Stimulation of erythropoiesis by thalidomide in multiple myeloma patients: its influence on FasL, TRAIL and their receptors on erythroblasts

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The expression of proteins of the tumor necrosis factor (TNF) family on erythroblasts was measured during thalidomide treatment in 29 patients with multiple myeloma (MM). A clinical response was observed in 17 patients (58.6%) and haemoglobin concentration increased in 22 patients (75.9%). The expression of FasL, Fas, TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL-R1 on erythroblasts decreased significantly during thalidomide treatment. Additional *in vitro* studies revealed that the apoptosis of erythroblasts and the expression of FasL, TRAIL, TRAIL-R1 and TRAIL-R2 was lower in cultures with thalidomide than in control cultures. Altogether our results suggest that thalidomide may stimulate erythropoiesis in MM patients by decreasing the expression of TNF-like ligands/receptors on erythroblasts.

Key words: multiple myeloma, thalidomide, anemia, FasL, TRAIL.

Haematologica 2006; 91:386-389

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Multiple myeloma (MM) constitutes about 10% of all hematologic proliferations. Anemia is found in most MM patients at diagnosis and it deteriorates the quality of life, especially in patients with advanced MM. Recent findings indicate that apoptosis has an important role regulating physiological erythropoiesis[1-2] and in defective erythropoiesis leading to anemia in MM.[3,4] Thalidomide has been used to treat refractory/relapsed MM patients[5] due to its antiangiogenic[6-7] and immunomodulatory[8] properties and it was shown to produce a response in 30-60% of patients.[9-12] Achieving remission was usually accompanied by an increase in hemoglobin concentration. In an earlier study we showed that the improvement in anemia was also observed in patients not responding to thalidomide therapy,[13] which suggests that this drug may directly or indirectly stimulate erythropoiesis independently of producing remission and decreasing bone marrow infiltration by monoclonal plasma cells. The aim of this study was to investigate selected parameters of the erythropoietic system in MM patients treated with thalidomide. We studied the expression of FasL, Fas, TRAIL, TRAIL-R1 and TRAIL-R2 on erythroblasts before and after 3 months of thalidomide therapy as well as the expression of tumor necrosis factor (TNF)-like receptors/ligands on erythroblasts and their apoptosis in short-term cultures of bone marrow mononuclear cells from MM patients.

Design and Methods

Patients

Twenty-nine patients with refractory/relapsed MM were given thalidomide (Grunenthal, Germany) at a dose of up to 400 mg/day. Bone marrow aspirates taken before and after 3 months of thalidomide treatment were studied in flow cytometry and used in short-term cultures. The time interval between the last chemotherapy and the beginning of thalidomide therapy ranged from 1 to 63 months and in most patients was at least 2 months. The patients did not receive any growth factors before or during the thalidomide treatment. The response to treatment was evaluated as previously described.[12] The study was approved by the local Ethics Committee and performed according to the S.T.E.P.S. program.[14]

Cultures of bone marrow mononuclear cells. Bone marrow mononuclear cells were isolated by density gradient centrifuging on Gradisol L (Aqua-Medica, Poland). Cell samples obtained from 15 patients before thalidomide treatment were used in short-term cultures. Isolated cells (2×10^6 cells/mL) were cultured in RPMI-1640 medium with 2 mM/L L-glutamine (Biomed, Poland) supplemented with 10% fetal calf serum (Biomed, Poland) and two antibiotics, penicillin 100 U/mL and streptomycin 100 μg/mL (Sigma, USA) at 37°C in a 5% CO_2 atmosphere. Cultures were performed with 10 μg/mL of thalidomide (Gruenenthal, Germany) in 0.1% DMSO (Sigma, USA), and with 0.1% DMSO alone as a control. After 72 hours cells were collected and examined for apoptosis and the expression of TNF-like receptors/ligands.

