Regulació de la producció de gelatinases (MMP2 i MMP9) pels limfòcits.

Implicació en malalties inflamatòries i síndromes limfoproliferatives

Tesi presentada per

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Efecte de la Talidomida en la Producció de Gelatinases induïda per Integrines en Línies Cel·lulars de Limfòcits B

TERCER ESTUDI:

Anàlisi de l'efecte de la talidomida sobre la producció i l'alliberament de gelatinases per línies cel·lulars limfocítiques de fenotip B. Estudi de l'acció de la talidomida sobre les vies de senyalització que vehiculitzen la inducció de gelatinases per integrines.

Com a resultat d'aquest treball hem elaborat el següent article: Thalidomide Disrupts Fibronectin-induced Gelatinase Production by Malignant B Lymphoid Cells through Interference with Integrin-mediated Signaling Pathways. Segarra M, Lozano E, Vilardell C, Cibeira MT, Esparza J, Izco N, Bladé J, Campo E, Cid MC. (Abstract presentat oralment al congrés Experimental Biology 2004 FASEB Meeting i manuscript enviat a publicar).

THALIDOMIDE DISRUPTS FIBRONECTIN-INDUCED GELATINASE PRODUCTION BY MALIGNANT B LYMPHOID CELLS THROUGH INTERFERENCE WITH INTEGRIN-MEDIATED SIGNALING PATHWAYS.

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Running title: Thalidomide decreases integrin-mediated gelatinase production

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ABSTRACT

Purpose: Thalidomide and its analogs have become relevant agents in the treatment of patients with multiple myeloma but the molecular mechanisms underlying their effects are poorly understood.

Methods: Given that gelatinases (MMP2 and MMP9) play a crucial role in tumor progression, 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: Thalidomide strongly inhibited gelatinase (MMP2 and MMP9) and MMP2-activator MMP14 production by B cell lines and by primary myeloma cells in response to fibronectin, which has been previously shown to be the most efficient inducer of gelatinases in cells of lymphoid origin. Accordingly, treatment with thalidomide resulted in decreased cell invasiveness. These effects were not due to changes in integrin expression but to disruption of integrin-mediated signaling. Exposure to thalidomide resulted, indeed, in severely reduced Src and ERK MAP kinase phosphorylation in response to fibronectin, crucial events not only in integrin-induced gelatinase expression but also in cell migration and survival.

Conclusion: Disruption of integrin-mediated signaling may be a crucial effect of thalidomide that, in addition to gelatinase production, may also impair additional relevant cell responses driven by the tumor microenvironment such as cell motility and cell growth.

INTRODUCTION

Thalidomide (Thd) has received a great deal of attention in recent years because of its therapeutic efficacy in the treatment of multiple myeloma (MM)[1]. Consequently, Thd and its analogs are now being investigated in other hematologic disorders for which rescue therapeutic strategies are needed such as mantle cell lymphoma[2], chronic lymphocytic leukemia[3] and myelodysplastic syndromes[4].

The mechanisms through which Thd exerts its therapeutic benefits are not well defined and appear to be highly complex. Understanding the molecular basis of its effects is essential to design more efficient and less harmful analogs[1]. Identifying new biologic effects is important in order to test Thd-derivatives in preclinical studies.

It is widely believed that Thd benefits on MM patients are related to the impact of Thd on MM microenvironment[1]. Thd inhibits TNFα production through various pathways[5,6] and disrupts the production of other cytokines produced in the MM milieu such as IL-6 and IL-10[1] which are able to promote tumor growth and survival[7]. Moreover, Thd inhibits angiogenesis and this has been thought to be one of the most important mechanisms underlying its therapeutic effects. However, correlation between the degree of bone marrow angiogenesis and response to Thd has not been consistently demonstrated[8]. In addition, extramedullary plasmacytomas do not respond to Thd in spite of being highly vascularized[9]. These data support that Thd may have many additional relevant effects in addition to angiogenesis inhibition and may also have direct effects on malignant cells. In this regard, Thd and its analogs may induce apoptosis of myeloma cells but the molecular mechanisms involved are not clearly understood[10].

