VEGF receptor
A  *in vivo* CAM assay: 4 days

Conditioned medium of MM plasma cells

- SFM
- Len
- + Len 1.75 μM

B  *in vitro* Matrigel assay: 18 hours

- SFM
- Len 0.25 μM
- Len 0.5 μM
- Len 1.75 μM
- Len 2.5 μM

C

- Inhibition of mesh area
- Inhibition of vessel length
- Inhibition of branching points
MMEC Wound Assay

Conditioned medium of MM plasma cells

- SFM
- - Len
- + Len 0.5 μM
- + Len 1.75 μM

De Luisi et al., Figure 2
**A**

<table>
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<tr>
<th>Len (µM)</th>
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<td>VEGF₁₆₅</td>
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**B**

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<tr>
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**C**

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<tr>
<td>Total NF-κB p65</td>
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**Results**

- VEGFR2
  - OD: $P < .03$, $P < .01$
- VE-cadherin
  - OD: $P < .01$
- ERK 1/2
  - pERK1/2: $P < .003$, $P < .001$
  - Total ERK1/2
- Src
  - pSrc: $P < .03$, $P < .01$
  - Total Src
- β-actin
  - OD: $P < .03$
De Luisi et al., Figure 6

1 Laminin-binding precursor/p40
2 Vimentin
3 Keratin 2C1
4 Dihydrolipoyl dehydrogenase
5 Glutamate dehydrogenase
6 Septin 2
7 Alpha Enolase
8 γ-Actin
9 Purine nucleoside phosphorylase
10 Proteasome subunit beta type-4
Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma

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