Polish Journal of Pharmacology
Pol. J. Pharmacol., 2001, 53, 709–713
ISSN 1230-6002

PRELIMINARY COMMUNICATION

INFLUENCE OF THALIDOMIDE ON Bcl_2 EXPRESSION AND PROANGIOGENIC CYTOKINE LEVELS IN SHORT-TERM CULTURE OF PERIPHERAL BLOOD AND BONE MARROW MONONUCLEAR CELLS OF MULTIPLE MYELOMA PATIENTS

Anna Dmoszyńska[1, #], Jacek Roliński[2], Agnieszka Bojarska-Junak[2], Joanna Mańko[1], Dariusz Jawniak[1], Adam Walter-Croneck[1], Maria Soroka-Wojtaszko[1], Marek Hus[1]

Haematooncology[1] and Clinical Immunology Department[2], University School of Medicine, Jaczewskiego 8, PL 20-950 Lublin, Poland

Influence of thalidomide on Bcl_2 expression and proangiogenic cytokine levels in short-term culture of peripheral blood and bone marrow mononuclear cells of multiple myeloma patients A. DMOSZYŃSKA, J. ROLIŃSKI, A. BOJARSKA-JUNAK, J. MAŃKO, D. JAWNIAK, A. WALTER-CRONECK, M. SOROKA-WOJTASZKO, M. HUS. Pol. J. Pharmacol., 2001, 53, 709–713.

Supernatants from short-term culture of peripheral blood and bone marrow mononuclear cells obtained from 22 multiple myeloma patients were used to measure the concentration of TNF-α, HGF, IL-6 and its soluble receptor (sIL-6R), VEGF and bFGF. Cells were cultured with or without thalidomide (THAL). We observed statistically significant decrease in TNF-α, HGF, IL-6, sIL-6R in supernatants from THAL cultures compared to cells cultured without THAL. Flow cytometry technique was applied to study the Bcl_2 expression on CD 4, CD 8 and CD 138 positive cells. The statistically significant decrease in Bcl_2 expression on myeloma cells (CD 138^+) was observed both in PB and BM cultures. THAL could inhibit the plasma cell growth both by diminishing proangiogenic cytokines production and enhancing myeloma cell apoptosis.

Key words: *multiple myeloma, thalidomide, Bcl_2, proangiogenic cytokines*

[#] *correspondence*; e-mail: dmoszann@free.med.pl

Despite of many therapeutic regimens introduced recently, patients suffering from treatment-resistant or relapsing multiple myeloma (MM) have a very poor prognosis. Therefore, novel approaches evoke great attention [1]. Several studies indicated that many cancers including MM progress together with an induction of angiogenesis and that high level of different proangiogenic cytokines may reflect this process [4, 10].

Recently, a growing number of studies have demonstrated that thalidomide (THAL) is an effective drug in MM patients [5, 9]. Some of these studies indicate that the THAL antimyeloma effect is associated with decreased vessel density [6]. Many factors and molecules have been reported to be involved in the angiogenesis such as tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β), interleukin-6 (IL-6) and its soluble receptor (sIL-6R) or hepatocyte growth factor (HGF) [2, 3]. The most potent and specific factors in this regard seem to be vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Levels of these factors appear to be related to angiogenic activity and increased microvessel density [7]. MM is often associated with an increased microvessel density of the bone marrow

Table 1. Clinical characteristics of patients treated with thalidomide (THAL)

	All patient	Responders to THAL therapy	Non-responders to THAL therapy
Total (n)	22	14	8
Male (n)	9	8	1
Female (n)	13	6	7
Age (years; median)	63	63	63
Refractory to chemotherapy (n)	5	3	2
Relapsed (n)	17	10	7
Number of previous chemotherapy cycles (median)	18	15.5	20.5

Table 2. Cytokine secretion in 72 h cultures of peripheral blood (PB) and bone marrow (BM) mononuclear cells with or without thalidomide (THAL) (mean ± SD)