Thd has also immunomodulatory functions that may influence host defense against malignant cells. It provides co-stimulatory signals for T cell activation by increasing CD28 phosphorylation[11] and increases IL-2 and IFNγ production[12]. Based on these functions it has been used to treat infectious and immune-mediated conditions such as erythema nodosum leprosum, Behçet's and graft-versus-host disease, among others[1].

Gelatinases (MMP2 and MMP9) have a crucial function in tumor progression[13,14] not only by breaking natural barriers such as basement membranes but by many other 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[13,14]. Integrin-mediated cell interaction with matrix molecules, particularly fibronectin, is the most powerful inducer of gelatinase production, release, and activation by cells of lymphoid origin[15,16].

Given the relevance of gelatinases in cell interactions with the microenvironment and ultimately in tumor progression, we investigated the effects of Thd on integrin-induced MMP production by malignant B lymphoid cell lines, in search for direct effects on malignant cells with relevant impact on their relationship with the surrounding milieu.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

Human B lymphoblastoid cell lines Raji (EBV positive Burkitt's lymphoma) and IM9 (plasma cell leukaemia) were acquired from the European Collection of Cell Cultures (Salisbury, UK). Primary MM cells were isolated from bone marrow aspirates obtained for diagnostic purposes from patients with MM. Cells were cultured as described[16].

EXPERIMENTAL PROCEDURES

Gelatin zymography of concentrated cell supernates was performed as described[15,16]. Total RNA was extracted with TRIzol® reagent (Invitrogen) and RT-PCR amplification of MMP2, MMP9 and MMP14 was done as previously reported[16]. Adhesion and invasion assays were performed as described[16].

Flow cytometry measurement of surface receptors was carried out as previously published[15] using the following monoclonal antibodies: anti-α4 integrin chain (clone HP2/1) (Immunotech, Marseille, France), anti-α5 integrin chain (clone SAM1) (Immunotech), anti-αvβ3 integrin (clone LM609) (Chemicon International, Inc., Temecula, CA), anti-β1 integrin chain (clone K20).

Protein phosphorylation was assessed by western-blot analysis as reported[16] using the following primary rabbit anti-human polyclonal antibodies: anti-phospho-p44/42 MAPK, anti-p44/42 MAPK, anti-phospho-Src at Y416 (active form), anti-phospho-Src at Y527 (inactive form), and anti-Src (Cell Signaling, Beverly, CA). MMP14 was detected with a polyclonal rabbit anti-human MMP14 (Chemicon).

Cell viability after exposure to chemicals was confirmed by trypan blue exclusion. All experiments were repeated at least 3 times with consistent results.

RESULTS AND DISCUSSION

THALIDOMIDE STRONGLY INHIBITS FIBRONECTIN-INDUCED GELATINASE (MMP2 AND MMP9) AND MMP14 PRODUCTION BY MALIGNANT B LYMPHOID CELLS

None of the cell lines used in this study had baseline expression of MMP2. IM9 but not Raji had slight constitutive expression of MMP9. As we have previously demonstrated in T cell lines, exposure to fibronectin strongly induced gelatinase (MMP2 and MMP9) and MMP14 expression by both IM9 and Raji cells. Interestingly, Thd dramatically inhibited fibronectin-induced gelatinase expression by both cell lines in a dose-dependent manner (figure 1A). Fibronectin-induced gelatinase production by primary myeloma cells was also reduced (figure 1B). Production of MMP2-activator MMP14, which is also up-regulated by fibronectin in lymphoid cells[15,16], was also inhibited by Thd treatment, particularly in its activated form (figure 1C). Thd decreased fibronectin-induced MMP2, MMP9 and MMP14 mRNA in both cell lines (figure 1D).

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[15]. 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 (data not shown). Thd significantly decreased Raji cell adhesion to fibronectin in a dose-dependent manner (figure 2A). Given the relevance of gelatinase production in cell invasiveness we next tested the effect of Thd on Raji cell invasion through

reconstituted basement membrane Matrigel. As shown in figure 2B, Thd significantly reduced Raji cell invasion through Matrigel-coated filters.

We next investigated whether Thd inhibitory effects on integrin-mediated responses were due to a decrease in integrin expression. Both cell lines significantly expressed fibronectin receptors α4 and α5 and the common β1 chain. In addition, IM9 cells expressed αvβ3. In the conditions used in our experiments, Thd did not significantly modify integrin surface expression as assessed by flow cytometry (figure 2C).

