The influence of thalidomide therapy on cytokine secretion, immunophenotype, BCL-2 expression and microvessel density in patients with resistant or relapsed multiple myeloma

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Thalidomide (THAL) is currently used as a novel drug in patients with chemotherapy resistant or relapsed multiple myeloma. THAL antitumor activity seems to be very complex, however the precise mechanisms of its action are still not fully understood.

The aim of this study was to assess some of possible mechanisms of THAL action both in *in vivo* analysis of immune cells phenotype and in *in vitro* cultures with THAL. The study involved 30 patients with relapsed or chemotherapy refractory multiple myeloma who were qualified to THAL treatment. We assessed immunothenotype of malignant plasma cells and T lymphocytes in both peripheral blood (PB) and bone marrow (BM) samples taken before and after 4 and 8 weeks of THAL treatment. Before therapy cytokine secretion (VEGF, HGF, bFGF, TNF, IL-6 an sIL-6R) and BCL-2 expression in PB and BM cell cultures with THAL were analyzed. We used flow cytometry technique and ELISA method. The clinical response to therapy was assessed after 4 and 8 weeks of treatment. We also investigated microvessel density (MVD) in bone marrow samples before the THAL treatment and after 6 months of therapy in the group of responding patients.

In cell cultures with THAL we detected statistically significant lowering of analyzed cytokines concentration and the decrease in BCL-2 expression by malignant plasma cells in BM and CD8+ T lymphocytes in BM and PB. In the group of patients responding to therapy we observed the decrease in the number of myeloma cells and significant increase of CD4+ and CD8+ cells in both PB and BM samples. There was statistically significant increase of CD3+/CD69+ cells in the course of therapy, while the percentage of CD3+/HLA-DR+ cells was significantly lower after 8 weeks of therapy. We also detected lowering of MVD after THAL therapy in responders group.

The obtained results demonstrate that THAL efficacy in MM is multidirected and included such mechanisms like down-regulation of proangiogenic cytokines, that could lead to lowering of MVD, induction of apoptosis and influence on malignant cells and T lymphocytes immunophenotype.

Key words: apoptosis, cytokines, BCL-2, mmicrovessel density, multiple myeloma, thalidomide

Multiple myeloma (MM) is an incurable neoplastic disease characterized by slow proliferation of malignant plasma cells and their accumulation within the bone marrow. Once the diagnosis is made the expected survival time does not exceed four years. The introduction of high-dose chemotherapy followed by autologuous peripheral stem cells transplantation has improved the effects of treatment, however only in a low number of patients long-term remission is achieved. Therefore much attention is put recently to discover a novel, less toxic and more efficient antimyeloma drugs for treatment of resistant or relapsed MM patients.

Thalidomide (THAL) is a drug that has been shown as having an antitumor activity effect in MM therapy [2 7, 17]. It is known as a sedative drug that was withdrawn from the usage when its teratogenic effects on the fetus were reported. THAL has an antiangiogenic activity and shows immunomodulatory effect on cytokine secretion [14, 20, 21). Some of the recent studies indicate that THAL antimyeloma effect is associated with decreased vessel density [8, 19]. On the other hand THAL modulates the function of T cell subsets, which

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can also influence the plasma cell growth [6, 9, 10] and has an influence on interaction and redistribution of B cells [1]. Such a panel of THAL effects qualifies it for MM therapy, however these activities should be well established.

The aim of this study was to assess some of the possible mechanisms of THAL action both in *in vivo* analysis of immune cells phenotype and in *in vitro* cultures with THAL. In cell cultures with THAL we analyzed the cytokine secretion and the expression of BCL-2 anti-apoptotic protein. At the same time the clinical response to THAL treatment was estimated and compared with obtained results. We also investigated microvessel density (MVD) in bone marrow samples before the THAL treatment and after 6 months of therapy in the group of responding patients.

Material and methods

Patients. The study was carried out at the Hematology Department of the Medical University in Lublin. It involved 30 patients with relapsed or refractory to chemotherapy multiple myeloma who were qualified to THAL treatment. The THAL therapy started from a dose of 200 mg daily, administered orally in two separate doses. The dose was then increased by 100 mg every seven days, reaching 400 mg per day in two doses 200 mg + 200 mg. Peripheral blood (PB) and bone marrow (BM) samples were taken before therapy, and after 4 and 8 weeks of treatment. All samples were obtained after informed consent and with approval of the local ethics committee.