	TNF-α (pg/ml)		HGF (pg/ml)		IL-6 (pg/ml)		IL-6R (pg/ml)		bFGF (pg/ml)		VEGF (pg/ml)	
Cultures	without THAL	THAL	without THAL	THAL	without THAL	THAL	without THAL	THAL	without THAL	THAL	without THAL	THAL
PB	43.0 ± 36.4	32.5 ± 17.9[a]	96.9 ± 127.6	33.6 ± 34.3[c]	404.0 ± 244.3	329.8 ± 116.4[b]	279.9 ± 267.9	248.9 ± 239.6[a]	2.6 ± 1.4	2.2 ± 1.2[b]	95.7 ± 161.5	67.4 ± 97.3[a]
BM	31.5 ± 16.9	25.8 ± 6.3[a]	203.8 ± 304.0	131.9 ± 190.6[b]	712.3 ± 506.7	703.5 ± 634.7	193.5 ± 180.0	165.6 ± 134.5	11.4 ± 19.2	9.1 ± 16.1	48.7 ± 35.4	39.1 ± 20.1

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ in comparison to culture without THAL in Mann-Whitney U-test

Table 3. Expression of Bcl_2 antigen on T-lymphocyte and malignant plasma cells in patients with MM (mean ± SD)

Material		Bcl_2+/CD4+		Bcl_2+/CD8+		Bcl_2+/CD138+	
		without THAL	THAL	without THAL	THAL	without THAL	THAL
Peripheral blood	% of positive cells	53.5 ± 16.7	41.1 ± 12.9	31.0 ± 10.0	21.5 ± 6.8*	–	–
	MFI	134.1 ± 10.2	131.1 ± 12.2	134.8 ± 7.0	131.7 ± 7.9	–	–
Bone marrow	% of positive cells	42.0 ± 16.9	33.8 ± 16.6	29.8 ± 9.2	22.4 ± 11.4	34.4 ± 12.0	16.6 ± 6.0
	MFI	130.5 ± 10.4	130.1 ± 8.6	135.8 ± 10.2	127.8 ± 9.1*	138.3 ± 9.7	121.2 ± 7.5*

* $p < 0.05$ in comparison to culture without THAL in Mann-Whitney U-test

Table 4. Cytokine secretion in 72 h cultures of peripheral blood (PB) and bone marrow (BM) mononuclear cells with or without thalidomide (THAL) (mean ± SD)

Cytokine	Cultures	Material	Responders (pg/ml)	Non-responders (pg/ml)
TNF-α	without THAL	*PB*	52.7 ± 47.8	32.7 ±14.3
		BM	28.5 ± 8.7	97.3 ± 132.4*
	THAL	*PB*	36.3 ± 22.8	27.1 ± 8.7
		BM	27.2 ± 7.4	83.3 ± 113.9
HGF	without THAL	*PB*	74.5 ± 82.3	67.1 ± 68.4
		BM	238.0 ± 395.5	160.9 ± 181.3
	THAL	*PB*	35.9 ± 33.0	18.5 ± 9.9
		BM	153.2 ± 217.9	114.2 ± 174.9
IL-6	without THAL	*PB*	362.9 ± 138.3	480.5 ± 360.1
		BM	598.6 ± 482.3	728.0 ± 443.1
	THAL	*PB*	300.4 ± 73.1	373.2 ± 161.8
		BM	612.0 ± 498.6	851.2 ± 818.1
IL-6R	without THAL	*PB*	254.7 ± 243.0	272.6 ± 329.3*
		BM	370.4 ± 612.2	198.4 ± 232.2
	THAL	*PB*	221.0 ± 216.5	265.3 ± 279.0
		BM	253.9 ± 338.1	182.8 ± 167.0
bFGF	without THAL	*PB*	2.62 ± 1.5	2.23 ± 1.3*
		BM	22.3 ± 34.4	6.69 ± 9.5
	THAL	*PB*	2.35 ± 1.5	1.99 ± 1.0
		BM	19.7 ± 31.7	14.2 ± 3.5
VEGF	without THAL	*PB*	119.3 ± 217.3	66.3 ± 43.4
		BM	111.0 ± 201.2	43.9 ± 26.3
	THAL	*PB*	86.7 ± 126.0	48.7 ± 46.7*
		BM	103.3 ± 215.1	39.9 ± 23.3*

* $p < 0.05$ in comparison to responder group in Mann-Whitney U-test

(BM) and this is an adverse prognostic factor [8]. The neovascularization may even persist after high--dose chemotherapy.

