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Thalidomide decreases gelatinase production by malignant B lymphoid cell lines through disruption of multiple integrin-mediated signaling pathways

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ABSTRACT

Background
Thalidomide (Thd) and its analogs are efficient agents in the treatment of multiple myeloma (MM). Since gelatinases (MMP2 and MMP9) play a crucial role in tumor progression, we explored the effect of Thd on gelatinase production by malignant B lymphoid cell lines.

Design and Methods
We investigated the effect of therapeutic doses of thalidomide on integrin-mediated production of gelatinases by malignant B lymphoid cell lines by gelatin zymography, western-blot, and RT-PCR and the ensuing invasive capacity through Matrigel-coated Boyden chambers. We also explored the effect of thalidomide on the activation status of the main signaling pathways involved in this process.

Results
Thd strongly inhibited gelatinase production by B cell lines and primary myeloma cells in response to fibronectin (FN), the most efficient gelatinase inducer identified in lymphoid cells. Thd disrupted integrin-mediated signaling pathways involved in gelatinase induction and release such as Src and MAP-kinase ERK activation, resulting in decreased cell motility and invasiveness. Unexpectedly, treatment with Thd elicited an increase in FN-induced Akt phosphorylation through PI3-kinase-independent pathways since Thd decreased FN-induced PI3-kinase phosphorylation and reversed the inhibition of Akt phosphorylation achieved by the PI3-kinase inhibitors wortmannin and LY294002.

Conclusions
Disruption of integrin-mediated signaling may be an important mechanism through which Thd and analogs impair tumor cell interactions with the microenvironment. The unexpected Thd effects on Akt activation indicate the need of further studies to elucidate whether interfering with Akt downstream effects would synergize with Thd anti-tumor activity.

Key words: matrix metalloproteinases, thalidomide, B cell malignancies, integrins.


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Introduction

Thalidomide (Thd) has received a great deal of attention in recent years due to its remarkable therapeutic efficacy in the treatment of multiple myeloma (MM). Thd and its analogs are also being investigated in other hematologic malignancies and hematologic disorders for which there is no effective treatment such as mantle cell lymphoma, chronic lymphocytic leukemia, and myelodysplastic syndromes. Thd is not directly cytotoxic and the mechanisms through which Thd exerts therapeutic benefits are not well defined and appear to be highly complex. Understanding the molecular basis of its effects is crucial in order to design more efficient and less harmful analogs.

Survival and progression of myeloma cells are highly dependent on stimuli from the bone marrow microenvironment. These include soluble factors, contact-dependent interactions with surrounding cells, and angiogenesis. IL-6, TNFα and IGF are pivotal in promoting proliferation and survival of myeloma cells. Contact-dependent interactions with bone marrow stromal cells, endothelial cells, and matrix proteins, including fibronectin, transduce survival signals, which may confer resistance to apoptosis induced by radiation, dexamethasone, or traditional chemotherapy. Angiogenesis is a crucial requirement for myeloma progression and the extent of angiogenesis inversely correlates with survival in patients with disseminated MM and also in patients with solitary plasmacytomas.

Thd was tried in MM on the basis of its ability to inhibit angiogenesis. Thd inhibits proliferation of endothelial cells and decreases FGF-2 and VEGF-induced angiogenesis in several models. In addition, Thd reduces the production of angiogenic factors VEGF, HGF and FGF-2 by endothelial cells obtained from bone marrow biopsies of MM patients. However, correlation between the extent of bone marrow angiogenesis and response to Thd has not been consistently demonstrated and extramedullary plasmacytomas are resistant to Thd in spite of being highly vascularized. These observations suggest that, besides inhibiting angiogenesis, Thd has additional therapeutic effects probably targeting interactions between tumor cells and the bone marrow microenvironment.

