Pharmacological Reports 67 (2015) 1193–1200

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

Thalidomide can promote erythropoiesis by induction of STAT5 and repression of external pathway of apoptosis resulting in increased expression of GATA-1 transcription factor

Norbert Grzasko [a,*], Sylwia Chocholska [a], Aneta Goracy [a], Marek Hus [a], Anna Dmoszynska [b]

[a] Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Lublin, Poland
[b] Department of Clinical Transplantology, Medical University of Lublin, Lublin, Poland

ARTICLE INFO

Article history:
Received 7 February 2015
Received in revised form 11 April 2015
Accepted 15 May 2015
Available online 29 May 2015

Keywords:
Thalidomide
Erythropoiesis
Apoptosis
Multiple myeloma

ABSTRACT

Background: Thalidomide was shown to stimulate erythropoiesis and increase hemoglobin level in multiple myeloma patients, but way of such activity remains unclear. The aim of the study was to investigate the mechanisms of thalidomide stimulating effect on erythroid differentiation.
Methods: Hematopoietic stem cells were isolated from bone marrow aspirates taken from myeloma patients and cultured with or without thalidomide. Then the generation of erythroid cells and the expression of STAT5, GATA-1, GATA-2, selected caspases and Bcl-2 family proteins in erythroid cells were assessed using flow cytometry and real-time PCR.
Results: The generation of erythroblasts was higher in thalidomide than in control cultures (63.9% *vs.* 55.8%, $p < 0.001$). The expression of caspase 3 (cytometry 947.3 *vs.* 1021.0, $p = 0.025$; PCR 12.9 *vs.* 16.3, $p = 0.025$) and caspase 8 (cytometry 1050.8 *vs.* 1168.5, $p = 0.033$; PCR 16.2 *vs.* 17.8, $p = 0.004$) was significantly lower in thalidomide than in control cultures. The expression of STAT5 (cytometry 331.5 *vs.* 276.1, $p = 0.015$; PCR 24.3 *vs.* 21.1, $p = 0.003$) and GATA-1 (cytometry 259.7 *vs.* 232.0, $p = 0.027$; PCR 18.9 *vs.* 16.5, $p = 0.003$) was higher in thalidomide than in control cultures.
Conclusion: Our results suggest that thalidomide enhances expression of STAT5 in response of erythroid cells to erythropoietin and as a result of caspase 3 suppression. Moreover it may exert inhibitory effect on an external pathway of caspases activation with consequent decreased degradation of GATA-1 transcription factor by downstream caspases.

Introduction

Apoptosis, or programmed cell death, has an important role in normal erythroid maturation [1,2]. It is mediated by caspases, proteases activated by different inducers of apoptosis, which initiate irreversible cell damage including cleavage of important cell proteins and DNA fragmentation [3,4]. There are two pathways of apoptosis: external mediated *via* death receptors which is associated with caspase 8 activation and mitochondrial associated with caspase 9 activation and regulated by the BLC-2 gene family. They both lead to the activation of downstream caspases with the most important caspase 3 [5].

The mechanisms promoting apoptosis of immature erythroid cells provide negative regulation which prevents excessive erythropoiesis in physiological conditions. TNF-family proteins like Fas, Fas ligand (Fas-L), TNF-related apoptosis-inducing ligand (TRAIL) and its two receptors TRAIL-R1 and TRAIL-R2 are considered potent negative regulators of erythropoiesis [1,2,6]. Immature basophilic-stage erythroblasts express Fas, TRAIL-R1 and TRAIL-R2 which are highly susceptible to Fas-L and TRAIL ligation. In contrast, mature erythroblasts are resistant to Fas- and TRAIL-induced apoptosis, although they express large amounts of Fas-L, TRAIL and their receptors. Apoptosis of immature erythroid progenitors resulting from the interaction with Fas-L and TRAIL-positive mature erythroblasts leads to the inhibition of erythropoiesis. Erythropoietin (EPO) is a positive regulator of erythropoiesis and the survival of immature erythroblasts is strictly dependent on EPO levels [7]. Interaction of EPO with its receptor (EPO-R) activates the

* Corresponding author.
E-mail address: norbertgrzasko@gmail.com (N. Grzasko).

http://dx.doi.org/10.1016/j.pharep.2015.05.011
1734-1140/

Janus-like kinase 2 (Jak2)/signal transducer and activator of transcription 5 (STAT5) pathway [8,9]. Phosphorylated active molecules of STAT5 dimerise and after moving to nucleus stimulate transcription of genes important for cell survival and proliferation, like apoptosis-regulating Bcl-2 family proteins [10,11] or GATA-1 and GATA-2, members of the GATA transcription factors family which are essential for erythroid proliferation and survival [6].

Anemia is found in most of multiple myeloma (MM) patients, especially in individuals with more advanced disease and its severity correlates with the disease stage at diagnosis [12]. Similar mechanisms as described above are involved in defective erythropoiesis leading to anemia in MM. Malignant plasma cells overexpress Fas-L and TRAIL, which enables them to avoid apoptosis induced by immune system cells and reflects their high degree of malignancy. In addition, this apoptogenic phenotype of plasma cells enhances apoptosis of erythroid cells leading to anemia [13,14]. Achieving remission in MM patients is usually accompanied by an increase in hemoglobin concentration, but such improvement was also described in some patients treated with thalidomide (THAL) and not responding to the therapy, which suggested that THAL could stimulate erythropoiesis independently of producing remission and decreasing bone marrow infiltration by monoclonal plasma cells [15,16]. Mechanism of erythropoiesis stimulation by THAL is unclear, although we previously showed that THAL might stimulate erythropoiesis in MM patients by decreasing the expression of TNF-like ligands/receptors on erythroblasts [16].

The aim of present study was to investigate detailed mechanisms of erythroid stimulation by THAL. We studied the generation of erythroid cells and their expression of STAT5, GATA-1, GATA-2, caspases and Bcl-2 family proteins in cultures of hematopoietic stem cells obtained from MM patients. The study was approved by the local Ethics Committee and was conducted in accordance with the Helsinki Declaration. All patients participating in the study signed informed consent prior to the study inclusion.

Materials and methods

Cultures of bone marrow hematopoietic stem cells

Bone marrow aspirates were taken from sternum or iliac bone from 20 MM patients at diagnosis after obtaining informed consent. In all patients a symptomatic MM was diagnosed with different stage of anemia. Patients' clinical charactaristics are summarized in Table 1.