Cytometric assessment of TNF-like proteins. The expression of TNF-like ligands/receptors on erythroblasts was measured before and after 3 months of treatment. Cells were assessed by double-color immunofluorescence analysis in a FACScan (Becton Dickinson, USA) using the

Table 1. Effect of thalidomide treatment on basic clinical and laboratory parameters.

A	B	C	D	E	F	G	H	I	L	M	N	O	P
1	52M	IgG κ	III	no	resistant	MR	-43	11.9	11.6	17	24	22.2	36.5
2	56F	IgG κ	II	yes	relapsed	PR	-91	8.4	12.8	7	34	200.0	77.2
3	56M	IgG κ	III	yes	relapsed	SD	-3	11.4	8.4	9	8	73.6	84.2
4	54F	IgG κ	III	yes	relapsed	PR	-94	11.5	11.7	16	14	7.4	12.4
5	42F	IgG κ	II	no	resistant	PR	-92	12.3	11.6	20	54	14.1	18.7
6	43F	IgG κ	II	no	resistant	SD	-22	9.2	10.1	12	41	71.3	62.7
7	64M	IgG κ	II	no	relapsed	SD	-24	10.0	11.0	11	24	55.0	41.7
8	57F	IgG κ	III	yes	relapsed	PR	-79	11.5	12.8	5	12	53.5	42.4
9	50M	IgG κ	III	no	resistant	SD	+2	12.5	13.9	17	18	39.2	32.2
10	59F	IgG κ	I	no	relapsed	PR	-63	11.2	11.8	10	39	25.0	28.5
11	33M	IgG κ	II	no	resistant	CR	-100	11.0	14.4	12	15	2.7	10.7
12	75F	IgG κ	III	no	resistant	MR	-29	10.8	13.3	20	47	36.1	14.8
13	41F	IgG λ	III	no	resistant	PD	+109	11.9	12.5	5	37	11.8	44.0
14	69F	IgG κ	III	no	relapsed	PR	-69	11.6	13.0	28	32	34.8	22.8
15	54M	IgA λ	II	no	resistant	PR	-80	10.1	13.3	17	18	27.5	21.7
16	64M	IgG κ	III	no	relapsed	SD	+4	13.3	13.7	36	34	11.7	39.6
17	65M	IgG λ	III	yes	relapsed	SD	-20	7.4	10.7	18	20	66.7	33.9
18	61M	IgG κ	III	yes	relapsed	SD	+17	6.7	9.8	6	26	200.0	161.0
19	73M	IgG κ	III	no	resistant	MR	-40	8.5	10.9	13	23	70.4	22.2
20	65F	IgG λ	III	yes	resistant	PR	-62	7.9	8.9	27	39	16.2	14.9
21	45M	IgG κ	III	yes	relapsed	CR	-100	11.8	13.5	13	31	22.5	30.5
22	64F	IgG κ	III	no	resistant	PR	-72	13.5	12.0	35	32	84.7	42.0
23	57F	l.ch.d.λ	III	no	resistant	PR	-58	13.0	12.7	11	4	13.4	21.9
24	68M	IgG κ	III	no	resistant	SD	-15	8.1	8.9	28	42	144.0	200.0
25	61F	IgG κ	II	no	resistant	SD	+9	13.0	13.6	27	36	93.3	86.1
26	42M	IgG λ	III	no	resistant	PD	+48	9.3	7.3	12	30	30.8	55.8
27	55M	IgG κ	III	yes	relapsed	SD	-22	9.1	10.7	4	10	43.2	33.6
28	68M	IgG λ	II	no	resistant	MR	-43	10.8	10.9	12	33	34.5	32.1
29	44F	IgG κ	III	no	resistant	PR	-52	11.6	11.6	22	17	26.8	25.9
mean±standard deviation								**10.5± 1.88**	**11.6± 1.75**	**16.2± 8.76**	**27.4± 12.37**	**52.8± 51.26**	**46.6± 42.47**