TREATMENT WITH THALIDOMIDE RESULTS IN DECREASED PHOSPHORYLATION OF INTEGRIN-ACTIVATED KINASES INVOLVED IN THE REGULATION OF GELATINASE PRODUCTION IN RESPONSE TO FIBRONECTIN

We have previously shown that fibronectin-induced gelatinase production by lymphoid cells is mediated by activation of Src and ERK1/2 MAP kinases[15,16]. Given that Thd did not elicit changes in integrin expression, we next investigated the effect of Thd on integrin-mediated signaling pathways. Fibronectin-induced Src activation was decreased by Thd as illustrated by the decrease in Src phosphorylation at Y416 elicited by Thd treatment. By contrast, Src phosphorylation at Y527 leading to Src inactivation remained unmodified (figure 2D). Similarly, Thd also inhibited ERK phosphorylation induced by fibronectin (figure 2E).

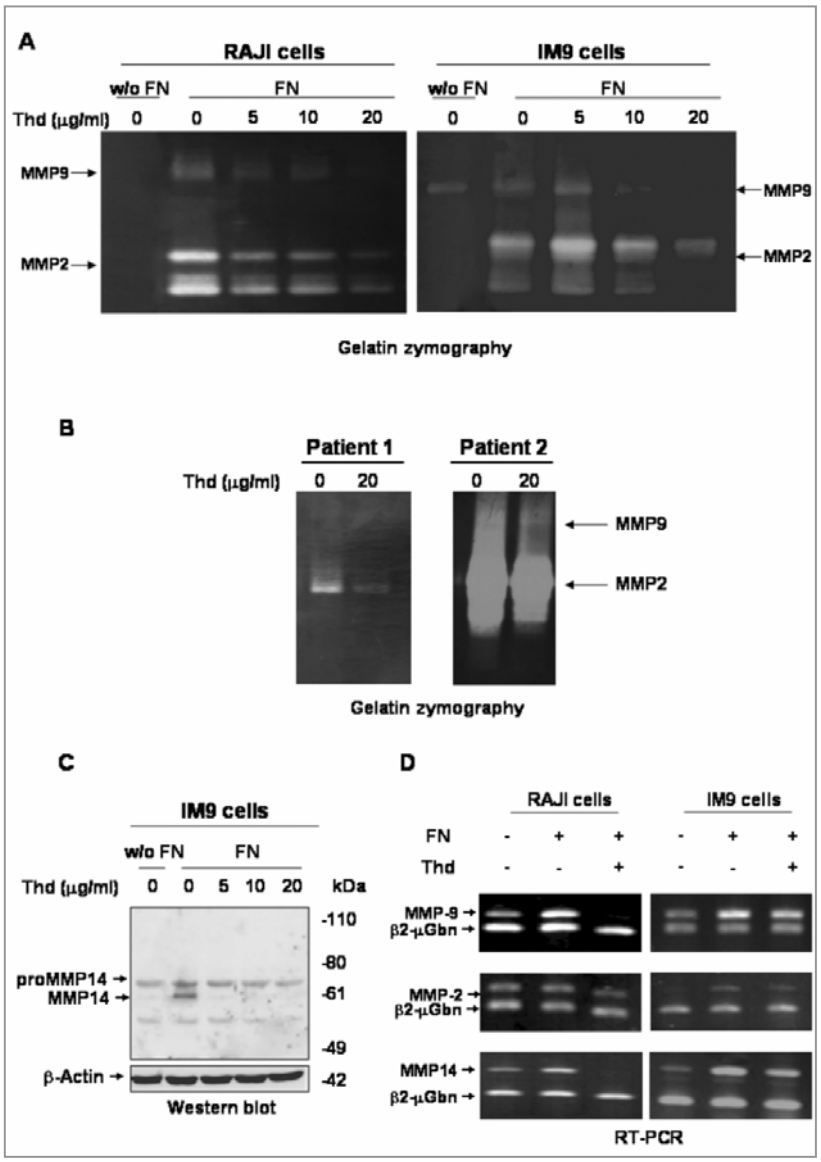
Thd-disrupted integrin-mediated pathways are involved not only in gelatinase production but also in other relevant functions such as cell motility and cell growth[17]. Src kinases are key enzymes in promoting tumor cell growth and invasiveness. Increased Src activity by v-Src has been one of the first recognized mechanisms of malignant transformation[18]. Src plays also a crucial role in cell motility and we an

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[16,19]. ERK are also involved in focal adhesion turnover and mediate integrin and growth factor-stimulated cell growth[17]. Disruption of integrin-mediated signaling and its consequences on endothelial cell growth, motility and MMP production may be also an important mechanism underlying the inhibition of angiogenesis achieved by Thd, given the relevance of cell-matrix interactions during new blood vessel formation[20].