Bone marrow hematopoietic stem cells were isolated from marrow aspirates using magnetic-activated cell sorting (MACS) method by means of the human CD34 microbeads kit according to the manufacturer's protocol (Miltenyi Biotec, Germany). Purified CD34-positive cells were cultured for 14 days in Iscove's Modified Dulbecco's Medium containing bovine serum albumins, recombinant human insulin and human transferrin (Stem Cell Technologies, Canada), 500 U/ml penicillin (Sigma, USA), 40 μg/ml streptomycin (Sigma, USA), 50 ng/ml stem cell factor (Sigma, USA), 4 U/ml erythropoietin (Sigma, USA), 10 ng/ml interleukin 3 (Sigma, USA) to induce erythroid differentiation. Cultures were performed at 37 °C in a 5% CO_2 atmosphere with 10^5 cells per 1 ml of medium [2]. Thalidomide (Sigma, USA) was diluted in 0.1% DMSO (Sigma, USA) and added to cultures at a concentration of 10 μg/ml; control cultures contained a respective amount of DMSO. Culture medium was changed every 3 days. After 14 days cells were collected and examined for expression of glycophorin A (GpA), CD71, STAT5, GATA-1, GATA-2, Bcl-2 family proteins and caspases 1, 3, 8 and 9.

Flow cytometric assessment

Cultured cells were stained with Anti-Human CD45 PE-Cyanine5 (eBioscience, USA), Anti-human CD235a/Glycophorin A FITC (eBioscience, USA), Anti-human CD235a/Glycophorin A PE (eBioscience, USA) and Monoclonal Anti-human Transferrin Receptor/CD71 Allophycocyanin (R&D Systems, USA) for detection of erythroblasts. After permeabilization with Fix&Perm Cell Permeabilization Reagents kit (Caltag, UK) cells were stained with the following fluorescence-conjugated antibodies: Caspase-1 FITC (Santa Cruz Biotechnology, USA), Fluorescein Active Caspase 3 Staining Kit (Abcam, UK), Fluorescein Active Caspase 8 Staining Kit (Abcam, UK), Fluorescein Active Caspase 9 Staining Kit (Abcam, UK), BCL-xL PE (Santa Cruz Biotechnology, USA), Bax PE (Santa Cruz Biotechnology, USA), Bcl-2 PE (Santa Cruz Biotechnology, USA), Anti-hGATA-1 Phycoerythrin (R&D Systems, USA), Anti-hGATA-2 Phycoerythrin (R&D Systems, USA), Anti-hSTAT5a Phycoerythrin (R&D Systems, USA). Fc receptors were blocked with normal mouse IgG (Caltag, UK) to avoid non-specific staining. Samples were

Table 1
Patients' clinical characteristics.

Patient no.	Sex/Age	Monoclonal protein	Hemoglobin (g/dl)	Creatinine (mg/dl)	Durie–Salmon/ISS staging	Cytogenetics	Difference in erythroblast generation between THAL and control cultures
1	M/78	IgG κ	8.3	1.23	II/3	Normal karyotype	+10.9%
2	F/67	IgG κ	11.1	2.17	II/1	Normal karyotype	−8.7%
3	M/68	IgA κ	10.0	0.89	I/2	Hyperdiploid	+9.6%
4	M/65	IgA κ	9.9	4.51	II/1	normal karyotype	+0.4%
5	M/64	IgG λ	9.1	2.29	III/3	Hyperdiploid, del13q14	+6.7%
6	F/65	LCD κ	9.8	0.80	III/2	Hyperdiploid	+4.5%
7	F/54	LCD κ	8.6	5.63	III/3	amp1q21	+13.9%
8	F/69	IgA κ	9.7	0.73	II/2	Normal karyotype	+7.1%
9	M/72	LCD λ	9.9	1.50	II/2	del13q14	+2.2%
10	M/58	IgG λ	11.5	0.75	II/3	del13q14, amp1q21	−9.2%
11	F/60	IgG λ	8.1	2.41	III/3	Hyperdiploid, del13q14, del17p13, amp1q21	+1.5%
12	F/79	LCD λ	10.5	3.78	III/3	amp1q21	+15.3%
13	F/64	IgG λ	11.1	0.85	III/2	Hyperdiploid	+24.4%
14	M/71	IgG κ	10.3	1.16	III/3	Hyperdiploid	−2.6%
15	F/61	LCD λ	8.1	2.36	III/3	del13q14, del17p13	+12.3%
16	F/45	IgA λ	9.7	0.79	III/2	Normal karyotype	+19.8%
17	F/65	IgG κ	10.7	0.84	III/3	t(4;14)	−11.5%
18	F/46	IgG κ	8.7	0.63	I/1	Normal karyotype	+20.8%
19	M/74	IgG λ	8.6	1.09	II/2	del13q14, amp1q21	+7.6%
20	M/66	IgG κ	9.6	0.95	II/3	del13q14, t(4;14)	+18.7%

assessed by four-color immunofluorescence analysis in a FACScan with graphic presentation in CellQuest software (Becton Dickinson, USA). In CD71-positive/GpA-positive/CD45-negative erythroid cells obtained after culturing, we measured the expression of GATA-1, GATA-2, STAT5 and caspases by the mean fluorescence intensity (MFI) calculated according to the formula MFI = $\Sigma X1/n$, where X1 is the linear value for each event and n is the number of events used in the calculation. Combinations of antibodies used in a flow cytometry assay are listed in Table 2.

Quantitative real-time PCR

After completion of culture, the cells in an amount of about 1×10^6 were suspended in RLT buffer (Qiagen, Germany) and stored at −80 °C until analysis. Total RNA was extracted using the QIAamp RNA Blood Mini Kit (Qiagen, Germany). The quantity and purity of RNA sample was assessed by spectrophotometric measurement using SmartSpec Plus (Bio-Rad, USA). Synthesis of cDNA was performed using High Capacity cRNA Reverse Transcription Kit (Applied Biosystems, USA). Reverse transcription was carried out in DNA Engine Peltier Thermal Cycler (Bio-Rad, USA). Using TaqMan Gene Expression Master Mix (Applied Biosystems, USA), gene-specific oligonucleotide primers (Applied Biosystems, USA), TaqMan MGB Probes (Applied Biosystems, USA), the expression of following genes was measured: 1/ transcription factors GATA-1, GATA-2 and STAT5; 2/ Bcl-2 family proteins; and 3/ caspases 1, 3, 8 and 9. Amplification of cDNA with specific primers for beta-2-microglobulin (B2M) gene of constitutive expression was used as internal control. The real time PCR (RT-PCR) reactions were run for 40 cycles under universal cycling conditions (95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min) on the 7300 Real Time PCR System (Applied Biosystems, USA). The expression of examined genes were normalized to B2M expression, analyzed using the threshold cycle (C_T) and presented as $2^{-\Delta CT}$. Delta CT (ΔC_T) was the difference between the C_T of the target gene (C_{Tt}) and the reference gene (C_{Tr}) ($\Delta C_T = C_{Tt} - C_{Tr}$).