THAL antitumor activity is very complex and probably separated from its antiangiogenic activity. THAL possesses several properties that could explain its antimyeloma effect. The drug increases count of CD 8 and CD 4 positive cells, alters adhesion molecule expression and enhances cell mediated immunity by direct stimulation of T cells. In turn, stimulated T cells produce IFN-γ and IL-12 what could lead to inhibition of angiogenesis [6] but precise mechanism of THAL action is still not fully understood.

The aim of our study was to assess some of possible mechanisms of THAL action in *in vitro* culture and to compare the obtained results with clinical response to THAL therapy. The criteria of clinical response and schedule of THAL treatment are described in details in our previous publication [5].

22 multiple myeloma patients who were relapsing or resistant to chemotherapy and were qualified to THAL therapy were enrolled to the study. None of studied patients showed the clinical sign of active infection. The short clinical characteristics of patients is shown in Table 1. Peripheral blood (PB) and BM samples were taken in the morning after fasting overnight. Mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Norway) and washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin. Isolated cells (2×10^6 cells/ml) were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (GIBCO), 2 mM/l L-glutamine (GIBCO), penicillin 100 U/ml (Sigma) and streptomycin 100 μg/ml (Sigma) at 37°C in a 5% CO_2 atmosphere, under sterile conditions. Each sample was cultured in 6-well plates (Nunc) with and without 10 μg/ml of thalidomide (Grünenthal, Germany) in 0.1% DMSO (Sigma). The supernatants were collected after 72 h of cell culture and stored at –80°C until assayed. The concentrations of proangiogenic cytokines were determined by ELISA assays according to manufacturer's instructions: VEGF, HGF and bFGF (R&D Systems), IL-6 and sIL-6R (Endogen).

Mitochondrial oncoprotein Bcl_2 was determined in fresh cells before and after adding 10 μg/ml of THAL. Mononuclear cells were isolated as described above. Double color immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluorescein (FITC) conjugated monoclonal antibodies. Monoclonal antibodies were obtained from Dako (Denmark). The following antibody combinations were used: IgG_1 FITC/IgG_2 PE negative control antibody, CD 3, CD 4, CD 8, CD138 PE, Bcl_2 FITC. 10^6 cells were incubated with antibodies for 30 min at 4°C and washed twice with

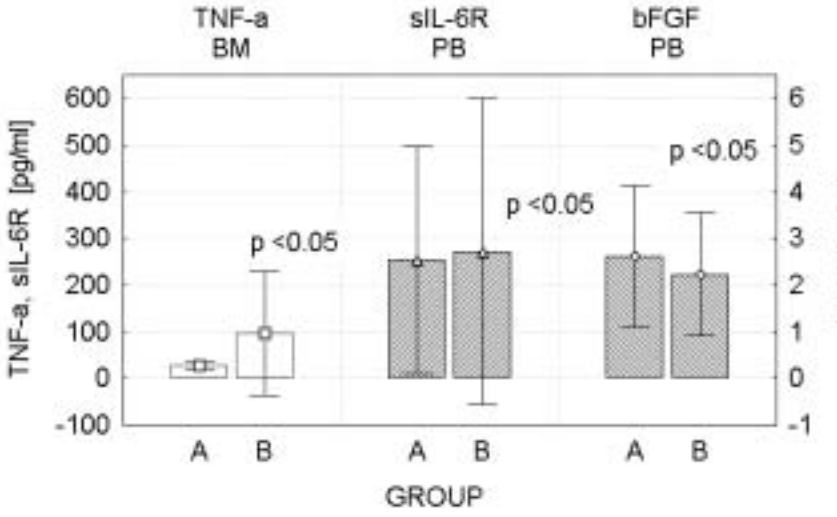

Fig. 1. Statistically significant differences in TNF-α, sIL-6R and bFGF concentration in supernatants of peripheral blood (PB) and bone marrow (BM) mononuclear cell cultures in responder (A) versus non-responder (B) group