Gelatinases (MMP2 and MMP9) have a crucial function in the progression of lymphoproliferative disorders. Gelatinases facilitate tumor progression, not only by breaking natural barriers such as basement membranes or interstitial matrix but through many additional mechanisms such as activation of cytokines and growth factors by proteolytic cleavage, release of matrix-bound growth factors and exposure of cryptic sites or release of active fragments from large matrix proteins. It has been recently shown that integrin-mediated cell interaction with matrix molecules, particularly fibronectin, is the strongest inducer of gelatinase production and activation in cells of lymphoid origin. Integrin engagement not only induces gelatinase production but also gelatinase activation through coordinated induction of MMP2 activator MMP14, and down-regulation of tissue inhibitor of metalloproteinases (TIMP)-2. Moreover, integrin-mediated signaling drives a rapid post-transcriptional release of gelatinases through pathways related to cell migration.

Given the relevance of gelatinases in cell interactions with the microenvironment and ultimately in tumor progression, we investigated the effects of thalidomide on matrix-induced MMP production by malignant lymphoid B cell lines, in search for direct effects on malignant cells with relevant impact on their relationship with the surrounding milieu. We found that Thd decreases gelatinase production in response to fibronectin by interfering with multiple integrin-mediated signaling pathways which are also involved in the regulation of cell motility and survival. Thd-mediated disruption of integrin signaling may then impair multiple contact-dependent tumor cell interactions with the bone marrow microenvironment which are thought to be crucial for malignant cell survival.

Design and Methods

Reagents

Thalidomide (Chemie Grünenthal, Germany) was dissolved in DMSO to give a stock solution of 10 mg/ml, and stored at -20°C. Wortmannin was purchased from Sigma-Aldrich (St Louis, MO, USA). LY294002 was obtained from Calbiochem (EMD Chemicals, Inc, Gibbstown, NJ, USA). Lenalidomide was a generous gift from Dr Dolors Colomer (Haematopathology Unit, Dpt of Haematology, Hospital Clinic, Barcelona, Spain).

Cells and cell culture

Human B lymphoblastoid cell lines RAJI (derived from an EBV positive Burkitt’s lymphoma) and IM9 (derived from a plasma cell leukaemia) were obtained from the European Collection of Cell Cultures (Salisbury, UK). RPMI 8226 and KMM1 myeloma cell lines, Hbl-2 (diffuse B-cell lymphoma) and MEC-1 (B-cell chronic lymphocytic leukemia) cell lines were kindly provided by Dr Dolors Colomer. Cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% Fetal Calf Serum (FCS) (Biological Industries, Israel), 2 mM L-glutamine and 50 μg/mL gentamycin at 37°C in 5% CO₂.

Primary MM cells were obtained from remaining bone marrow aspirates obtained for diagnostic purposes from patients with MM. The use of this tissue was approved by the Ethics Committee and patients signed informed consent. Mononuclear cells were isolated by Ficoll-Histopaque density centrifugation. Adherent cells were removed by allowing cells to adhere to a 0.1% gelatin-coated dish at 37°C in 5% CO₂ for 24 hours. Non-adherent cells were collected, cultured in RPMI 1640 at 10% FCS and used for experiments. Unless otherwise indicated, cells were exposed to chemicals for 4 hours and cell viability was confirmed by trypan blue exclusion.
Gelatin zymography

Cells were resuspended in serum-free RPMI 1640 medium at 0.5×10⁶ cells/ml. 5×10⁶ cells per condition were preincubated with chemicals at the indicated concentrations for 30 minutes at 37°C before the addition of FN at 10 μg/ml. The supernatant fluid was collected 4 hours later and concentrated 200-fold with Unifil-10 concentrator devices (Millipore, Molsheim, France). Concentrated samples were subjected to gelatin zymography as previously described.20,21

RT-PCR

RNA was extracted from 5x10⁶ cells using TRizol, Reagent (GIBCO). One μg of RNA was used for cDNA synthesis with Superscript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), using oligo d(T)s priming. MMP2, MMP9 and MMP14 cDNAs were amplified as previously described.20,21 Thirty-five reaction cycles were run, each consisting of 3 steps of 45 seconds at 94°C, 57°C (for MMP2 and MMP9) or 60°C (MMP14), and 72°C respectively, followed by an elongation period of 10 minutes at 72°C. PCR products were analyzed in 1.2% agarose gels (Invitrogen). Multiplex amplification of β2-microglobulin was used as internal control.