Statistical analysis

Data were presented as median. Statistical significance was assessed by the Wilcoxon signed-rank test with $p < 0.05$ considered to be statistically significant. Statistica 7.0 (StatSoft, USA) and GraphPad Prism 5 (GraphPad Software, USA) software were used for calculations.

Results

Flow cytometric assessment of erythroblasts generation and expression of transcription factors, caspases and Bcl-2 family proteins

Cells obtained after culturing were stained for the presence of CD71 and GpA proteins, which are the major surface antigens of erythroid progenitor cells. The generation of CD71-positive/GpA-positive/CD45-negative cells considered erythroblasts was higher in THAL cultures than in control cultures in 16 from 20 cases. The median percentage of CD71-positive/GpA-positive/CD45-negative erythroid progenitor cells was significantly higher in THAL than in control cultures (63.9% *vs.* 55.8%, $p < 0.001$). There were no clear differences in terms of clinical parameters such as age, sex, type of monoclonal protein, stage according to Durie–Salmon and ISS, creatinine level and detected cytogenetic changes between patients whose cells did not respond to THAL and those with a higher percentage of erythroblasts in THAL cultures compared to control cultures. However, there were evident differences in hemoglobin level: in all patients with no response to THAL it was higher than 10 g/dl with a median of 10.9 g/dl (range 10.1–11.5 g/dl); among the remaining 16 patients whose cells responded to treatment with THAL, hemoglobin level was 10 g/dl or more only in 3 patients with a median of 9.65 g/dl (range 8.1–11.1 g/dl). Unfortunately, due to the small sample size it was not possible to carry out reliable statistical analysis.

CD71+/GpA+ erythroid cells were further investigated for the expression of activated phosphorylated form of STAT5 and GATA-1 and GATA-2 transcription factors, which are generated after stimulation of EPO-R with EPO and have a crucial role in erythroid cells survival. The expression of proteins was measured as MFI. Among tested antigens, the median expression of activated phosphorylated form of STAT5 and transcription factor GATA-1 was significantly higher in THAL cultures than in control cultures, while the difference in the expression of GATA-2 did not reach statistical significance.

Caspases expression measured as MFI was lower in THAL than in control cultures. The median MFI of caspase 1 was 212.8 in THAL cultures and 252.8 in control cultures and the median MFI of caspase 9 was 1861.7 and 2010.5, respectively. However, both differences did not reach the statistical significance ($p > 0.05$ for both). In contrast, the median MFI of caspase 3 (947.3 *vs.* 1021.0, $p = 0.025$) and caspase 8 (1050.8 *vs.* 1168.5, $p = 0.033$) was significantly lower in THAL than control cultures. The expression of Bcl-2 gene family proteins did not differ significantly between THAL and control cultures.

The detailed results of flow cytometry analysis are summarized in Table 3 and Fig. 1. The flow cytometric analysis on an exemplary culture is shown in Fig. 2.

Quantitative real-time PCR assessment of transcription factors, caspases and Bcl-2 family proteins

The expression of analyzed proteins mRNA was measured by RT-PCR and normalized to B2M. The results supported those obtained with flow cytometry. The median expression of STAT5 and GATA-1 was significantly higher in THAL cultures than in control cultures. The expression of caspases was lower in THAL than in control cultures, although differences reached the

Table 2
Combinations of antibodies used in a flow cytometry assay.

Measured expression	Fluorescein (FITC)	Phycoerythrin (PE)	Phycoerythrin-Cyanine5 (PE-Cy5)	Allophycocyanin (APC)
Caspase 1	Anti-caspase 1	Anti-CD235a/Glycophorin	Anti-CD45	Anti-CD71
Caspase 3	Anti-caspase 3	Anti-CD235a/Glycophorin	Anti-CD45	Anti-CD71
Caspase 8	Anti-caspase 8	Anti-CD235a/Glycophorin	Anti-CD45	Anti-CD71
Caspase 9	Anti-caspase 9	Anti-CD235a/Glycophorin	Anti-CD45	Anti-CD71
Bcl-2	Anti-CD235a/Glycophorin	Anti-Bcl-2	Anti-CD45	Anti-CD71
Bcl-xL	Anti-CD235a/Glycophorin	Anti-Bcl-xL	Anti-CD45	Anti-CD71
Bax	Anti-CD235a/Glycophorin	Anti-Bax	Anti-CD45	Anti-CD71
GATA-1	Anti-CD235a/Glycophorin	Anti-GATA-1	Anti-CD45	Anti-CD71
GATA-2	Anti-CD235a/Glycophorin	Anti-GATA-2	Anti-CD45	Anti-CD71
STAT5	Anti-CD235a/Glycophorin	Anti-STAT5	Anti-CD45	Anti-CD71

Table 3
The expression of analyzed proteins in the flow cytometric assay (results shown as MFI; the Wilcoxon's signed-rank test).

	Thalidomide cultures		Control cultures		*p* value
	Median	Range	Median	Range	
Caspase 1	212.8	82.9–1039.4	252.8	87.6–726.6	>0.05
Caspase 3	947.3	254.9–1574.6	1021.0	171.2–1666.6	0.025
Caspase 8	1050.8	258.6–1825.1	1168.5	192.6–1847.1	0.033
Caspase 9	1861.7	164.2–4526.5	2010.5	158.8–5126.1	>0.05
Bcl-2	206.3	92.1–514.5	233.7	82.3–608.9	>0.05
Bcl-xL	339.5	144.3–812.2	366.2	99.7–825.2	>0.05
Bax	220.9	106.2–670.4	239.9	95.6–444.5	>0.05
GATA1	259.7	195.0–401.8	232.0	171.3–362.8	0.027
GATA2	154.2	90.0–270.8	136.5	78.4–284.1	>0.05
STAT5	331.5	218.1–429.5	276.1	196.0–411.5	0.015

statistical significance only for caspase 3 and caspase 8. The expression of Bcl-2 gene family proteins mRNA did not differ significantly between THAL and control cultures.

The detailed results of RT-PCR analysis are summarized in Table 4 and Fig. 3.