Cell separation and culture. Ten millilitres of peripheral venous blood was collected in tubes containing heparin then diluted in phosphate buffered saline (PBS) at a ratio 1:1. The bone marrow aspirates were collected in tubs with heparin and diluted in PBS at a ratio 1:2. Both PB and BM mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Norway), and then washed twice in PBS. The isolated cells at a concentration of $2x10^6$ cell/ml were cultured in RPMI1640 medium supplemented with 10 % fetal calf serum (Gibco, USA), 2 mM/l L-glutamine (Gibco, USA), 100 U/ml penicillin (Sigma, Germany), 100 µg/ml streptomycin (Sigma, Germany) at 37 °C in a CO_2 atmosphere, under sterile conditions. Each sample was cultured with and without 10 µg/ml THAL (Grnenthal, Germany) resuspended in 0.1 % DMSO (Sigma, Germany).

Analysis of BCL-2 expression. The expression of mitochondrial oncoprotein BCL-2 was determined in analyzed samples before and after 72 h period of cell culture. Double color flow cytometry studies were performed using combinations of phycoerythrin (PE) and fluorescein isothiocynate (FITC) conjugated monoclonal antibodies (MoAbs). The following MoAb combinations were used: IgG1FITC/IgG2PE negative control, CD3PE/BCL-2FITC, CD4PE/BCL-2FITC, CD8PE/BCL-2FITC, CD138PE/BCL-2FITC. All MoAbs were purchased from Dako, Denmark. The cells in amount of 10^6 per tube were incubated with PE conjugated anti-extracellular antigens MoAbs for 20 min at room temperature (RT) and washed twice with PBS afterwards. Then the cells were fixed and permeabilized with 0.25 % paraformaldehyde (15 min at RT) and subsequently in cold 70 % methanol (60 min at 4 °C) and incubated with BCL-2 FITC MoAb. The stained samples were analyzed on Cytoron – Ortho Diagnostic Systems flow cytometer. The data were shown as percentage of positive cells and the mean fluorescence intensity (MFI) measured on histogram of BCL-2 expression from the upper limit of negative control.

Detection of cytokine levels. The supernatants from each sample in cell culture were collected after 72 h period and stored at –80 °C until assayed. The concentration of analyzed cytokines were determined with use of enzyme-linked immunoabsorbent assay (ELISA) method according to manufactures' instructions: VEGF, HGF, bFGF and TNF with use of R&D Systems kits, IL-6 an sIL-6R with use of Endogen kits.

Analysis of immunophenotype. To assess the influence of Thal therapy on cells immunophenotype PB and BM samples were taken before and after 4 and 8 weeks of THAL treatment. The cells isolated as described before were incubated with following MoAbs combinations: CD38FITC/CD138PE, CD20FITC/CD138PE, CD4FITC/CD8PE, CD3FITC/CD69PE, CD3FITC/CD25PE, CD3FITC/HLA-DR PE. Then the double color flow cytometry assessment was performed.

Assessment of clinical response. The all studied patients were examined after 4 and 8 weeks of THAL treatment and classified as responders or non-responders. The responders fulfilled criteria of complete remission (total disappearance of the serum monoclonal protein and 5 % of plasma cells in the bone marrow biopsy, normalization of hemoglobin, albumin and calcium level) or partial response (50 % or greater reduction in the pre-treatment value of monoclonal protein, normalization of serum calcium level).

Assessment of bone marrow MVD. MVD estimation in bone marrow samples was performed before the THAL treatment and after 6 months in the group of responding patients (n=18). BM specimens were decalcified in a solution of 10 buffered formalin, with 10 % (10 ng/100 ml) ethylenediaminetetraacetic (EDTA) and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE), Giemsa, periodic acid Schiff (PAS) and Gemori's stain for reticulin fibers. Immunostaining was performed using the labeled streptavidin-biotin (LSAB) method using anti-von Willebrand factor VIII (vWF) MoAb and anti-CD34 MoAb (Dako, Denmark).

MVD was determined according to Weidner et al and Vermeulen et al. Each slide was first scanned at x100 to determine 3 "hot spots" defined as areas with the maximum number of microvessels. The slides were then examined at x400 to count the microvessels. MVD was estimated by determining the average number of vessels in each of 3 hot spots. The results were expressed as number of vessels/mm^2.

Statistical analysis. The results obtained in the study were

statistically analyzed with use of STATISTICA 5.0 for Windows software. Statistical significance was set at $p<0.05$.