Adhesion assay

Ninety-six-well plates (Nalgene Nunc International, Denmark) were coated with FN (50 μg/well) overnight at 4°C. Cells were suspended in serum free medium, plated on FN-coated wells at 0.15x10⁶ cells/well and incubated at 37°C for 1 hour. Non-adherent cells were aspirated and the remaining cells were fixed and stained with 0.2% crystal violet (Sigma) in 20% methanol, washed with distilled H2O and air-dried. The dye was solubilized with 1% SDS and optical density was read with a spectrophotometer at 600 nm wavelength. Conditions were tested in quadruplicate wells.

Matrigel invasion assay

10 μm-pore polycarbonate filters (Nucleopore, Toronto, Canada) were coated with Matrigel (kindly provided by Dr Hynda K Kleinman, NIH, Bethesda, MD) diluted in RPMI 1640 at 1.25 mg/ml, and placed between the lower and the upper compartment of 48-well Boyden chambers (Neuro Probe Inc, Gaithersburg, MD). The lower compartments were filled with 25 μL RPMI 1640 with 10% FCS and 0.1x10⁶ cells in serum free medium with the indicated concentrations of thalidomide were loaded onto the upper chambers. After 6 hour-incubation at 37°C, the filters were removed, fixed with methanol and stained with hematoxylin. Cells on the upper side were swept and cell number/field in the lower side was counted under an inverted microscope in 6 randomly selected fields/well. Experiments were performed in quadruplicate wells.

Flow cytometry analysis

0.5x10⁶ cells per condition were incubated for 30 min at 4°C with 2 μg of the following mAb diluted in 100 μL of RPMI-1% BSA: anti- integrin α4 chain (clone HP2/1) (Immunotech, Marseille, France), anti- integrin α5 chain (clone SAM1) (Immunotech), anti-εβ3 integrin (clone LM609) (Chemicon International, Inc., Temecula, CA, USA), anti-integrin β1 chain (Clone K20) (Immunotech) or HUTS-21 (β1 activated epitope), a generous gift from Dr F Sánchez-Madrid (Hospital La Princesa, Madrid). Cells were subsequently washed with cold PBS-1% BSA and incubated for 30 min at 4°C with goat anti-mouse IgG Alexa Fluor™ 488 secondary antibody (Molecular Probes, Leiden, The Netherlands) at 1:200 dilution. After two washes with cold PBS-1% BSA, cells were fixed in 200 μL of 5% formaldehyde in PBS and fluorescence measured using FACScan™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Analysis of the expression of HUTS-21 was done in the absence and in the presence 200 μM of Mn2⁺ divalent cations.22

Western-blot

5x10⁶ cells per condition were incubated with the indicated concentration of chemicals with or without subsequent exposure to FN at 10 μg/ml in serum-free RPMI 1640 medium for 4 hours. Cells were lysed in 0.5 mL of modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X-100, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) supplemented with freshly added protease inhibitors (Complete™, Boehringer Mannheim, Germany) and NaVO₃ at 200 μM. Protein content of lysates was measured with the BCA protein assay (Pierce, Rockford, IL, USA). Twenty μg of lysate per condition were subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with the appropriate primary antibody: ERK MAP kinase activation state was evaluated using anti-phospho-p44/42 MAPK. The nitrocellulose membrane was then stripped and reprobed with p44/42 MAPK rabbit polyclonal IgG antibodies (Cell Signaling, Beverly, MA) at 1:1000 dilution. Src activation was evaluated with anti-phospho-Src at Y416 (activated form), then reprobed with anti-phospho-Src at Y527 (inactive form) and finally reprobed with anti-Src rabbit polyclonal IgG antibodies (Cell Signaling) at 1:1000 dilution. Akt activation state, combination of two anti-phospho-Akt (Ser473 and Thr308) were used and the membrane reprobed with AKT rabbit polyclonal IgG antibodies (Cell Signaling) at 1:1000 dilution. To assess Akt activation state, combination of two anti-phospho-Akt (Ser473 and Thr308) were used and the membrane reprobed with AKT rabbit polyclonal IgG antibodies (Cell Signaling) at 1:1000 dilution. Activation state of PTEN was evaluated with anti-phospho-PTEN rabbit polyclonal IgG antibody (Cell Signaling) at 1:1000. Anti-phospho-mTOR and anti-mTOR antibodies were also obtained from Cell Signaling. Monoclonal mouse anti-human β-actin antibody (clone AC-15) (Sigma) was used at 1:2000 dilution. MMP14 was detected with a polyclonal rabbit anti-human MMP14 (Chemicon) at 1:1000 dilution.