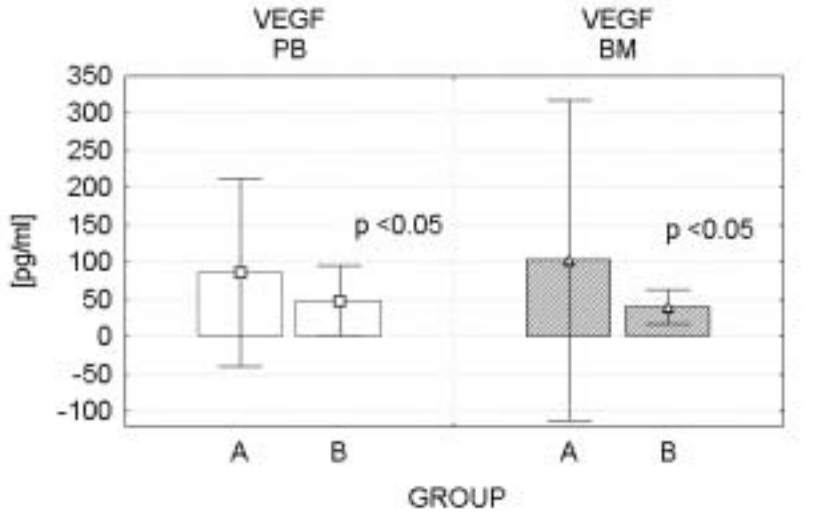

Fig. 2. Statistically significant differences in VEGF concentration in supernatants of peripheral blood (PB) and bone marrow (BM) mononuclear cell cultures in responder (A) versus non-responder (B) group

PBS afterwards. For the immunodetection of the intracellular Bcl-2 protein, the cells were fixed and permeabilized with 0.25% paraformaldehyde (15 min at room temperature) and then in cold 70% methanol for 60 min at 4°C. Permeabilized cells were stained with FITC conjugated Bcl_2 mouse monoclonal antibody or IgG_1 FITC negative control antibody. All samples were tested on flow cytometr (Cytoron – Ortho Diagnostic System). Ten thousand cells were analyzed per test. In order to quantitate the levels of fluorescence, the mean fluorescence intensity (MFI) of the Bcl_2 positive cells were calculated. The MFI was measured on histogram of the Bcl_2 expression from the upper limit of the negative control.

The results of proangiogenic cytokine secretion in supernatants of 72 h culture of PB and BM mononuclear cells with or without THAL are shown in Table 2. The concentration of all assayed cytokines were significantly lower in THAL cultures compared to cultures without THAL. Expression of Bcl_2 antigen and mean fluorescence intensity is shown in Table 3. The statistically significant decrease in both Bcl_2 expression and MFI was observed in MM cells ($CD138^+$) and in CD 8^+ cells. Then we correlated the level of proangiogenic cytokines with clinical response to THAL treatment after 8 weeks of therapy (the time to optimal response to THAL therapy). We found that in responder group (14 of 22 patients) the concentration of TNF-α in BM and sIL-6R in PB cultures were significantly lower compared to non-responder group (8 patients), see Figure 1. On the contrary, the concentrations of VEGF both in PB and BM cultures,

and bFGF only in BM cultures were significantly higher in responder group than in non-responders. The values were: for VEGF 119.3 vs. 66.0 pg/ml in PB cultures and 86.7 vs. 48.6 pg/ml in BM cultures (Fig. 2), respectively; for bFGF 2.6 vs. 2.2 pg/ml in PB cultures and 2.4 vs. 1.9 pg/ml in BM cultures (see Table 4 and Figure 1).