Results

The expression of BCL-2 by malignant plasma cells and T lymphocytes with and without THAL. We detected statistically significant decrease in both percentages of BCL-2 positive cells and MFI of BCL-2 expression by BM malignant plasma cells (CD138+) as well as BM and PB CD8+ T lymphocytes in cell cultures with THAL in comparison to those without THAL. There was no statistically significant difference in percentage and expression of BCL-2 by CD4+ cells between samples before and after THAL adding. The obtained results are shown in Table 1.

The cytokine secretion in 72 h cell culture of PB and BM with and without THAL. The concentrations of all assayed cytokines were statistically significantly lower in THAL cultures compared to cultures without THAL (Tab. 2). The comparison of cytokines level in cell cultures with THAL and clinical response to THAL treatment indicated in responder group statistically significant lower concentration of TNFα in BM and sIL-6R in PB cultures in comparison to non-responder group. On the contrary, the concentration of VEGF in BM and PB and bFGF in BM were significantly higher in responders than in non-responders (Fig. 1).

The immunophenotype of BM and PB malignant plasma cells and T lymphocytes before and after THAL therapy. We observed the changes in the number of myeloma cells CD38+/CD138+ and CD20+/CD138+ in both PB and BM in course of THAL treatment. In the group of patients responding to therapy there was statistically significant decrease of these cells percentage after 4 and 8 weeks of therapy in comparison to results obtained before THAL application (Tab. 3). Such changes were not detected in the group of non-responding patients. On the contrary, there was statistically significant increase of CD4+ and CD8+ cells in both PB and BM samples in course of THAL in responders group. We did not detect such an increase in non-responder group (Tab. 3).

We detected the changes in percentage of CD3+ cells with expression of early activation markers CD69 and HLA-DR. There was statistically significant increase of CD3+/CD69+ cells in both BM and PB after 8 weeks of THAL therapy with comparison to the samples before therapy. The percentage of CD3+/HLA-DR+ cells in BM was statistically significantly lower after 4 and 8 weeks of therapy in comparison to results before therapy. There were no changes in percentage of CD3+/CD25+ cells in analyzed BM and PB samples in course of THAL treatment. (Tab. 4).

MVD in BM samples. We investigated MVD in bone marrow samples before the THAL treatment and after 6 months of therapy in the group of responding patients (n=18). The obtained results are shown in Table 5 and in Figure 2. We detected lowering of MVD after THAL therapy in this group of patients.

Discussion

THAL has a number of properties that can make it a part of effective regimen for treating MM patients [4, 5, 17]. THAL

Tab. 1. BCL-2 expression by PB and BM malignant plasma cells and T lymphocytes in cell cultures with and without THAL

	CD138+/BCL2+		CD4+/BCL2+		CD8+/BCL2+	
	Without THAL	THAL	Without THAL	THAL	Without THAL	THAL
Peripheral blood % of 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 cells	34.34±12.0	16.6±6*	42.0±16.9	33.8±16.6	29.8±9.2	22.4±11.4
MFI	138.3±9.7	121.2±7.5*	130.5±10.4	130.1±8.6	135.8±10.2	127.8±9.1*

* statistically significant ($p<0.05$)

Tab. 2. The cytokines level (pg/ml) in PB and BM 72h cell culture with and without THAL

TNFα		HGF		IL-6		IL-6R		bFGF		VEGF	
Without THAL	THAL	Without THAL	THAL	Without THAL	THAL	Without THAL	THAL	Without THAL	THAL	Without THAL	THAL
Peripheral blood											
43.0 ±36.3	32.5 ±17.9*	96.9 ±127.6	33.6 ±34.3***	404.0 ±244.3	329.8 ±116.4**	279.9 ±267.9	248.9 ±239.6*	2.6 ±1.4	2.2 ±1.2**	95.7 ±161.5	67.4 ±97.3*
Bone marrow											
31.5 ±16.9	25.8 ±6.3	203.8 ±304.0	131.9 ±190.6**	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

* statistically significant ($p<0.05$), ** statistically significant ($p<0.01$), *** statistically significant ($p<0.005$)

Tab. 3. The immunophenothype of malignant cells and T lymphocytes in patients responding and non-responding to THAL therapy before and after 4 and 8 weeks of treatment