Discussion

THAL was successfully used in MM therapy [17] but its mechanism of action is still not fully understood. It was initiated to treat MM on the basis of its antiangiogenic [18,19] and immunomodulatory [20–22] properties and it was successfully used alone or in combination with glycocorticoids or chemotherapy in patients with refractory/relapsed [23–26] as well as newly diagnosed MM [27–29]. Few studies focused on the influence of THAL on erythropoiesis and the hemoglobin level in MM patients so far. Most of investigators suggested that an increased concentration of hemoglobin during THAL therapy was closely related to response and decreased infiltration of bone marrow by monoclonal plasma cells [17,30]. On the other hand there are also reports showing an improvement in the hemoglobin level in selected patients who did not respond [23,31]. In our earlier studies we showed that the amelioration of anemia was also observed in patients not responding to THAL therapy [15,16], which suggested that THAL may directly or indirectly stimulate erythropoiesis independently of its antimyeloma effect and decreased bone marrow infiltration by monoclonal plasma cells. The ability of THAL to affect erythropoiesis and to increase the hemoglobin level was also confirmed in myelodysplastic syndromes [32,33] and myelofibrosis with myeloid metaplasia [34]. The discrepancy of results regarding hemoglobin level obtained in MM patients may be caused by different THAL doses and treatment duration, as it was usually changed early in non-responders, whereas our results suggested that patients with stable disease could benefit from prolonged THAL treatment and their hemoglobin level might increase after about 3 months of therapy [15,16]. The hypothesis seems to be supported by the results of studies on patients with myelodysplastic syndromes in whom responses were observed after at least 3 months, and in some individuals even after 5 months of therapy [33].

The mechanism by which THAL stimulates erythropoiesis and increases the hemoglobin level remains unclear. There are reports suggesting that there were early changes in bone marrow morphology during THAL treatment and that its cellularity increased with the reappearance of erythroblasts and myeloid cells in various phases of differentiation [35,36]. In previous study

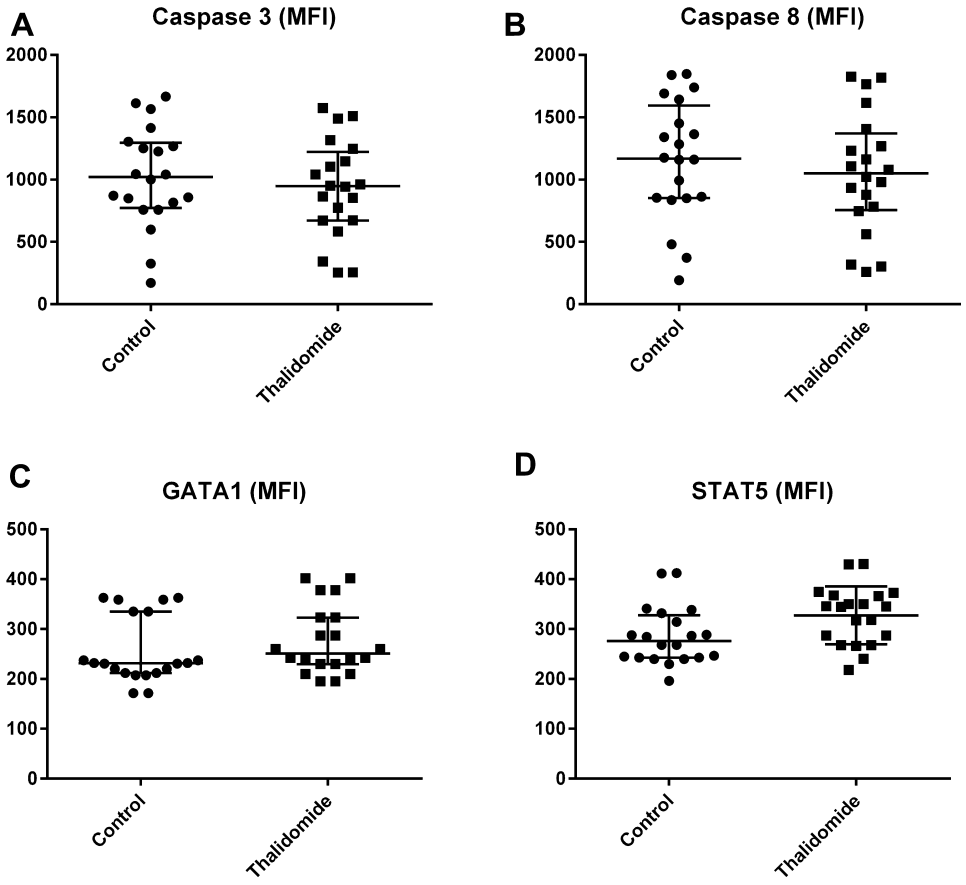

Fig. 1. Comparison of the expression of selected proteins in the flow cytometric assay. The median expression of caspase 3 (A) and caspase 8 (B) was significantly lower in THAL cultures than in control cultures. The median expression of GATA-1 (C) and STAT5 (D) was significantly higher in THAL cultures than in control cultures.

