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ARTICLE

Detection of TNFα Expression in the Bone Marrow and Determination of TNFα Production of Peripheral Blood Mononuclear Cells in Myelodysplastic Syndrome

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TNFα is a highly active cytokine which plays an important role in the regulation of apoptotic cell death, a mechanism involved in the pathophysiology of myelodysplastic syndrome (MDS). In this study we investigated the expression of TNFα of the bone marrow trephine biopsies by immunohistochemical method and the TNFα production of peripheral blood mononuclear cells by ELISA method in 15 patients affected by MDS. Five of seven patients without excess of blasts showed high or intermediate TNFα expression in the bone marrow biopsies, whereas two patients with excess of blasts were negative and one had low expression. The five CMML patients revealed low or intermediate expression. The production of TNFα by the PBMC was analysed in 10 patients, four patients with RA and two with CMML produced higher level of TNFα which increased after stimulation with phorbol myristic acetate, but none of the RAEB patients revealed increase in TNFα production. In conclusion we suppose that increased TNFα expression and production by PBMC may be an indirect evidence of the role of increased apoptosis in low risk MDS patients. (Pathology Oncology Research Vol 6, No 1, 18–23, 2000)

Keywords: Myelodysplastic syndrome, MDS, apoptosis, TNFα

Introduction

The myelodysplastic syndromes (MDS) are clonal hematopoietic disorders, characterized by the clinical symptomes of peripheral cytopenia despite of normo- or hypercellular bone marrow. The functional bone marrow failure is thought to be due to ineffective hematopoiesis, probably associated with increased premature intramedullary cell death, apoptosis.[19,20]

Normal hematopoietic proliferation and differentation are regulated by the complex interaction of stimulatory and inhibitory cytokines which are produced by the accessory cells of the bone marrow microenvironment and by the mononuclear blood cells – T lymphocytes, monocytes and macrophages. Stimulatory signals include hematopoietic growth factors (G-CSF, GM-CSF), interleukin-1α, (IL-α1), IL-3, IL-6, erythropoietin and others, whereas TNFα, TGFβ and interferons have inhibitory effects.[12,22]

The pathogenetic role of the abnormalities of these regulations in the myelodysplastic process has been suggested and widely examined in the last decade. The abnormalities in either hematopoietic growth factor (HGF) and inhibitory factor production or responsiveness to them seem to be an important pathogenetic factor in MDS.

The aim of our study was to investigate the role of TNFα in the hematopoietic failure of MDS patients. We studied the cytokine producing cells in the bone marrow by immunohistochemical method, and the ability of TNFα production of peripheral mononuclear cells by ELISA method without and after stimulation with a non specific stimulator of cytokine production.

Patients and methods

The study was carried out in 15 MDS patients. Controls for immunohistochemical studies were bone marrow biopsies with normal morphology from patients with non

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Hodgkin' lymphoma (n=6) and healthy volunteers for peripheral blood testing (n=4). According to FAB classification the MDS patients included six with refractory anemia (RA), one with refractory anemia with ringed sideroblasts (RARS), two with refractory anemia with excess of blasts (RAEB), one with RAEB in transformation (RAEB-t), and five with chronic myelomonocytic leukemia (CMML). No patients received specific therapy at the time of the study, and non of them suffered from infection. The patients' clinical characteristics, age, sex, date of diagnosis, hematological data at the time of the study and the duration of survival are shown in *Table 1.*

Immunohistochemical detection of TNFα in BM

The indirect immunoperoxidase technique was performed on deparaffinized bone marrow tissue sections. Endogenous peroxidase was blocked with 1mg/ml phenylhydrazine (Sigma) in phosphate buffered saline (PBS) for 20 min. After two washing steps (in PBS) the background was blocked with 5% BSA/PBS buffer for 30 minute, followed by the incubation with mouse anti-human TNFα monoclonal antibody (mAb) (Serotec Cat. no: MCA-1385) or isotype control mAb supernatant for 30 min. at room temperature. After three washing steps in PBS anti-mouse-Ig-HRPO was applied in appropriate dilution for further 30 min. The colour reaction was developed with aminoethylcarbasole (AEC) (2).