Interference with these and other protein kinase activities may contribute to most of the identified effects which are thought to contribute to the therapeutic benefit of Thd. Changes in protein phosphorylation are indeed on the basis of the known co-stimulatory effects and decreased NFκB activation elicited by Thd in T cells[5,11].

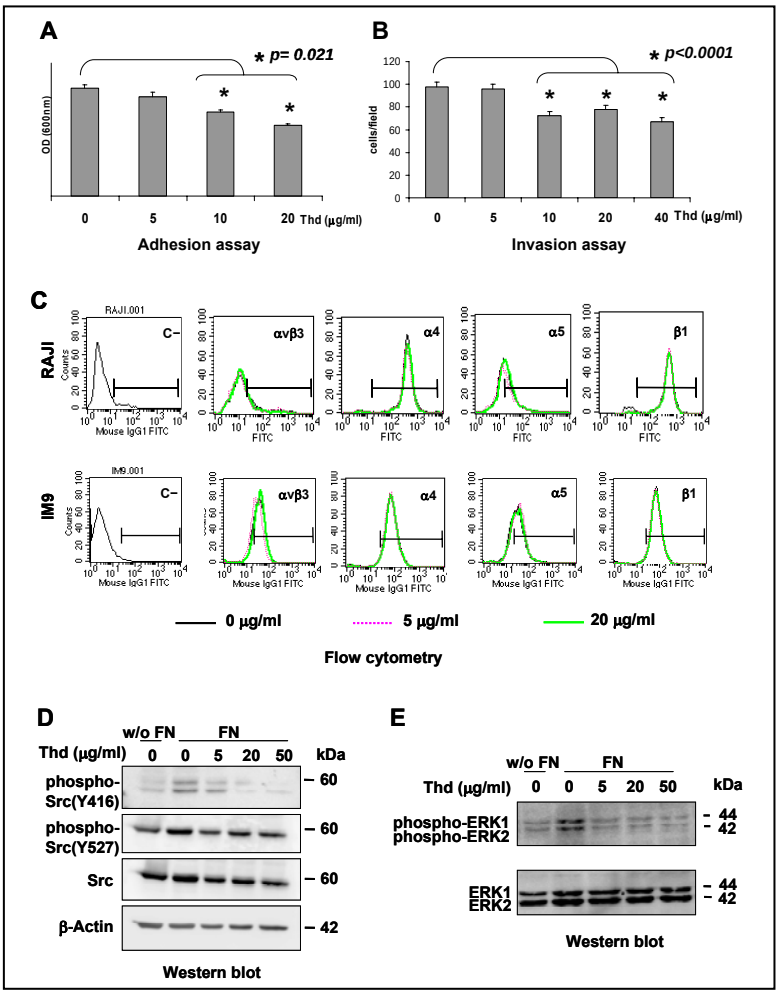
In summary, our data indicate that thalidomide has direct effects on tumor cells disrupting multiple integrin-mediated signaling pathways driven by tumor cell interactions with the surrounding milieu and underline the importance of the tumor microenvironment for the anti-myeloma effect of thalidomide, as supported by our clinical findings[9]. The signaling pathways interfered by thalidomide are known to regulate not only gelatinase production but also additional cell functions crucial for tumor progression such as cell motility and cell growth.

FIGURE 1: Effect of Thd on gelatinase (MMP2 and MMP9) and MMP14 expression by lymphoid cells.