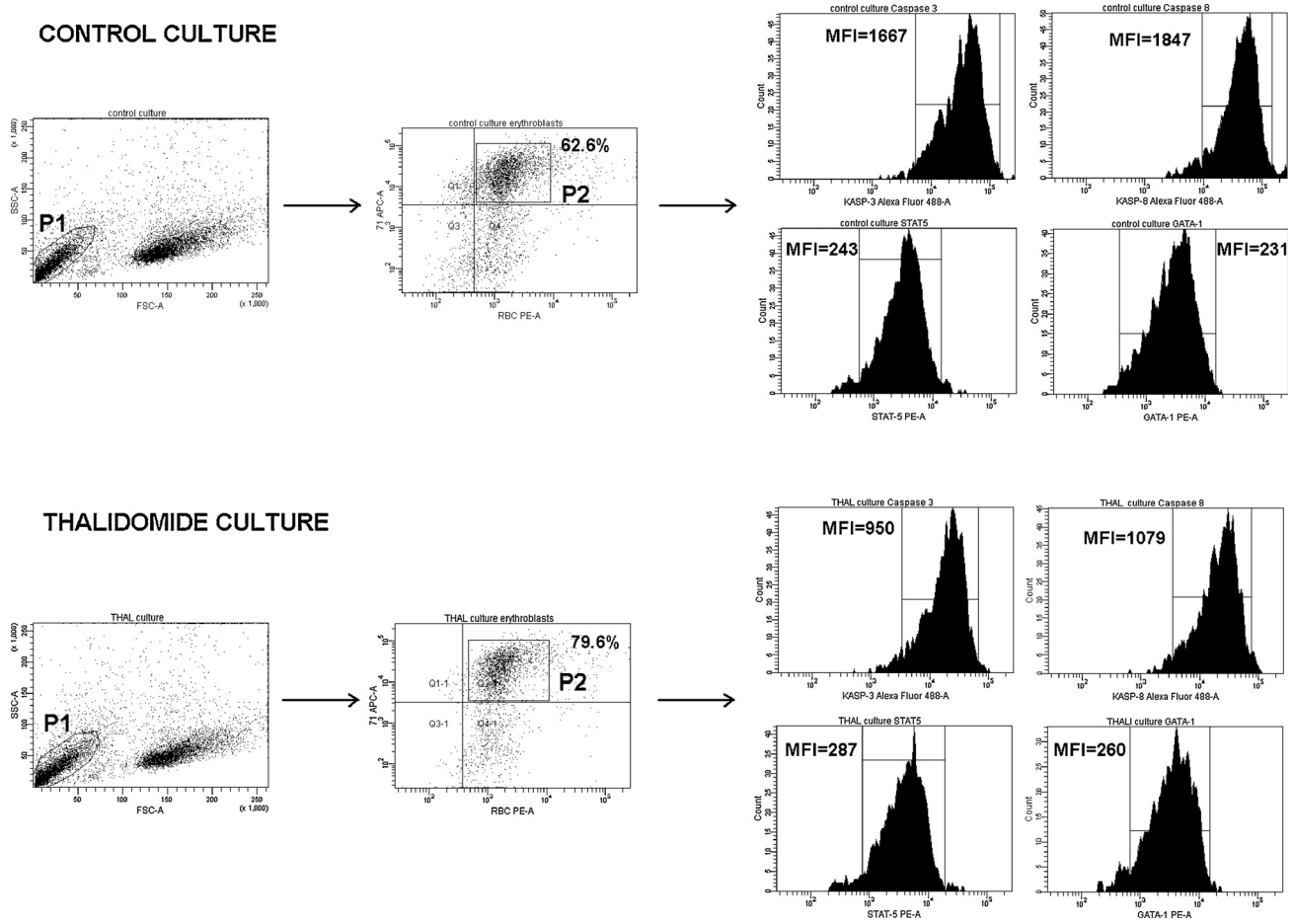

Fig. 2. The representative plots and histograms illustrating the analysis method for expression of caspase 3, caspase 8, GATA-1 and STAT5. An acquisition gate including mononuclear cells was established based on Forward Scatter (FSC) and Side Scatter (SSC). Then gated events were analyzed for the presence of CD71, CD235a/Glycophorin and CD45; events in the upper right quadrant on the dot plot represents CD71+/CD235a+/CD45− erythroid cells. Histograms shows MFI analysis of caspase 3, caspase 8, GATA-1 and STAT5 in CD71+/CD235a+/CD45− cells. In the presented exemplary cultures from the patient no. 20 the percentage of CD71+/CD235a+/CD45− erythroid cells was 62.6% in control cultures and 79.6% in THAL cultures. MFI of caspase 3, caspase 8, STAT5 and GATA-1 in control culture was 1667, 1847, 243 and 231 respectively; in THAL culture 950, 1079, 287 and 260 respectively.

we showed that THAL induced changes in the expression of TNF-like receptors/ligands on erythroblasts, which contributed to increased erythroblasts survival, stimulation of erythropoiesis and improvement of hemoglobin level in patients with refractory/relapsed MM. This effect was separated from antimyeloma activity of THAL, as it was observed also in non-responders. Moreover, *in vitro* experiments also supported such mechanism of THAL's influence on erythropoiesis [16].

Table 4
The expression of analyzed proteins in real-time PCR (results presented as $2^{-\Delta CT}$; the Wilcoxon's signed-rank test).

	Thalidomide cultures		Control cultures		*P* value
	Median	Range	Median	Range	
Caspase 1	20.1	12.0–41.3	20.7	9.7–40.7	>0.05
Caspase 3	12.9	9.1–37.2	16.3	8.9–33.1	0.025
Caspase 8	16.2	7.3–23.7	17.8	9.0–27.1	0.004
Caspase 9	17.9	9.8–31.7	17.8	8.9–41.4	>0.05
Bcl-2	21.5	5.9–42.5	22.0	7.4–47.3	>0.05
Bcl-xL	21.4	11.4–35.2	21.8	10.1–34.0	>0.05
Bax	18.3	8.7–42.2	19.3	10.1–38.5	>0.05
GATA1	18.9	8.1–28.9	16.5	9.2–25.6	0.003
GATA2	15.2	9.1–26.8	14.6	8.9–21.2	>0.05
STAT5	24.3	13.7–36.8	21.1	11.5–37.2	0.001

Derivatives of THAL also have an ability to stimulate erythropoiesis. Lenalidomide (LEN) was the first targeted therapy for myelodisplastic patients with chromosome 5q deletion. It was shown to produce rapid response including increased hemoglobin level, transfusion independence and reversal of cytogenetic abnormality [37], which was associated with increased number of erythroid precursor cells produced from CD34+ stem cells [38]. Lenalidomide was also effective in patients with low- and intermediate-risk myelodisplastic patients who were transfusion dependent and refractory to erythropoiesis stimulating agents [39,40]. Pomalidomide, another derivative of THAL, was efficient in anemic patients with myelofibrosis [41].

In the present study the generation of erythroblasts was significantly higher in cultures treated with thalidomide, however there were some discrepancies. In cell cultures derived from four patients the percentage of erythroblasts was lower in cultures treated with THAL. All patients in this group had mild anemia and hemoglobin level was above 10 g/dl in all cases, whereas in the remaining 16 patients whose cells responded to the treatment with THAL, hemoglobin levels were clearly lower with only 3 patients with values above 10 g/dl. No differences were observed in terms of other basic clinical characteristics. On this basis, one can speculate that the stimulating effect of THAL on erythropoiesis is more pronounced in patients with more severe anemia and this group may benefit more in terms of increased hemoglobin levels.