A: Patient no.; B: Age/Sex; C: Type of MM; D: Salmon-Durie stage; E: Prior PBSCT; F: indication to THAL therapy; G: effect of therapy; H: change of M-protein (%); I: Hb before treatment (g/dL); L: Hb after 3 months of treatment (g/dL); M: erythroblasts in bone marrow before treatment (%); N: erythroblasts in bone marrow after 3 months of treatment (%); O: EPO before treatment (mIU/mL); P: EPO after 3 months of treatment (mIU/mL). CR: complete remission; PR: partial response; MR: minimal residual disease; SD: stable disease; PD: progressive disease.

following monoclonal antibodies: negative control IgG_1 FITC/ IgG_{2a} PE (Dako, Denmark), anti-GpA FITC (Dako, Denmark), anti-FasL PE (Santa Cruz Biotechnology, USA), anti-Fas PE (Dako, Denmark), anti-TRAIL PE (BD Pharmingen, USA), anti-TRAIL-R1 PE (R&D Systems, USA) and anti-TRAIL-R2 PE (R&D Systems, USA). Fc receptors were blocked with normal mouse IgG (Caltag Laboratories, USA) to avoid non-specific staining. In erythroblasts obtained after culturing, we measured the expression of TNF-like ligands/receptors indicated by the mean fluorescence intensity (MFI) calculated according to the formula $MFI=\Sigma X_i/n$, where X_i is the linear value for each event and n is the number of events used in the calculation.

Assessment of erythroblast apoptosis in vitro. The death of erythroblasts was evaluated in seven cultures using an immunofluorescence assay that detects exposure of phosphatidylserine by apoptotic erythroblasts. Cultured cells were treated with FITC-conjugated annexin V (Dako, Denmark) and PE-conjugated anti-GpA antibody (BD Pharmingen, USA). $AnnexinV^+/GpA^+$ cells were considered as apoptotic erythroblasts. Moreover the expression of BAX and BCL-2 indicated by MFI, was measured using the Intra Prep Kit (Immunotech, France) with permeabilization procedures and monoclonal antibodies: anti-intracellular BAX (Santa Cruz Biotechnology, USA) or BCL-2 (Dako, Denmark) conjugated with FITC and PE-conjugated anti-GpA antibody. We then calculated the BAX/BCL-2 ratio, which reflects the cells' sensitivity to apoptotic signals.

Results and Discussion

Clinical response to thalidomide treatment

Of the 29 patients, 34% were aged >60 years, 62% had stage III disease according to the Salmon-Durie classification, 32% had β-microglobulin >3 mg/L, 10% had received more than 3 years of prior therapy, and 31% had received prior high-dose therapy with stem cell support. The patients' profile and the effect of thalidomide on selected parameters are shown in Table 1. Seventeen patients (58.6%) responded to the treatment. Hemoglobin concentration increased in 22 patients (75.9%), decreased in six patients (20.7%) and did not change in one patient (3.4%). In the group of 17 responders, an increase in hemoglobin concentration was observed in 12 cases and the mean hemoglobin concentration in this group rose from 11.02±1.54 g/dL before treatment to 12.16±1.28 g/dL after 3 months (p=0.0200).