The following evaluation system was used to approximately quantitate the expression of TNFα (examined by two independent observers):

negative: absent or occasional staining

low: rare, scattered positive cells

Table 1. **Clinical data**

Patient No.	*Age/sex*	*FAB Dg*	*WBC G/l*	*HGB G/l*	*PLT G/l*	*Duration of survival (month)*
1	69/F	RA	2,63	122	218	28
2	68/F	RA	5,41	114	154	21
3	70/F	RA	2,06	106	51,9	56
4	72/F	RA	3,08	104	58	9
5	81/M	RA	2,4	81	147	12
6	41/M	RA	3,11	51,2	6,53	8 dead
7	56/F	RARS	3,3	77,3	228	60
8	84/M	RAEB	0,747	79,6	14,7	4
9	56/F	RAEB	0,757	85,7	125	11
10	71/M	RAEB-t	1,7	51,8	134	9 dead
11	75/M	CMML	5,15	90,5	42,1	10
12	75/M	CMML	7,14	113	101,2	11
13	61/M	CMML	11,8	112	12,7	32
14	62/M	CMML	5,79	131	70,4	4
15	72/M	CMML	21,2	95,7	47,6	20

Table 2. **BM TNFα expression and PBMC TNFα production**

Pat. No	*FAB Dg*	*BMP*	*BMC TNF alpha production*	
		TNF alpha immunohistochemistry	*without stimulation*	*after stimulation with PMA*
1	RA	intermediate	950	1220
2	RA	low	1350	1750
3	RA	high	460	145
4	RA	high	590	1420
5	RA	low	ND	ND
6	RA	high	ND	ND
7	RARS	intermediate	ND	ND
8	RAEB	negative	75	15
9	RAEB	low	60	65
10	RAEBt	negative	ND	ND
11	CMML	low	185	115
12	CMML	intermediate	410	490
13	CMML	intermediate	198	700
14	CMML	low	810	2150
15	CMML	low	ND	ND
control	n:4	negative	175	581

intermediate: small groups of positive cells, or more frequently scattered positive cells as in group low

high: large groups of positive cells, or high number of scattered cells.

TNFα ELISA

Stimulation of PBMCs – peripheral blood mononuclear cells (PBMC) were separated on FicollPaque gradient (Pharmacia) from fresh, heparinized blood samples. 10^6 cells/ml in 24 well tissue culture plates were stimulated under sterile conditions in DMEM 10% FCS containing 50 ng/ml phorbol myristatic acetate (PMA, Sigma, Cat. no:C-9275) for 24 hours in a CO_2 incubator. The control samples were incubated without PMA. The supernatant of the cells was used for immunoserology.

Sandwich ELISA (HyCult biotechnology b.v. HK307) – 100 µl/well supernatant of the stimulated and control samples and the positive control standards (1–8) were added to the precoated wells in duplicates and incubated for 1 hour at room temperature. After three washing steps 100 µl of diluted biotinilated secondary antibody was added to each well for 1 hour followed by the streptavidin-peroxidase conjugate also for 1 hour. After four washing steps freshly prepared TMB solution was added in substrate buffer to the wells and incubated for 30 min ot room temperature. After blocking the reaction the absorbance was measured at 450 nm in a Dynatech MR7000 reader. The detection limit was 20 pg/ml.