	Responders n=18			Non- responders n=12		
	Before	4 weeks	8 weeks	Before	4 weeks	8 weeks
			Peripheral blood			
CD38+/CD138+	2.5 ±0.6	1.9±0.3**	1.2 ±0.3**	2.3 ±0.8	22.0 ±0.6	2.0 ±0.4
CD20+/CD138+	1.4 ±0.8	0.9 ±0.4	0.2 ±0.2**	1.2 ±0.8	1.9 ±0.6	1.2 ±0.3
CD4+	22.7 ±8.2	29.5 ±14.2**	34.9 ±17.5**	23.1 ±10.5	19.5 ±9.9	19.3 ±8.1*
CD8+	26.5 ±10.5	44.0 ±17.4**	45.3 ±17.5**	27.3 ±11.6	27.2 ±12.6	29.7 ±12.8
			Bone marrow			
CD38+/CD138+	32.0 ±14.2	6.4 ±2.1**	3.2 ±1.1**	30.4 ±13.2	26.2 ±10.7	23.2 ±10.8
CD20+/CD138+	5.5 ±1.8	1.3 ±0.5**	0.4 ±0.2**	4.90 ±2.0	4.3 ±1.5	4.0 ±1.9
CD4+	19.4 ±9.2	25.6 ±6.2**	26.8 ±12.4	19.6 ±6.9	13.6 ±5.7*	13.4 ±9.8*
CD8+	26.5±16.4	44.1 ±19.3*	46.0 ±19.3**	28.5 ±13.8	30.7 ±14.8	32.2 ±12.9

* statistically significant p<0.05 in comparison to the phenotype before therapy, ** statistically significant p<0.01 in comparison to the phenotype before therapy, *** statistically significant p<0.005 in comparison to the phenotype before therapy

Tab. 4. Expression of activation markers by T cells in PB and BM before and after 4 and 8 weeks of treatment

	Before	4 weeks	8 weeks
		Peripheral blood	
CD3+/CD69+	3.2 ±1.8	4.2 ±2.9	4.8 ±2.1*
CD3+/CD25+	7.6 ±4.2	8.6 ±3.9	6.8 ±4.1
CD3+/HLA-DR+	5.6 ±3.7	6.8 ±2.9	5.2 ±3.2
		Bone marrow	
CD3+/CD69+	4.3 ±3.1	5.1 ±2.6	5.3 ±3.8*
CD3+/CD25+	7.4 ±4.5	8.8 ±4.9	7.0 ±5.2
CD3+/HLA-DR+	9.9 ±3.2	5.8 ±4.7*	3.4 ±1.9**

* statistically significant p<0.05 in comparison to the phenotype before therapy, ** statistically significant p<0.01 in comparison to the phenotype before therapy

Tab. 5. Microvessel density (MVD) in bone marrow samples before and after 6 months of THAL treatment in the group of responding patients. The results are expressed as vessels/mm^2

	Before	6 months
Anti-vWF MoAb	31.1	19.3
Anti-CD34 MoAB	32.1	20.1

antitumour activity is very complex, however the precise ways of its action are still not fully understood. Thus the aim of our study was to assess some possible mechanisms of THAL effects and to compare the obtained results with clinical response to THAL therapy.

It is proposed that the THAL antimyeloma effect is associated with inhibition of angiogenesis and lowering of vessel density [8]. Many factors and molecules have been reported to be involved in the angiogenesis such as tumor necrosis factor (TNF), transforming growth factor β (TGFβ), interleukin 6 (IL-6), and its soluble receptor (sIL-6R), hepatocyte growth factor (HGF) [3]. Among the factors and molecules being reported to be involved in angiogenesis the most important and specific seems to be vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Levels of these cytokines appear to be related to the angiogenic activity and increased microvessel density [15]. MM is often associated with an increased microvessel density of the bone marrow, and it is an adverse prognostic factor [16]. The neovascularization may even persist after high dose of chemotherapy. In this study we detected lowering of MVD after THAL therapy in responders group in comparison to the results before therapy. Results obtained using immunostaining with anti-vWF MoAb comparing to anti-CD34 MoAb were similar, that confirms usefulness of both methods in BM MVD studies.