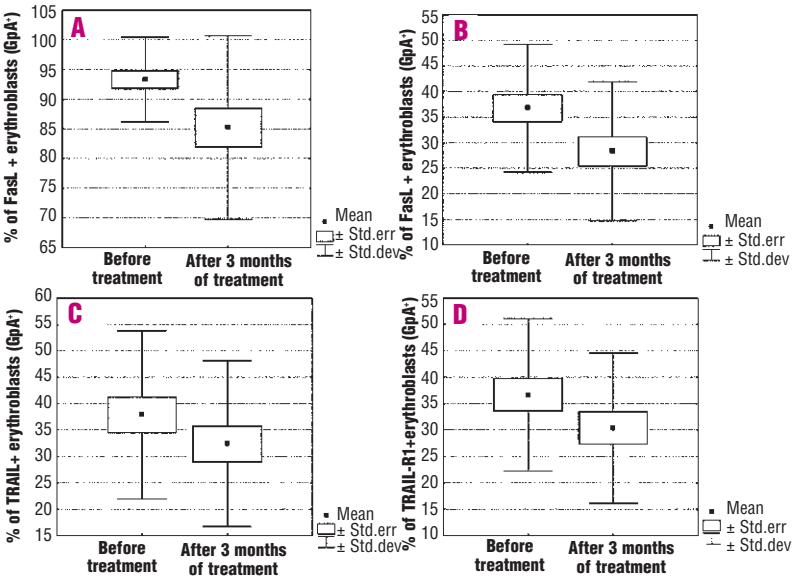

Figure 1. Changes in the expression of Fas, FasL, TRAIL and TRAIL-R1 in erythroblasts in the group of patients with an increased hemoglobin level during thalidomide treatment. A: mean expression of Fas decreased from 93.3±7.15% to 85.2±15.48% (p=0.0007), B: mean expression of FasL decreased from 36.7±12.50% to 28.3±13.61% (p=0.0031), C: mean expression of TRAIL decreased from 37.9±15.87% to 32.4±15.69% (p=0.0393); D: mean expression of TRAIL-R1 decreased from 36.7±14.4% to 30.4±14.2% (p=0.0186). Statistical significance was calculated using the Wilcoxon's test.

Among the 12 patients who did not respond, hemoglobin concentration increased in ten patients (nine with stable disease and one with progressive disease) and the mean hemoglobin level in this group rose from 9.73±2.12 g/dL before treatment to 10.68±2.00 g/dL after 3 months (p=0.0844). The mean percentage of erythroblasts in bone marrow increased during thalidomide treatment in both responders and non-responders – from 16.8±7.9% to 27.5±13.31% (p=0.0070) and from 15.4±10.17% to 27.2±11.48% (p=0.0076), respectively.

Expression of TNF-like proteins on erythroblasts in vivo

The *in vivo* expression of TNF-like proteins on erythroblasts was calculated in patients whose hemoglobin concentration increased during thalidomide treatment (group A) and in patients whose hemoglobin concentration did not increase (group B). In group A the mean percentage of erythroblasts expressing TNF-like ligands/receptors was significantly higher before thalidomide treatment than after 3 months: FasL 36.7±12.5% vs 28.3±13.6% (p=0.0031), Fas 93.3±7.2% vs 85.2±15.5% (p=0.0007), TRAIL 37.9±15.9% vs 32.4±15.7% (p=0.0393) and TRAIL-R1 36.7±14.4% vs 30.4±14.2% (p=0.0186). The decrease in the TRAIL-R2 expression was not statistically significant, although the expression was lower after 3 months of thalidomide treatment (35.0±15.3% vs 30.4±17.4%, p=0.0619). In contrast, there were no significant changes in the percentage of GpA^+ cells expressing these proteins in group B. The *in vivo* expression of selected TNF-like receptors/ligands are shown in Figure 1. We also observed a lower expression of TNF-like receptors/ligands on erythroblasts in cultures with thalidomide than in control cultures without thalidomide. Differences in the expressions of FasL, TRAIL, TRAIL-R1 and TRAIL-R2 were statistically significant. The difference in Fas expression was not statistically significant, although the expression was distinctly lower on GpA^+ cells from the cultures containing thalidomide.

Table 2. Results of *in vitro* experiments (mean ± SD, Wilcoxon's test).