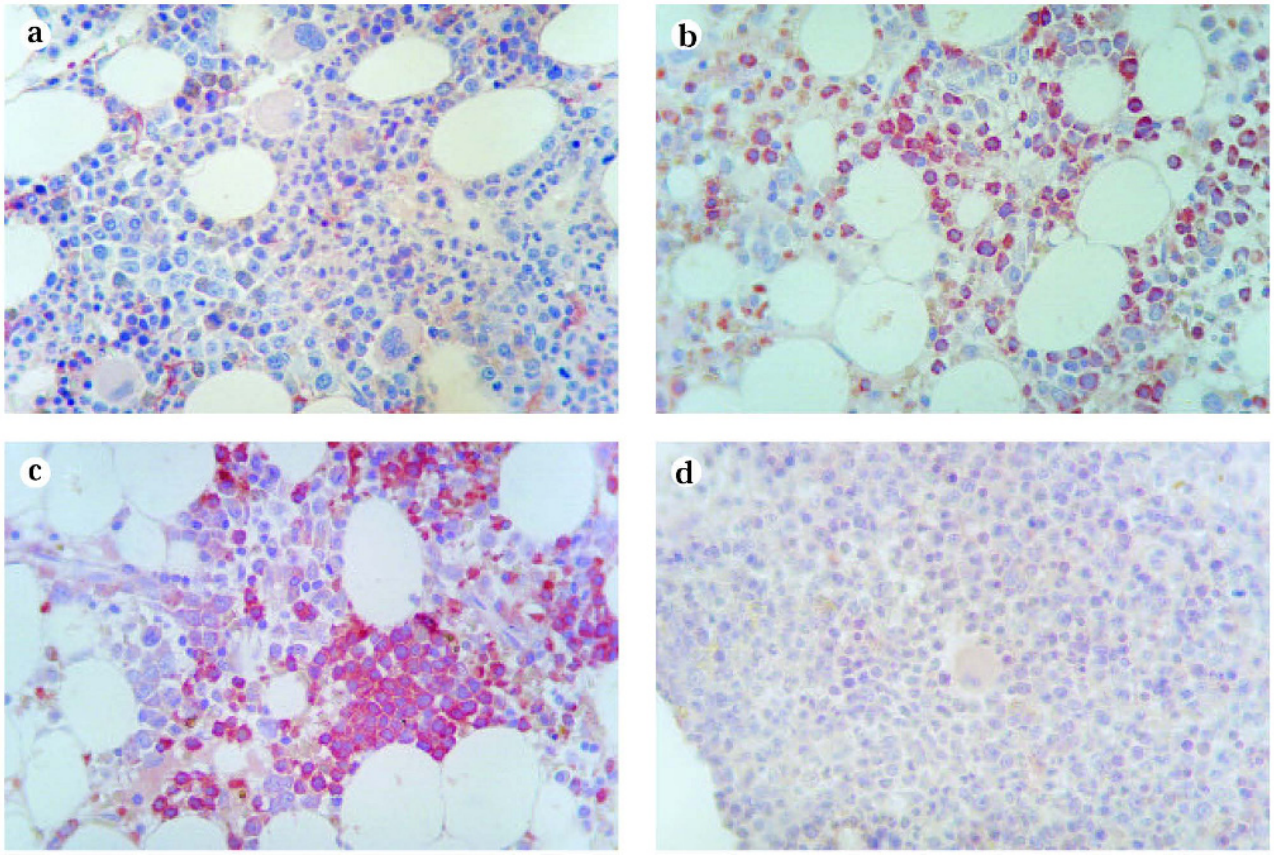

Figure 1. *TNFα immunohistochemistry of the BM sections (original magnification: 120X) A: Control bone marrow, normal morphology, negative TNFα expression. B: Patient 1, dg: RA: intermediate expression. C: Patient 3, dg: RA: high reactivity. D: Patient 8, dg RAEB: negative reaction.*

Results

Table 2 describes in detail the degree of the positivity of TNFα immunhistochemistry in the bone marrow and the amount of TNFα in the supernatant of PBMC without and after stimulation with PMA.

The morphologically normal bone marrow biopsies of control patients showed quite an absence of TNFα (*Figure 1A*). TNFα was expressed in varied amounts in the bone marrow biopsies of all patients without excess of blasts (six RA and one RARS), five showed intermediate or high expression, and two were low, whereas two patients with excess of blasts (one RAEB, one RAEB-t) were negative, and the third showed low reactivity. Three of the five CMML patients revealed low and the remaining two had intermediate TNFα expression (*Figure 1B-D*).