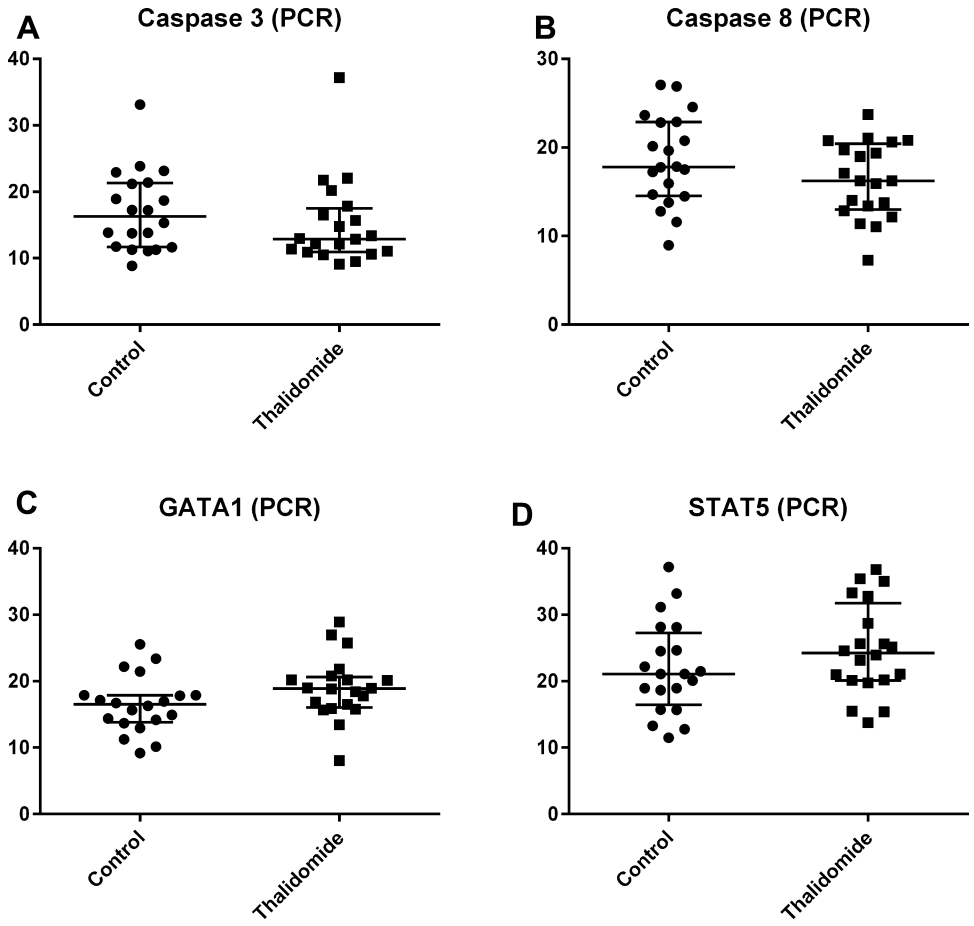

Fig. 3. Comparison of the expression of selected genes in real-time PCR. The median expression of caspase 3 (A) and caspase 8 (B) was significantly lower in THAL cultures than in control cultures, whereas the median expression of GATA-1 (C) and STAT5 (D) was significantly higher in THAL cultures than in control cultures.

However, the presented results are only preliminary *in vitro* studies and ultimately such conclusions should be drawn only after proper clinical trials.

The results obtained our present study suggest that at least two mechanisms are involved in the stimulating effect of THAL on erythropoiesis. The first seems to be associated with increased sensitivity of erythroid precursors to EPO. EPO binding to EPO-R causes receptor dimerization and subsequent activation of JAK-2 kinase, which phosphorylates tyrosine residues in cytoplasmic domain of EPO-R [7]. As a result numerous signaling pathways are activated, but the central pathway in EPO-R signaling is the activation of STAT5. Molecules of STAT5 bind to phosphotyrosine residues on cytoplasmic domain of activated EPO-R and they are phosphorylated by Jak2. Upon phosphorylation STAT5 dimerise and translocates to nucleus, where it acts as a transcription factor binding to promoter regions of numerous genes [11–13]. The importance of STAT5 in erythropoiesis was clearly showed in mice lacking STAT5, which displayed increased apoptosis of erythroid cells and as a result fetal anemia [42,43]. Moreover, a total knockout of STAT5 in mice was associated with severe anemia *in utero* and perinatally lethal [44]. On the other hand constitutively activated mutant of STAT5 was able to promote erythropoiesis both in Jak2$^{-/-}$ and EPO-R$^{-/-}$ cells [45]. In our experiments there was a significantly higher percentage of CD71+/GpA+ erythroid cells in THAL cultures compared to control cultures with a higher expression of phosphorylated active form of STAT 5 in these cells. Increased STAT5 activation at the same concentrations of EPO suggests that hematopoietic stem cells treated with THAL are more sensitive to EPO stimulation, which resulted in increased generation and survival of erythroid precursors.

The second mechanism of THAL's stimulating effect on erythropoiesis seems to be associated with changed regulation of caspase expression. Caspase 8 is known as an initiator caspase during external activation pathway mediated by TNF-family receptors [5] and it is very important in triggering apoptosis of erythroid progenitor cells [46]. Our results indicate that THAL decreases the expression of caspases in erythroid cells, particularly caspase 3 and 8. This observation is consistent with the results showed in our previous report on THAL's influence on TNF-family proteins expression on erythroblasts [16]. We found previously that the expression of FasL, Fas, TRAIL and TRAIL-R1 decreased significantly during THAL treatment. Moreover, culture studies revealed that the apoptosis of erythroblasts and the expression of TNF-family proteins on the cells was lower in THAL cultures compared to controls. The results suggested that the decreased expression of TNF-like proteins on erythroblasts induced by THAL made the cells less susceptible to apoptotic signals and contributed to their increased survival, stimulation of erythropoiesis and improvement of the hemoglobin level regardless of response achieved during THAL treatment. It is known that binding death ligands to TNF-like receptors results in the formation of death-induced signaling complex (DISC) [47], which transforms procaspase 8 to active form. Therefore it is possible that previously proven lower expression of TNF-family receptors and ligands on erythroblasts caused by THAL could result in decreased activation of caspase 8.

In contrast to caspase 8, changes in the expression of caspase 9 observed in our experiments, although slightly lower in THAL than control cultures, did not reached statistical significance. Activation of caspase 9 in the complex formed by ATP, cytochrome *c*, apoptotic protease activating factor 1 (Apaf-1) and procaspase 9 is a key event in the mitochondrial pathway of apoptosis [48], so it seems not to be essential for stimulation of erythropoiesis by THAL. This conclusion is supported by the results of Bcl-2, Bcl-xL and Bax expression, which did not differ significantly between THAL and control cultures. Listed proteins are involved in the regulation of mitochondrial pathway of apoptosis acting as inhibitors, such as Bcl-2 and Bcl-xL, and as activators, such as Bax [5,49]. The lack of influence on caspase 9 and Bcl-2 family proteins expression supports the hypothesis that mitochondrial pathway of apoptosis activation is not a key point in stimulating effect of THAL on erythropoiesis in contrast to external pathway represented by caspase 8.