Some of the studies provide evidence of THAL antivascular activity mediating by regulation of cytokines secretion. ROWLAND et al [13] indicated that THAL inhibited IL-6 and TNF secretion by PBMC in cell culture and had a slight or no effect on IFNγ, IL-2, IL-4 and IL-10 secretion. The inhibition of IL-6 and TNF production was observed on transcription level. The decrease of IL-6 and TNF as well as IL-10 production and no differences in IFNγ, IL-2 and IL-12 secretion were also reported by MOREIRA et al [11]. MCHUGH et al [9] indicated that THAL induced cytokines production by Th2 cells at the same time inhibiting Th1 function. In our study we noticed that in the short term culture of mononuclear cells with THAL there is decrease in proangiogenic cytokines such as VEGF, bFGF, HGF, TNF, IL-6, sIL-6R. It seems to support the hypothesis that one of the possible antitumor action of THAL is an inhibition of angiogenesis. We also detected statistically significant lower concentration of TNF in BM and IL-6R in PB in the group of patients responding to THAL therapy in comparison to the non-responding group. On the contrary, in our study the concentration of VEGF in BM and PB and bFGF in BM were significantly higher in responders than in non-responders. It could be expected that the level of these proangiogenic cytokines should decrease in this group of patients. However, the patients were examined after 8 weeks of therapy, while the lowering of VEGF and bFGF levels may require more time. There was also reported that high pretreatment plasma bFGF concentration is associated with a good response to THAL in cases of progressive myeloma [12].

THAL antitumor activity seems to be very complex and to involve mechanisms separated from its antiangiogenic activity. The drug has an influence on CD8 and CD4 positive cells,

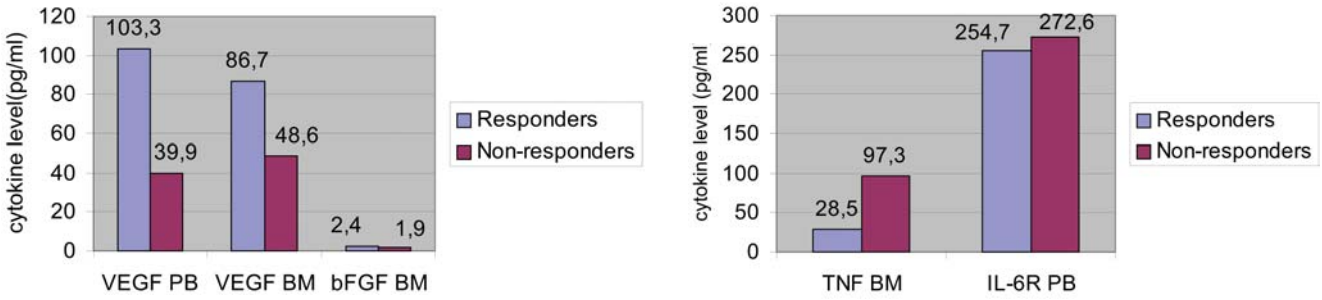

Fig. 1. The concentration of particular cytokines in patients responding and non-responding to THAL therapy.