Expression on GpA+ cells	*Control cultures*	*Thalidomide cultures*
EPOR (MFI, n=15)	34.45±26.80	31.57±16.94
FasL (MFI, n=15)	35.82±26.10[1]	28.05±14.49[1]
Fas (MFI, n=15)	93.49±80.78	75.90±49.99
TRAIL (MFI, n=15)	35.38±19.62[2]	27.07±14.78[2]
TRAIL-R1 (MFI, n=15)	35.48±17.32[3]	30.75±15.52[3]
TRAIL-R2 (MFI, n=15)	31.05±14.68[4]	27.47±11.91[4]
BAX/BCL-2 ratio (MFI, n=7)	1.32v0.42	1.18v0.38
GpA+/AnnV+ (%, n=7)	48.8±20.78	42.7±25.33

[1]p=0.0268; [2]p=0.0171; [3]p=0.0356; [4]p=0.0178.

Apoptosis of erythroblasts in vitro

The mean BAX/BCL-2 ratio was 1.32±0.42 in control cultures and 1.18±0.38 thalidomide-containing cultures (p=0.0747). The mean percentage of Gpa^+/$AnnexinV^+$ apoptotic erythroblasts was 48.8±20.78% in control cultures and 42.7±25.33% in thalidomide-containing cultures (p=0.1282). In experiments with mononuclear bone marrow cells from five patients whose hemoglobin level increased during thalidomide treatment, the mean percentage of GpA^+/$AnnexinV^+$ apoptotic erythroblasts was significantly lower in thalidomide-treated cultures than in control cultures (38.8±9.29% vs 49.9±10.14%, p=0.0431). For this group of patients the BAX/BCL-2 ratio was 1.25±0.22 in control cultures and 1.03±0.27 in the cultures containing thalidomide (p=0.0679). The results of the *in vitro* experiments are summarized in Table 2. Few studies have focused on the influence of thalidomide on erythropoiesis and the hemoglobin level in MM patients so far. Most investigators suggested that an increased concentration of hemoglobin was closely related to a response and to decreased infiltration of bone marrow by plasmocytes,[5,15] although they did not analyze non-responders carefully. Moreover, therapy was usually changed early in

non-responders, whereas our results suggest that patients with stable disease may benefit from thalidomide treatment and that their hemoglobin level may increase after about 3 months of the therapy. On the other hand in some studies there was also an improvement in the hemoglobin level in selected patients who did not respond.[9,16] In our previous studies we observed that thalidomide may improve the hemoglobin level in MM patients, in both responders and non-responders.[13,17] In the present study we have obtained results that support our preliminary observation.

The precise mechanisms of thalidomide's action remain unknown, especially with regard to stimulation of erythropoiesis. There are reports suggesting that early changes in bone marrow morphology occur, with the reappearance of erythroblasts in various phases of differentiation during thalidomide treatment.[18,19] Our results suggest that changes in the expression of TNF-like receptors/ligands on erythroblasts induced by thalidomide make these cells less susceptible to apoptotic signals and contribute to increased erythroblast survival, stimulation of erythropoiesis and improvement of the hemoglobin level in MM patients. Although experiments evaluating erythroblast apoptosis were performed on a limited number of cultures, it seems that the decrease in the expression of TNF-like receptors/ligands on erythroblasts is sufficient to prevent these cells' apoptosis. To our knowledge this is the first report presenting evidence that thalidomide may stimulate erythropoiesis in MM patients by decreasing the expression of TNF-like ligands/receptors on erythroblasts. Further investigations, including assessement of the transcription factor GATA-1, which is responsible for erythroblast survival, are warranted to confirm whether these changes correlate with apoptosis and proliferation of erythroid cells.

NG: interpretation of data, drafting the article, final approval of the version to be published; AD: conception and design of the study, interpretation of data, revising the article, final approval of the version to be published; MH and MSW: interpretation of clinical data; PK and AG: performance of laboratory tests, interpretation of laboratory data. We thank Piotr Klimek and Aneta Goracy for excellent technical assistance. The authors declare that they have no potential conflicts of interest.

The work was supported by a grant from the State Committee for Scientific Research, No. KBN3PO5B04623.

Manuscript received June 3, 2005. Accepted December 28, 2005.

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