Immunodetection was performed by incubating membranes with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit HRP-conjugated, Cell Signaling or anti-mouse HRP-conjugated, Transduction Laboratories, Lexington, KY, USA) at 1:2000 dilution. Chemiluminescence signals were detected with LUMIGLO™, reagent (Cell Signaling). Membranes were exposed to LAS3000 chemiluminiscence detector (Fuji

Thalidomide decreases integrin-mediated gelatinase production
Results

**Thalidomide strongly decreases fibronectin-induced gelatinase (MMP2 and MMP9) and MMP14 production by malignant B lymphoid cells**

Fibronectin (FN) is a matrix protein present in the bone marrow microenvironment which is crucial in promoting tumorigenesis through several mechanisms including stimulation of cell migration, invasiveness and survival. These responses are mainly mediated by integrin engagement and signaling. We used two B lymphoid cells, IM9 and Raji, to study gelatinase production induced by fibronectin. As we have previously demonstrated in T cell lines, exposure to FN strongly induced metalloproteinase (MMP9, MMP2 and MMP14) expression and release by both IM9 and Raji cells (Figure 1 and 2). Interestingly, Thd decreased fibronectin-induced gelatinase production in a dose-dependent manner (Figures 1 and 2A and 2B). Production of MMP2 activator, MMP14, which is also up-regulated by fibronectin in lymphoid cells, was strongly inhibited by Thd, particularly in its activated form (Figure 1C). Therefore, Thd not only reduced FN-induced MMP expression, but also restrained gelatinase activation and release.

Raji and IM9 cells used in this study come from different lymphoid malignancies, suggesting that the effects of Thd on integrin signaling is not restricted to a particular cell line. Accordingly, Thd impairment of gelatinase production in response to fibronectin was also confirmed in primary multiple myeloma cells obtained from bone marrow aspirates of 2 patients (Figure 2D). Patient 1 had 100% of plasma cell infiltration whereas patient 2 had 50%. The increased gelatinolytic signal observed in cells from patient 2 may indicate contribution of additional cells (i.e. from the myelomonocytic lineage) which have higher ability to produce gelatinases than cells of lymphoid origin and suggest that Thd may also decrease MMP production by other cell types present in the MM microenvironment.

**Thalidomide decreases Raji cell adhesion and invasiveness**

We have previously shown that fibronectin-induced gelatinase production by lymphoid cells is mediated by integrins α4, α5 and αv. Given that Thd decreased integrin-mediated induction and release of gelatinases, we assessed the effect of Thd on additional integrin-mediated functions, such as cell adhesion to matrix proteins. Preliminary experiments showed that Raji cells significantly adhered to fibronectin whereas adhesion to other matrix proteins such as collagen I, collagen IV or laminin was poor (data not shown). Thd significantly decreased Raji cell adhesion and spreading onto fibronectin in a dose-dependent manner (Figure 3A). Moreover, as shown in Figure 3B, Thd significantly reduced Raji cell invasion through the reconstituted basement membrane Matrigel. As reported by others, IM9 cells were not naturally adherent to FN. Although IM9 cells interacted with fibronectin and efficiently produced gelatinases in response to FN engagement, these cells were unable to complete additional steps required for cell attachment and migration and therefore were not tested in these systems. To confirm that Thd analogs elicit similar responses, and to verify activity in other cell lines, we explored the effects lenalidomide in other cell types including a MM derived cell line, RPMI 8226. As shown in Online Supplementary Figure 1, lenalidomide was highly effective in reducing cell adhesion, and gelatinase production in response to FN.
Thalidomide decreases integrin-mediated gelatinase production