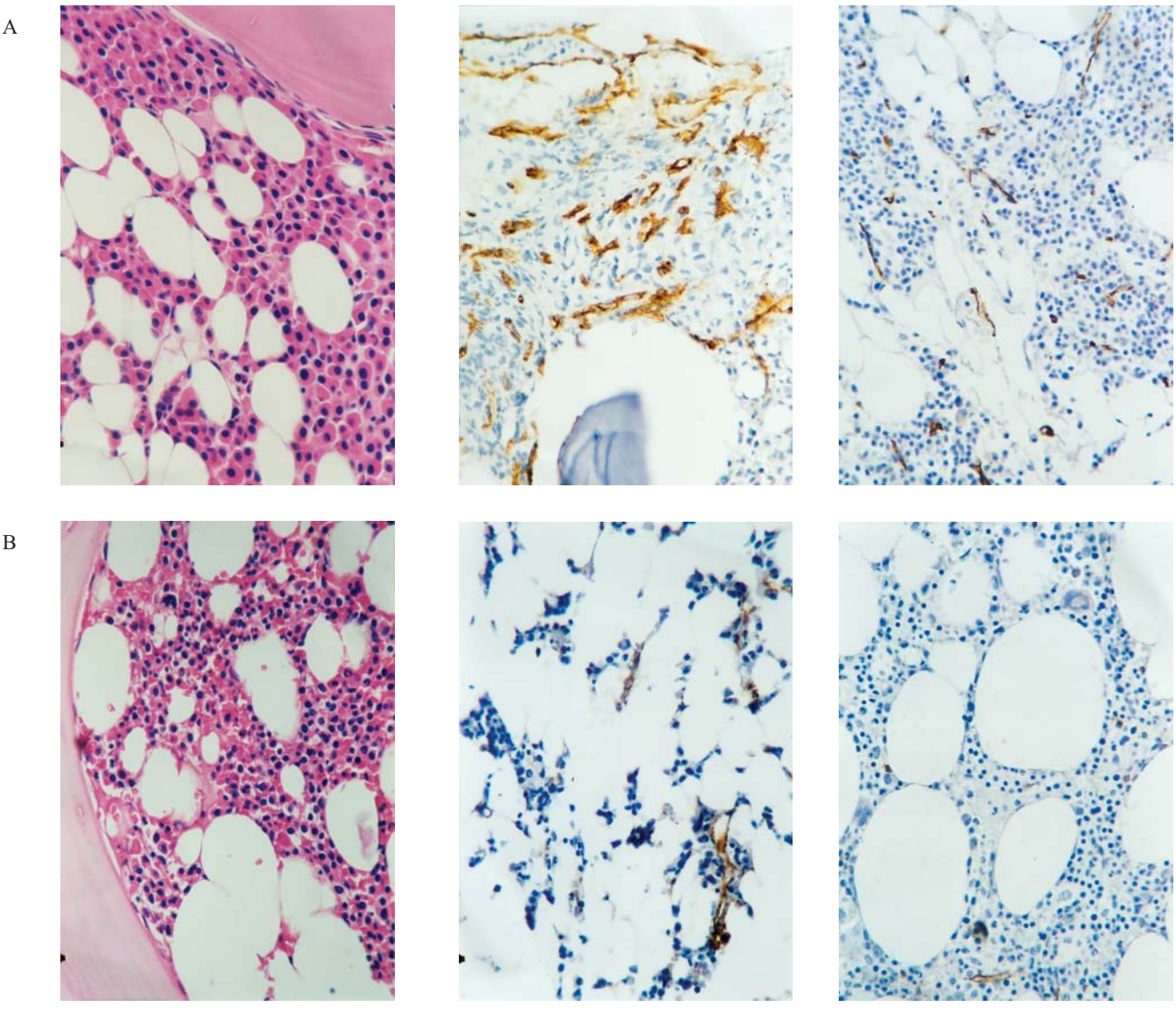

Fig. 2. Microvessel density (MVD) in bone marrow samples before (A) and after 6 months (B) of THAL treatment in the group of responding patients (n=18); a – hematoxylin and eosin (HE) staining; b – anti-von Willebrand factor VIII (vWF) immunostainning; c – anti-CD34 immunostaining.

alters adhesion molecules expression and enhances cell-mediated immunity by direct stimulation of T cells. In our study, after 4 and 8 weeks of THAL treatment significant increase in CD4+ and CD8+ cells both in PB and BM samples was observed in patients clinically responding to THAL treatment. It seems probable that THAL may exert its effect on malignant cells by inducing cell mediated immune response. We observed significant increase in expression of T cell early activation marker CD69 and decrease of the other activation marker HLA-DR. There was no difference in expression of late activation marker CD25 on T cells. It indicates that THAL may provide early activation signals to T lymphocytes promoting T cell response. Different expression of T activation markers suggests existing of the different pathways of signals transduction, but further studies need to be done. Interestingly in our study the percentage of CD38+/CD138+ and CD20+/CD138+ were significantly decreased in patients responding to THAL therapy. It may suggest that among many mechanisms of THAL action the direct influence on malignant cells is also important.

The impaired regulation of apoptosis process is known to be a key event in several malignancies. On the other hand, the modulation of particular stages of apoptosis by drugs is very important treatment approach [18, 22]. Thus in this study the expression of well-known apoptosis regulating protein BCL-2 by malignant plasma cells and T lymphocytes was detected. We noticed significant decrease in BCL-2 expression by CD138+ malignant plasma cells after 72 h culture with THAL. Among T cell populations such a decrease in BCL-2 expression was detected in CD8+ T lymphocytes. So it seems that one of potential mechanisms of THAL action is inducing apoptosis both in malignant cells and T lymphocytes that regulate malignant cell growth.

It is clear that much needs to be discovered about the mechanism of THAL action and its interaction with human immune system. However, the obtained results demonstrate that THAL efficacy in MM is multidirected and includes such mechanisms like down-regulation of proangiogenic cytokines, induction of apoptosis and influence on malignant cells and T lymphocytes immunophenotype.

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