Inhibition of caspase 3 and induction of GATA-1 transcription factor by THAL found in our study seems to be a logical consequence of its effect on caspase 8. Caspase 3, as well as caspases 6 and 7 represent the downstream or executor caspases which cleave a variety of substrates including members of GATA transcription factors family [5,6]. GATA-1 and GATA-2 are particularly important during erythropoiesis. GATA-1 is necessary for differentiation of early erythroid cells and absence of GATA-1 triggers development arrest and apoptosis of these cells [50,51]. GATA-2 has similar importance for erythroid differentiation and its deficiency resulted in severe anemia leading to embryonic death [52]. It was shown that GATA-1 was cleaved by caspases 3, 7 and 8 after stimulation of immature erythroblasts with FasL, TRAIL or TNF and this observation proved that negative regulation of erythropoiesis is controlled by caspase-mediated GATA-1 cleavage after death receptors stimulation [7]. Our results support this mechanism as decreased expression of caspase 3 in THAL cultures resulted in increased expression of GATA-1. STAT5 is also well-known substrate for caspase 3 [53]. For this reason, decreased expression of caspase 3, observed in THAL cultures can lead to increased expression of the STAT5, which may explain not only upregulated phosphorylated form of STAT5 detected with flow cytometry, but also increase in total STAT5 shown in PCR. Thus, the stimulatory effect of THAL on erythropoiesis associated with increased expression of STAT5 may result from two processes: a reduced degradation due to the inhibition of caspase 3 and an increased sensitivity of EPOR to stimulation with EPO leading to enhanced phosphorylation and thereby enhanced expression of the active form of STAT5.

The mechanism of the stimulatory effect of immunomodulatory drugs on erythropoiesis has not been widely studied so far and there are quite a few reports on the subject. It was demonstrated that THAL caused increased colony formation from erythroid progenitors [54] and induced the expression of gamma-globin *via* increasing intracellular reactive oxygen species levels and acetylation of histone H4 and activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway [55]. Enhanced proliferation of immature erythroid cells and fetal hemoglobin synthesis was also observed after the application of lenalidomide and pomalidomide [56]. These results are partially consistent with increased generation of erythroid cells after treatment with THAL observed in our study, however the above mentioned studies aimed mostly to investigate the potential use of immunomodulatory drugs in hemoglobinopathies like sickle cell disease or thalassemia. In contrast, the detailed effects of thalidomide on erythropoiesis in myeloma patients has not been previously studied and to our knowledge this is the first report, which describes the changes in the expression of caspases, STAT5 and GATA-1 caused by THAL.

In conclusion, our results enable to further understand the mechanism of stimulating effect of THAL on erythropoiesis. Firstly, it seems to enhance a response of erythroid cells to ligation of EPO with its receptor, which results in increased expression of active phosphorylated form of STAT5. Secondly, THAL has inhibitory effect on external pathway of caspase activation resulting in decreased degradation of GATA-1 transcription factor by downstream caspases. Increased generation of erythroid cells is a result of described mechanisms.

Conflict of interest

The authors declare that there are no know conflict of interest associated with this publication that have influenced on its outcome.

Funding

The study was supported by a research grant from the Polish Ministry of Science and Higher Education, Republic of Poland, No. N N402 287236.

References

[1] De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, et al. Apoptotic role of Fas/Fas-ligand system in the regulation of erythropoiesis. Blood 1999;93:796–803.
[2] Zamai L, Secchiero P, Pierpaoli S, Bassini A, Papa S, Alnemri ES, et al. TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. Blood 2000;95:3716–24.
[3] Nicholson DW. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ 1999;6:1028–42.
[4] Stennicke HR, Salvesen GS. Catalytic properties of the caspases. Cell Death Differ 1999;6:1054–9.
[5] Smolewski P, Darzynkiewicz Z, Robak T. Caspase-mediated cell death in hematological malignancies: theoretical considerations, methods of assessment, and clinical implications. Leuk Lymphoma 2003;44:1089–104.
[6] De Maria R, Zeuner A, Eramo A, Domenichelli C, Bonci D, Grignani F, et al. Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. Nature 1999;401:489–93.
[7] Tilbrook PA, Klinken SP. The erythropoietin receptor. Int J Biochem Cell Biol 1999;31:1001–5.
[8] Ward A, Touw I, Yoshimura A. The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood 2000;95:19–29.
[9] Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA. STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 2002;99:95–101.
[10] Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promoteerythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. Blood 1996;88:1576–82.
[11] Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, et al. Erythropoietin can reduce the expression of Bcl-xl through Stat 5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 1999;274:22165–69.
[12] Tucci M, Grinello D, Cafforio P, Silvestris F, Dammacco F. Anemia in multiple myeloma: role of deregulated plasma cell apoptosis. Leuk Lymphoma 2002;43:1527–33.
[13] Silvestris F, Tucci M, Cafforio P, Dammacco F. Fas-L up-regulation by highly malignant myeloma plasma cells: role in the pathogenesis of anaemia and disease progression. Blood 2001;97:1155–64.
[14] Silvestris F, Cafforio P, Tucci M, Dammacco F. Negative regulation of erythroblast maturation by Fas-L+/TRAIL+ highly malignant plasma cells: a major pathogenic mechanism of anaemia in multiple myeloma. Blood 2002;99:1305–13.
[15] Grzasko N, Dmoszynska A, Krawczyk S, Hus M, Soroka-Wojtaszko M, Ciepłuch H, et al. Evaluation of blood morphology in patients with refractory multiple myeloma treated with thalidomide. Pol Arch Med Wewn 2001;106:573–9.
[16] Grzasko N, Dmoszynska A, Hus M, Soroka-Wojtaszko M. Stimulation of erythropoiesis by thalidomide in multiple myeloma patients: its influence on FasL, TRAIL and their receptors on erythroblasts. Haematologica 2006;91:386–9.
[17] Singhal S, Metha J, Desikan R, Ayers D, Roberson P, Eddlemon P, et al. Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 1999;341:1565–71.
[18] D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 1994;91:4082–5.
[19] Dmoszynska A, Bojarska-Junak A, Domanski D, Rolinski J, Hus M, Soroka-Wojtaszko M. Production of proangiogenic cytokines during thalidomide treatment of multiple myeloma. Leuk Lymphoma 2000;43:401–6.