A) Gelatin zymography of concentrated supernates of RAJI and IM9 cells ($5x10^6$ cells per condition) exposed to thalidomide (Thd) (Chemie Grünenthal, Germany) at the indicated concentrations for 4 hours in the absence (w/o) or in the presence of fibronectin (FN) (10 μg/mL). **B)** Gelatin zymography of the concentrated supernates of plasma cells obtained from bone-marrow aspirates from patients with multiple myeloma. Ficoll-Histopaque separated cells were exposed to Thd at 20 μg/ml for 4 hours in the presence of FN (10 μg/mL). Patient 1 had 100% plasma cell infiltration whereas patient 2 had 50%, as assessed by cytology examination. **C)** Western-blot analysis of whole cell lysates obtained with modified RIPA buffer[16] (20 μg protein per lane) obtained from IM9 cells exposed to Thd at the indicated doses for 4 hours in the absence or in the presence of FN. Immunoreactivity to β-actin after stripping is displayed as control for loading. Similar results were obtained with RAJI cells. **D)** RT-PCR of RNA obtained from RAJI and IM9 cells cultured in the absence (-) and in the presence (+) of FN and exposed to Thd at 20 μg/ml for 4 hours. Multiplex amplification of β2-microglobulin (β2-μGbn) was used as internal control.

FIGURE 2: Effect of Thd on lymphoid cell migration and invasion and on crucial signaling pathways involved in these processes.



A) Raji cells suspended in serum-free medium, exposed to Thd at the concentrations displayed were allowed to adhere to fibronectin-coated wells for 1 hour at 37°C. Adherent cells were stained with crystal violet, solubilized in 1% SDS, and the optical density measured at 600 nm wavelength[16]. **B)** Raji cells were treated with the indicated doses of Thd and laid in the upper compartment of Boyden chambers containing Matrigel-coated polycarbonate filters and incubated at 37°C for 6 hours[16]. Bars represent mean ± SEM in both figures. Mann Whitney U test was used for statistical comparison. As previously reported, IM9 cells were not naturally adherent to FN. Although IM9 cells interacted with FN and efficiently responded to it in gelatinase production, these cells were unable to complete additional cascades required for cell attachment and were not tested in these systems. **C)** Flow cytometry analysis of integrin surface expression by Raji and IM9 cells at baseline and after exposure to Thd at the indicated concentrations for 6 hours. Identical results were obtained at earlier time-points (1 hour). **D)** Western-blot analysis of cell lysates obtained from Raji cells exposed to Thd for 4 hours in the absence or in the presence of FN. Membranes were incubated with phospho-specific anti-Src antibodies, stripped and reprobed with anti-Src antibodies. Similar results were obtained with IM9 cells. The lower molecular species identified with anti-phospho Y416 Src antibody probably corresponds to phosphorylated Src-type tyrosine kinase Lyn present in B cell lines for which the antibody used has known cross-reactivity. **E)** Western-blot analysis of whole cell lysates obtained from Raji cells treated as in D. Membranes were incubated with anti-phospho ERK, stripped and reprobed with anti-ERK antibody. Comparable results were obtained with IM9 cells.