We measured the TNFα production of the peripheral mononuclear cells without and after stimulation with PMA, a polyclonal activator for stimulating cytokine producing cells, in the supernatants of PBMC. The basic TNFα production – incubation for 24 hours without a stimulator – was different in controls and in MDS subgroups. Elevated levels have been observed in four RA and two CMML patients, but in none of the patients with excess of blasts. After simulation we observed in controls, three out of four RA patients a moderate increase, but none of the RAEB patients cells revealed increase in TNFα production *(Figure 2).* In the case of patient 2 with RA and patient 14 with CMML we found an inverse relation between the frequency of intramedullary TNFα positive cells and the production of TNFα by PBMC. The BM biopsies revealed low activity, otherwise the PBMC produced spontaneousely and after stimulation high amount of TNFα. Patients 8 and 9 with RAEB had low TNFα activity both in the bone marrow and PBMC, the WBC counts were low, but the production of TNFα was measured by identical number of PBMC.

Discussion

Recently it has been hypothesized that in the pathophysiology of the myelodysplastic syndromes the increase of intramedullary apoptosis plays an important role. Increased rate of premature cell death was observed by some authors in the bone marrow of MDS patients which can explain the paradoxon between the functional bone

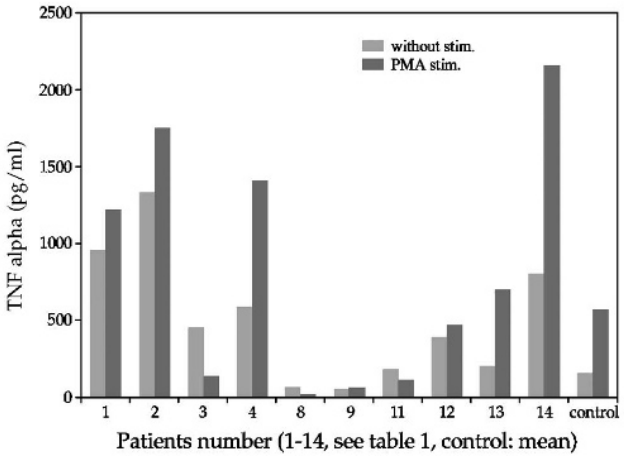

Figure 2. TNF alpha concentrations in the supernatants of PBMC from MDS patients

marrow failure and the cellular marrow. The first demonstration of massive apotosis was made by Raza et al in 1995, while other studies found that increased apoptosis predominates in RA and RARS, it was lower in RAEB and RAEBt and absent in AML.[4,16,19,20,21,29]

The pathomechanism of increased apoptosis in MDS is not clear. Several findings have been made, and the role of proapoptotic (c-myc) and antiapoptotic (bcl2) gene expressions, inhibitory – survival (HGF-s) – and stimulatory factors (TNFα, TGFβ) is supposed.[1,16,18,20,24,26,29] The correct examination of the degree of apoptosis has many difficulties which could explain the discrepancies between the results of different laboratories.[1,29]

TNFα is a highly active cytokine, it is released by monocytes, macrophages and lymphocytes, which suppresses in vitro colony formation.[3] TNFα regulates Fas antigen (CD95) expression. Fas mediated signals play a central role in apoptosis. The activation of Fas antigen by its ligand (Fas-L) results in the transduction of a signal for cell death.[11]

Gersuk et al in a recent study investigated TNFα and Fas mediated signals and their effect on hematopoiesis in patients with MDS and demonstrated up-regulation of TNFα, Fas and Fas-L. TNFα protein and sTNFα-R1 levels in the marrow plasma were higher, Fas expression was increased in CD34+ cells, Fas, Fas-L and TNFα mRNA were also elevated in MDS patients. In marrow cultures, after the blocking of Fas or TNF mediated signals by antibodies the number of colonies was increased.[6]

Recently elevated serum TNFα levels have been reported in MDS patients[1,13,17,23,25,27,30] which had prognostic significance. Verhoef et al. measured the serum concentration of different stimulatory and inhibitory cytokines in 75 patients with various manifestations of MDS and 25 healthy controls, and found elevated EPO and TNFα levels mainly in patients with transfusion dependency.[27] Alexandrakis et al. measured 4 cytokines in the sera of 82 MDS patients and concluded that elevated serum IL-6 and TNFα values were mainly seen in patients with high risk disease.[1]

The results of the TNFα measurements are summarised in *Table 3*, which represents the different and controversial observations. The predictive value of the cytokine determination in patients' sera is uncertain. It reveals only the actual situation which is influenced by many factors – underlying infections, other diseases, treatments, sample taking and storing.