[20] Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor α production by stimulated monocytes. J Exp Med 1991;173:699–703.
[21] Rowland TL, McHough SM, Deighton J, Dearman RJ, Ewan PW, Kimber I. Differential regulation by thalidomide and dexamethasone of cytokine expression in human peripheral blood mononuclear cells. Immunopharmacology 1998;400:11–20.
[22] McHough SM, Rifkin IR, Deighton J, Wilson AB, Lachmann PJ, Lockwood CM, et al. The immunosuppressive drug thalidomide induces T helper cell type 2 (Th2) and concomitantly inhibits Th1 cytokine production in mitogen- and antogen-stimulated human peripheral blood mononuclear cell cultures. Clin Exp Immunol 1995;99:160–7.
[23] Kneller A, Raanani P, Hardan I, Avigdor A, Levi I, Berkowicz M, et al. Therapy with thalidomide in refractory multiple myeloma patients – the revival of an old drug. Br J Haematol 2000;108:391–3.
[24] Juliusson G, Celsing F, Turesson I, Lenhoff S, Adriansson M, Malm C. Frequent good partial remissions from thalidomide including best response ever in patients with advanced refractory and relapsed myeloma. Br J Haematol 2000;109:89–96.
[25] Barlogie B, Desikan R, Eddlemon P, Spencer T, Zeldis J, Munshi N, et al. Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients. Blood 2001;98:492–4.
[26] Hus M, Dmoszynska A, Soroka-Wojtaszko M, Jawniak D, Legiec W, Ciepluch H, et al. Thalidomide treatment of resistant or relapsed multiple myeloma patients. Haematologica 2001;86:404–8.
[27] Weber D, Rankin K, Gavino M, Delasalle K, Alexanian R. Thalidomide alone or with dexamethasone for previously untreated multiple myeloma. J Clin Oncol 2003;21:16–9.
[28] Cavo M, Zamagni E, Tosi P, Cellini C, Cangini D, Tacchetti P, et al. First-line therapy with thalidomide and dexamethasone in preparation for autologous stem cell transplantation for multiple myeloma. Haematologica 2004;89:826–831.
[29] Zervas K, Dimopoulos MA, Hatzicharissi E, Anagnostopoulos A, Papaioannou M, Mitsouli Ch. et al. Primary treatment of multiple myeloma with thalidomide, vincristine, liposomal doxorubicin and dexamethasone (T-VAD doxil): a phase II multicenter study. Ann Oncol 2004;15:134–8.
[30] Palumbo A, Giaccone L, Bertola A, Pregno P, Bringhen S, Rus C, et al. Low-dose thalidomide plus dexamethasone is an effective salvage therapy for advanced myeloma. Haematologica 2001;86:399–403.
[31] Kakimoto T, Hattori Y, Okamoto S, Sato N, Kamata T, Yamaguchi M, et al. Thalidomide for the treatment of refractory multiple myeloma: association of plasma concentrations of thalidomide and angiogenic growth factors with clinical outcome. Jpn J Cancer Res 2002;93:1029–36.
[32] Gersuk GM, Beckham C, Loken MR, Kiener P, Anderson JE, Farrand A, et al. A role for tumour necrosis factor-alpha, Fas and Fas-Ligand in marrow failure associated with myelodysplastic syndrome. Br J Haematol 1998;103:176–88.
[33] Raza A, Meyer P, Dutt D, Zorat F, Lisak L, Nascimben F, et al. Thalidomide produces transfusion independence in long-standing refractory anemias of patients with myelodysplastic syndromes. Blood 2001;98:958–65.
[34] Piccaluga PP, Visani G, Pileri SA, Ascani S, Grafone T, Isidori A, et al. Clinical efficacy and antiangiogenic activity of thalidomide in myelofibrosis with myeloid metaplasia. A pilot study. Leukemia 2002;16:1609–14.
[35] Zappasodi P, Lorenzi A, Corso A. Thalidomide in refractory myeloma patients: early changes in bone marrow cellularity. Haematologica 2001;86:448.
[36] Corso A, Lorenzi A, Zappasodi P, Invernizzi R, Vanelli L, Lazzarino M. Early changes in bone marrow morphology induced by thalidomide in patients with refractory myeloma. Haematologica 2003;88:958–60.
[37] List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N Engl J Med 2006;355:1456–65.
[38] Ximeri M, Galanopoulos A, Klaus M, Parcharidou A, Giannikou K, Psyllaki M, et al. Effect of lenalidomide therapy on hematopoiesis of patients with myelodysplastic syndrome associated with chromosome 5q deletion. Haematologica 2010;95:406–14.
[39] Raza A, Reeves JA, Feldman EJ, Dewald GW, Bennett JM, Deeg HJ, et al. Phase 2 study of lenalidomide in transfusion-dependent, low-risk, and intermediate-1 risk myelodysplastic syndromes with karyotypes other than deletion 5q. Blood 2008;111:86–93.
[40] Sibon D, Cannas G, Baracco F, Prebet T, Vey N, Banos A, et al. Lenalidomide in lower-risk myelodysplastic syndromes with karyotypes other than deletion 5q and refractory toerythropoiesis-stimulating agents. Br J Haematol 2012;156:619–25.
[41] Begna KH, Mesa RA, Pardanani A, Hogan WJ, Litzow MR, McClure RF, et al. A phase-2 trial of low-dose pomalidomide in myelofibrosis. Leukemia 2011;25:301–4.
[42] Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 1999;98:181–91.
[43] Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in STAT5a−/−5b−/− mice due to decreased survival of early erythroblasts. Blood 2001;98:3261–73.
[44] Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX, et al. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol Cell Biol 2004;24:8037–47.
[45] Grebien F, Kerenyi MA, Kovacic B, Kolbe T, Becker V, Dolznig H, et al. STAT5 activation enables erythropoiesis in the absence of EpoR and JAK2. Blood 2008;111:4511–22.
[46] Dai C, Krantz SB. Interferon-(induces upregulation and activation of caspases 1, 3 and 8 to produce apoptosis in human erythroid progenitor cells. Blood 1999;93:3309–16.
[47] Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, et al. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 1997;16:2794–804.
[48] Bernardi P, Petronilli V, Di Lisa F, Forte M. A mitochondrial perspective on cell death. Trends Biochem Sci 2001;26:112–7.
[49] Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci 2001;26:61–6.
[50] Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 2001;349:257–60.
[51] Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc Natl Acad Sci USA 1995;92:9623–7.
[52] Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 1994;371:221–6.
[53] Pietschmann K, Bolck HA, Buchwald M, Spielberg S, Polzer H, Spiekermann K, et al. Breakdown of the FLT3-ITD/STAT5 axis and synergistic apoptosis induction by the histone deacetylase inhibitor panobinostat and FLT3-specific inhibitors. Mol Cancer Ther 2012;11:2373–83.
[54] Fard AD, Kaviani S, Noruzinia M, Soleimani M, Abroun S, Chegeni R, et al. Evaluation of H3 histone methylation and colony formation in erythroid progenitors treated with thalidomide and sodium butyrate. Lab Hematol 2013;19:1–5.
[55] Aerbajinai W, Zhu J, Gao Z, Chin K, Rodgers GP. Thalidomide induces gamma-globin gene expression through increased reactive oxygen species-mediated p38 MAPK signaling and histone H4 acetylation in adult erythropoiesis. Blood 2007;110:2864–71.
[56] Moutouh-de Parseval LA, Verhelle D, Glezer E, Jensen-Pergakes K, Ferguson GD, Corral LG, et al. Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells. J Clin Invest 2008;118:248–58.