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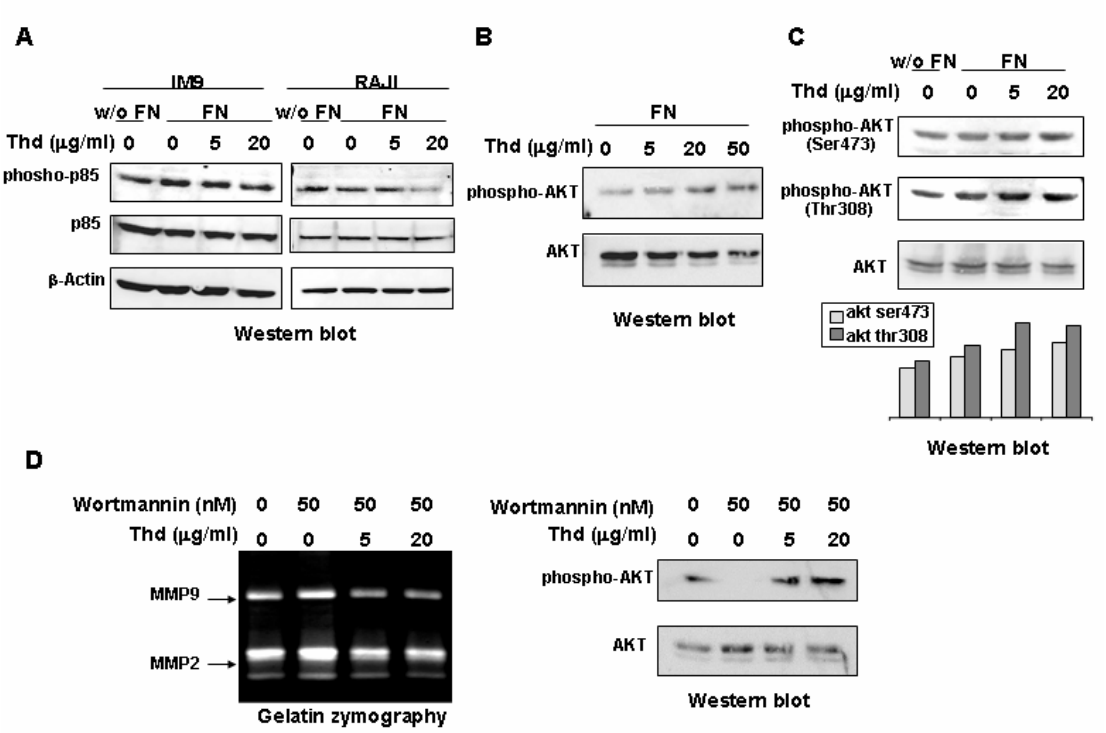
EXPERIMENTS ADDICIONALS (altres vies de senyalització)

La talidomida incrementa la fosforilació de Akt induïda per integrines a través d'una via independent de PI3K.

Donat que la talidomida redueix la fosforilació de diverses cinases importants en la senyalització integrina, vam voler estudiar si la talidomida també afectava l'activitat de fosfatidilinositol 3-cinasa (PI3K). PI3K és una altre enzim clau en la senyalització mitjançada per factors de creixement i per integrines i la seva activació es regula per la fosforilació en tirosines de la subunitat p85. L'activació de PI3K genera la formació de fosfatidilinositol trisfosfat (PIP3) el qual recluta Akt cap a la membrana cel·lular, on Akt pot ser fosforilada per PDK1 (cinasa dependent de fosfatidilinositol 1).[1,2] Nosaltres prèviament havíem demostrat que, de manera similar a ERK, la inhibició de PI3K amb wortmannin donava lloc a la reducció de la transcripció dels gens de les gelatinases (dades no publicades), però per contra, resultava en un ràpid increment de l'alliberament de gelatinases en resposta a la fibronectina.[3,4] Hem explorat els efectes de la talidomida en aquesta via de senyalització i hem observat que la talidomida redueix la fosforilació de la subunitat p85 de PI3K, però sorprenentment la talidomida augmenta l'activitat de Akt (figures 1A, 1B i 1C). A més, la talidomida no només redueix l'increment en l'alliberament de gelatinases aconseguit per l'inhibidor específic de PI3K, wortmannin, sinó que també reverteix l'efecte repressor de wortmannin sobre la fosforilació de Akt (figura 1D). Aquests resultats indiquen que la talidomida incrementa la fosforilació de Akt a través d'una via independent de PI3K, com ha estat recentment demostrat en altres sistemes.[5] D'acord amb aquest concepte, l'efecte de la talidomida sobre la fosforilació en el residu treonina és més evident que la fosforilació en serina (figura 1C) com ha estat descrit en les vies d'activació de Akt per mecanismes independents de PI3K.[5] L'augment en Akt pot semblar inconsistent amb l'activitat anti-tumoral de la talidomida, atès que Akt transdueix senyals anti-apoptòtiques[1,6]. No obstant, l'activació de Akt impulsada per la talidomida pot ser

compensada per la interferència amb altres vies de senyalització importants que estimulen el creixement cel·lular com Src i MAP cinases.