The mechanism and the cellular source of increased TNFα effect is not clear. A possible explanation may be the autocrine production by normal monocytes or transformed MDS progenitor cells.[14,24] The data about the production of TNFα by BM macrophages are controversial. The TNFα producing cells are mainly BM macrophages as Kitagawa et al demonstrated by double immunostaining. These authors investigated the expression of TNFα and IFNγ – the other inhibitory cytokine – mRNA by PCR and observed an enhanced expression of the TNFα mRNA in 79% and of IFNγ mRNA in 42% of MDS patients, whereas no enhancement was found in control, AML and CML cases. There was no difference between MDS subgroups.[8] Visani et al demonstrated the impairment of GM-CSF production but not the TNFα and IL-6 production of LPS stimulated BM macrophages.[28] Ohmori et al investigated the stimulatory activity of MDS derived BM macrophages on the in vitro growth of normal and MDS progenitor cells (CFU-GM). The MDS macrophage derived conditioning media suppressed the colony growth of normal progenitors, which was suppressed by antiTNFα antibody.[15]

The TNFα production of the peripheral mononuclear cells in MDS has scarcely been investigated.[14] Koike et al studied spontaneous IL-6, IL-1β and TNFα production by

***Table 3.* Summary of TNFα measurements in sera of MDS patients**

Author	*Ref.*	*MDS patients*	*TNF alpha (pg/ml)*
Zoumbos	30	n=42	297+/–65
		Group A (RA/RARS)	306+/–77
		Group B (others)	290+/–101
Verhoef	27	n=75	
		transfusion dep.	22,4+/–3,1
		transfusion indep.	9,1+/–0,7
		transf. indep. Hb: 10 g/l	18,8+/–2,9
Seipelt	23	n=15	14,2 (highest in 2 cases of RAEB)
Stasi	25	n=25	80,5 (EPO responders: lower)
Musto	13	n=93	37+/–8 (higher in CMML)
Peddie	17	n=15	Between 4–14,5

PBMC obtained from MDS and aplastic anemia patients, and found higher level of these cytokines in AA and RA but not in patients with excess blasts.[9] These findings are in contrast with the results of the more elevated cytokine levels in the sera of high risk MDS, whereas they are in agreement with our results.[1,23,27] Koike et al observed the change of cytokine production in a case during transformation from AA to RAEB which was markedly decreased. Their conclusions, – that the cytokine production is a secondary, reactive response in hypocellulary BM – do not agre agree with some of the most recent studies which suppose the role of apoptosis in the pathogenesis of MDS.[9]

In the present study we investigated the presence of TNFα producing cells in the bone marrow and the production of TNFα by peripheral mononuclear cells. We have found high basic TNFα levels in the majority of patients with RA and in one CMML patient and the production of TNFα enhanced after stimulation. Bone marrow immunohistochemistry has shown intensive reaction in patients with early MDS and less intensive in CMML cases. However, we have found an inverse relation between the frequency of intramedullary TNFα producing cells and the ability of TNFα production of PBMC in two cases. Patients 2 and 14 showed high basic and stimulated TNFα levels and low immunoreactivity in the BM. The decrease of TNFα producing cells in the bone marrow may be an early sign of disease progression. The patients with an advanced disease (RAEB, RAEB-t and progressive CMML) revealed low basic levels, no response to stimulation and mainly negative BM immunohistochemistry. Our results provide a further indirect evidence about the role of increased apoptosis in the pathogenesis of MDS first of all in patients without excess of blasts.

TNFα promotes apoptosis via intracellular oxygen free radical production and oxidation of DNA and proteins. Peddie et al using single gel electrophoresis identified oxidized pyrimidine nucleotides in CD34+ cells from MDS patients which were associated with elevated plasma TNFα levels. The therapeutic effect of antioxidant amifostine perhaps involves this mechanism.[17]

The modulation of TNFα production may be a promising therapeutic possiblity. Either amifostine or pentoxifylline modulating TNFα production via inhibition of TNFα mRNA transcription with corticosteroids seems to be an effective combination.[5,7,10]

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