We next investigated whether Thd inhibitory effects on integrin-mediated responses were due to a decrease in integrin expression. As displayed in Figure 3C, both cell lines significantly expressed fibronectin receptors \( \alpha v \beta 3, \alpha 4 \) and \( \alpha 5 \). According to previously published data, these integrins mediate fibronectin-induction of gelatinases.\(^{29}\) In these experimental conditions, Thd did not significantly down-regulate integrin surface expression as disclosed by flow cytometry (Figure 3C) nor did it modify the expression of \( \beta 1 \) integrin chain-activation related epitope recognized by the monoclonal antibody HUTS-21\(^{22}\) which was weakly expressed at baseline but strongly induced by \( \text{Mn}^{2+} \). Taken together, these findings indicate that Thd effects on FN-induced adhesion and gelatinase production do not primarily depend on changes in integrin expression and suggest that Thd may influence integrin-mediated signaling pathways.

**Treatment with thalidomide results in decreased phosphorylation of integrin-activated kinases involved in the regulation of gelatinase production in response to fibronectin**

In previous studies, we have shown that fibronectin-induced gelatinase expression and release by lymphoid cells is mediated by Src, PI3-kinase and MAP kinases ERK1/2.\(^{20,21}\) However, while inhibition of Src by PP2 also results in decreased gelatinase synthesis and secretion, inhibition of ERK phosphorylation by PD98059 and of PI3-kinase with wortmannin leads to increased fibronectin-induced rapid gelatinase release into the medium followed by a reduction in gelatinase mRNA.\(^{20,21}\) Similar effects were observed with B cell lines used in this study (data not shown). We next investigated the effect of Thd on these pathways. Fibronectin-induced Src activation was decreased by Thd as illustrated by the decrease in Src phosphorylation at Y416 elicited by Thd treatment (Figure 4A).\(^{25}\) By contrast, Src phosphorylation at Y527 leading to Src inactivation remained unmodified (Figure 4A). Thd also resulted in strongly decreased ERK phosphorylation induced by FN (Figure 4B).

**Thalidomide increases integrin-induced Akt phosphorylation in a PI3-kinase-independent pathway**

Previous studies have shown that PI3-kinase participates in fibronectin-induced release of MMPs.\(^{25}\) Given that Thd reduced phosphorylation of several kinases crucial to integrin-signaling, we next explored the effect of Thd on PI3-kinase activation and ensuing downstream effects. When activated by tyrosine phosphorylation of its p85 subunit, PI3-kinase catalyzes phosphatidylinositol trisphosphate (PIP3) formation. Then PIP3 recruits Akt to the cell membrane where it can be phosphorylated by active phosphoinositide-dependent kinase 1 (PDK1).\(^{26,27}\) Akt activation promotes cell survival by stimulating anti-apoptotic pathways and this mechanism is considered to be a pivotal downstream effect of PI3-kinase function.\(^{26,27}\) Exposure to Thd decreased PI3-kinase activation in both cell lines (Figure 5A).

We also explored the effects of thalidomide on PTEN activation. PTEN is a lipid phosphatase which dephosphorylates PIP3, decreasing Akt activation.\(^{20,21}\) PTEN is considered to be a tumor suppressor gene given that loss of PTEN activity by deletion or mutation is commonly found in solid tumors. However, although PTEN mutations can be found in some multiple myelomas,\(^{20}\) loss of PTEN function is an uncommon pathway of malignant transformation in lymphoproliferative disorders.\(^{6,31}\) We found that Thd reduced fibronectin-induced PTEN phosphorylation in IM9 cells (Figure 5B). Raji cells lacked detectable PTEN protein (data not shown). Since PTEN is inhibited by phosphorylation, this finding indi
cates that Thd increases PTEN activation, which may reduce even more downstream effects of PI3-kinase in IM9 and cells expressing functional PTEN. The increase in PTEN activity could also contribute to the anti-tumoral effects of Thd since PTEN inhibits cell proliferation through Akt-dependent or independent pathways.