MM is incurable disease, nevertheless, high--dose chemotherapy supported by PB stem cell transplantation improved the rate of complete remission and overall survival but for older, relapsing or therapy-resistant patients treatment options are very limited. We and others found that THAL treatment of resistant or relapsing MM is very promising therapeutic option for these patients [5, 9]. In this study, we have shown that short-term culture of mononuclear cells with THAL causes significant decrease in different proangiogenic cytokines such as VEGF, bFGF, HGF, TNF-α. We have also noted significant decrease in Bcl_2 expression after 72 h culture with THAL.

There is clearly much to discover about the mechanism of THAL action and its interaction with human immune system. It was recently shown that adhesion of MM cells to stromal cells up-regulates VEGF secretion by these cells [7]. So, it seems that VEGF could be one of important growth factors for MM cells, and THAL lowering VEGF concentration can also prolong survival time of treated patients but it should be proven in further randomized studies, on a larger group of patients.

Although THAL is a potent antiangiogenic agent, there are many other potential mechanisms of action, and one of these mechanisms includes apoptosis and down-regulation of proangiogenic cytokine secretion. Our data demonstrate that the efficacy of THAL in MM patients may be linked to modulation of secretion of cytokines by the normal cells and that THAL acts not only on MM cells, but also on accessory PB and BM cells.

Acknowledgment. This study was supported by grant DS 227/01 from the University Medical School in Lublin.

REFERENCES

1. Anderson K.C., Kyle R.A., Dalton W.S., Landowski T., Shain K., Jove R., Hazlehurst L., Berenson J.: Multiple Myeloma: Insight and Therapeutic Approaches. 42nd ASH Meeting, San Francisco 1–5 December 2000, Educational Book, 147–165.
2. Dankbar B., Padro T., Leo R., Feldmann B., Kropff M., Mesters R.M., Serve H., Berdel W.E., Kienast J.: Vascular endothelial growth factor and interleukin 6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood, 2000, 95, 2630–2636.
3. Dankbar B., Padro T., Mesters R.M. et al.: VEGF is expressed by myeloma cells and stimulates Il-6 secretion by microvascular endothelial and marrow stromal cells. Blood, 1998, 92, Suppl. 1, 2807.
4. Di Raimondo F., Azzaro M.P., Palumbo G.A., Bagnato S., Giustolisi G., Giustolisi R.: Angiogenic factors in multiple myeloma: higher levels in bone marrow than in peripheral blood. Haematologica, 2000, 85, 800–805.
5. Hus M., Dmoszyńska A., Soroka-Wojtaszko M., Jawniak D., Legieć W., Ciepłuch H., Hellman A., Wolska--Smoleń T., Skotnicki A., Mańko J.: Thalidomide treatment of resistant or relapsed multiple myeloma patients. Haematologica, 2001, 86, 418–422.
6. Marriot J.B., Muller G., Dalgleish A.G.: Thalidomide as an emerging immunotherapeutic agent. Immunol. Today, 1999, 20, 538–554.
7. Sezer O., Jacob Ch., Eucker J., Niemöller K., Gatz F., Wernecke K.D., Passinger K.: Serum levels of angiogenic cytokines, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in multiple myeloma. Eur. J. Haemat., 2001, 86, 83–88.
8. Sezer O., Niemöller K., Jakob K.: Bone marrow microvessel density is a prognostic factor for surrvival in patients with multiple myeloma. Ann. Hematol., 2000, 79, 574–577.
9. Singhal S., Mehta J., Desikan R.: Antitumor activity of thalidomide in refractory multiple myeloma. N. Eng. J. Med., 1999, 3421, 1565–1571.
10. Vacca A., Ribatti D., Presta M.: Bone marrow neovascularization plasma cell angiogenic potential and matrix metalloproteinase 2 secretion parallel progression of human multiple myeloma. Blood, 1999, 93, 3064––3073.

Received: August 24, 2001; in revised form: November 12, 2001.