Figura 1: **La talidomida incrementa la fosforilació de Akt induïda per fibronectina a través d'una via independent de PI3K.**



A) Detecció per western blot de la fosforilació de la subunitat p85 de PI3K en lisats de les línies cel·lulars IM9 i Raji després del tractament amb les dosis indicades de la talidomida (Thd) durant 4 hores en absència (w/o) o en presència de fibronectina (FN) (10 μg/ml). **B)** Detecció per western blot de la fosforilació de Akt en cèl·lules IM9 després de l'exposició a Thd a dosis creixents en presència de FN. **C)** Comparació entre la fosforilació en serina i treonina de Akt utilitzant anticossos específics en lisats cel·lulars de IM9 obtinguts en les mateixes condicions que A. La gràfica representa la proporció entre la intensitat de la senyal obtinguda per densitometria de cada anticós fosfoespecífic de Akt respecte el nivell corresponent de Akt total. Es van obtenir resultats similars amb cèl·lules Raji. **D)** Zimografia de gelatina i detecció per western blot de la fosforilació de Akt en sobrenedants i lisats de cèl·lules Raji respectivament, després de l'exposició a Thd a les concentracions indicades, en absència o en presència de wortmannin a 50 nM. Es van obtenir resultats similars amb cèl·lules IM9.

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RESUM DELS RESULTATS

1. La proteïna de la matriu extracel·lular fibronectina estimula la producció i activació de gelatinases (MMP2 i MMP9) i MMP14 en línies cel·lulars derivades de tumors limfoïdes B.

2. La talidomida redueix l'expressió, alliberament i activació de MMP2, MMP9 i MMP14 en línies limfocítiques B exposades a la fibronectina.

3. La inhibició de la producció de MMPs amb el tractament amb talidomida es demostra també *ex vivo*, amb la reducció de l'alliberament de gelatinases produïdes per les cèl·lules plasmàtiques infiltrants del moll d'os de pacients amb mieloma múltiple.

4. La talidomida produeix una reducció de l'adhesió a fibronectina i una menor invasivitat de les cèl·lules. Aquests fenòmens no són deguts a canvis significatius en l'expressió d'integrines en la superfície del limfòcits.

5. La talidomida interfereix amb les vies de senyalització mitjançades per integrines que condueixen a la producció de gelatinases. D'una banda, inhibeix la transcripció possiblement a través de reduir l'activació de Src i ERK MAPK; i d'altra banda disminueix l'alliberament de gelatinases a través de la inhibició de la forma catalíticament activa de Src.

6. La talidomida també intervé en la senyalització de la via PI3K/Akt. D'una banda la talidomida redueix l'activació p85, subunitat regualadora de PI3K, però d'altra banda la talidomida incrementa la fosforilació d'Akt, especialment en la treonina 308 que és clau per al domini catalític. A més, la talidomina també activa a Akt encara que les cèl·lules es tractin amb un inhibidor específic de PI3K (wortmannin). Aquest fet indicaria que la talidomida està intervenint en l'activació d'Akt de forma independent a PI3K.

CONCLUSIONS

La talidomida és un fàrmac que ha demostrat tenir efectes terapèutics beneficiosos en el tractament de síndromes limfoproliferatives, especialment el mieloma múltiple, i d'algunes malalties inflamatòries cròniques. No obstant, no es coneixen amb exactitud els mecanismes moleculars a través dels quals actua. Estudis previs en els quals hem col·laborat demostren l'eficàcia de la talidomida en el tractament del mieloma múltiple està condicionada pel microentorn tumoral. La producció de MMPs juga un paper molt important en la progressió de les malalties tumorals. Com hem dit, les integrines regulen les senyals procedents del microentorn que controlen l'alliberament de MMPs en coordinació amb els mecanismes de migració limfocitària, donant lloc a la invasió cel·lular. Els resultats obtinguts ens condueixen a les següents conclusions:

1. La disminució de la producció de gelatinases induïda per senyals del microentorn i la conseqüent reducció de la capacitat invasiva dels limfòcits pot contribuir a l'eficàcia del tractament amb talidomida en les síndromes limfoproliferatives i malalties inflamatòries cròniques.

2. La talidomida interfereix en les senyals modulades per integrines que actuen sobre la producció de MMPs. Aquestes senyals, com hem vist, estan coordinades amb les vies de senyalització que regulen la migració cel·lular.

3. En conjunt, els efectes de la talidomida sobre les senyals vehiculitzades per integrines poden tenir impacte sobre diversos aspectes relacionats amb la progressió de les síndromes limfoproliferatives com la supervivència i proliferació cel·lular, l'angiogènesi o la invasió tumoral.