Akt phosphorylation is a crucial downstream effect of PI3-kinase activation. We hypothesized that reduced PI3-kinase activation and increased PTEN activity induced by Thd might then result in decreased Akt activation, in accordance with Thd anti-tumor effects. Unexpectedly, and in spite of reduced PI3-kinase phosphorylation and increased activity of PTEN in IM9 cells, Thd increased FN-induced phosphorylation of Akt in both cell lines (Figure 6A) and was able to overcome the inhibitory effect of the PI3-kinase inhibitor wortmannin on Akt phosphorylation (Figure 6B). This finding was confirmed with the more specific PI3-kinase inhibitor LY294002 (Figure 6C). These findings indicate that Thd increases Akt phosphorylation through a PI3-kinase-independent pathway. Supporting the functional relevance of the increase in Akt phosphorylation, exposure to Thd resulted in an increased phosphorylation of Akt substrate mTOR in both cell lines (Figure 6C).

The increase in Akt phosphorylation induced by Thd was confirmed in myeloma cell lines RPMI 8226 and KMM1 (Online Supplementary Figure 2). In these cell lines total Akt content and Akt phosphorylation both in response to FN and to Thd were less intense than in Raji or IM9 cells, which are derived from more aggressive malignancies.

**Discussion**

Our data indicate that Thd has strong direct effects on tumor cells leading to disruption of integrin-mediated interactions with the surrounding microenvironment. Interfering with integrin-mediated signaling may also contribute to previously identified Thd effects such as angiogenesis inhibition and T-cell co-stimulation since cell-cell and cell-matrix interactions mediated by integrins are crucial in these processes. The signaling pathways disrupted by thalidomide regulate crucial cell functions for tumor progression such as cell motility, cell-cell and cell-matrix interactions, gelatinase production, and cell growth. Src kinases and ERK are key enzymes in promoting tumor cell growth and invasiveness. Increased Src activity by v-Src has been one of the first recognized mechanisms of malignant transforma-
Src also plays a crucial role in cell motility and we and others have shown that Src-family kinases are key regulators of integrin-mediated gelatinase production and rapid release through multiple interactions with focal adhesion kinase (FAK) and FAK-associated signaling molecules. Src not only mediates gelatinase production but also post-transcriptional rapid release of gelatinases in response to fibronectin. Therefore Src and ERK inhibition, may both be important mechanisms through which Thd decreases cell motility and MMP production in response to matrix proteins. Changes in protein phosphorylation elicited by thalidomide suggest that interference with these and other protein kinase and/or phosphatase activities may lead to additional alterations in cell function which may contribute to its anti-angiogenic, anti-inflammatory and anti-tumoral effects.

However, the increase in Akt activity may seem inconsistent with the anti-tumor activity of thalidomide, given that activated Akt transduces powerful anti-apoptotic signals and increase in Akt activation is crucial in survival of many tumors including multiple myeloma. Recent trials have shown that while Thd delays progression in multiple myeloma, advance is accelerated in some patients when progression occurs, supporting that Thd may activate, indeed, some survival pathways. Thalidomide-mediated survival signals through Akt activation may be partially overcome by interference with other important pathways stimulating cell growth and migration such as Src or MAP kinases in response to extracellular signals. Interestingly, increased Akt activity renders cells more sensitive to the suppressive effects of mTOR inhibitors on cell survival and on angiogenesis. The effect of Thd on Akt and mTOR activation suggests that inhibitors of mTOR may potentiate Thd anti-tumor effects as suggested by experimental animal models.

In conclusion, functional testing of new Thd analogs is currently based on their ability to elicit known Thd effects such as T-cell co-stimulatory function or inhibition of cytokine production and angiogenesis. The newly recognized effects of Thd reported here may help in the screening of new therapeutic agents.

Authorship and Disclosures

MS, EL, MC-B, CV, M-TC and JE performed the laboratory work. MS, NI, JB and MCC participated in the design of the research. MS and MCC analyzed the data and wrote the paper. The authors reported no potential conflicts of interest.

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