International Journal of
HEMATOLOGY

Biological Significance of Proliferation, Apoptosis, Cytokines, and Monocyte/Macrophage Cells in Bone Marrow Biopsies of 145 Patients With Myelodysplastic Syndrome

Krishnan Allampallam, Vilasini Shetty, Suneel Mundle, Diya Dutt, Howard Kravitz, Poluru L. Reddy, Sairah Alvi, Naomi Galili, Gurveen S. Saberwal, Shalini Anthwal, Maliha W. Shaikh, Aaron York, Azra Raza

Rush Cancer Institute, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois, USA

Received October 23, 2001; received in revised form November 12, 2001; accepted November 16, 2001

Abstract

Labeling index (LI), apoptosis, levels of 2 pro-apoptotic cytokines tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), and the number of monocyte/macrophage cells that are the likely source of the cytokines were simultaneously measured in plastic-embedded bone marrow (BM) biopsy sections of 145 patients with myelodysplastic syndromes (MDS). TNF-α was correlated with TGF-β (P = .001) and with monocyte/macrophage cells (P = .003). Patients with excess blasts in their marrows had a higher TGF-β level (P = .01) and monocyte/macrophage number (P = .05). In a linear regression model, TGF-β emerged as the most significant biological difference between patients who have excess of blasts and those who do not (P = .01). We conclude that in addition to TNF-α, TGF-β also plays a significant role in the initiation and pathogenesis of MDS, and that a more precise definition of its role will likely identify better preventive and therapeutic strategies. *Int J Hematol.* 2002;75:289-297.

Key words: Myelodysplastic syndromes; Cytokines; Labeling index; Apoptosis; Tumor necrosis factor-α; Transforming growth factor-β; Monocyte/macrophage cells

1. Introduction

The human bone marrow (BM) is an extremely dynamic compartment, constituting approximately 2.5% of total body weight, with 25% to 75% being the hematopoietic cells and the rest (yellow or fatty marrow) forming a reserve capable of mobilization during stress conditions [1,2]. In addition to blood cells, the marrow produces a number of cytokines responsible for regulating and coordinating the complex functions of its many components. Myelodysplastic syndromes (MDS), comprising a group of clonal hematopoietic diseases that present with the apparent incongruity of variable cytopenias despite a generally cellular BM [3,4], are marked by excessive intramedullary apoptotic death of hematopoietic cells belonging to all 3 lineages [5,6].

A substantial body of evidence has also been compiled now that demonstrates an increase in a variety of proinflammatory cytokines in the majority of MDS patients. This list includes 3 prominent members, namely, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and transforming growth factor-β (TGF-β) [7-12]. In addition, increases in the levels of IL-6 and macrophage colony-stimulating factor (M-CSF) have been documented [13,14]. The precise mechanism involving cytokine-induced apoptosis in the hematopoietic cells remains unknown, although signaling via the Fas/Fas ligand pathway, attenuation of Fap-1, and

Correspondence and reprint requests: Azra Raza, MD, Rush Cancer Institute, Rush-Presbyterian-St. Luke's Medical Center, 2242 West Harrison Street, Suite 108, Chicago, IL 60612;312-455-8474;fax: 312-455-8479 (e-mail: araza@rush.edu).

K.A. and V.S. have contributed equally to the manuscript.

activation of the caspase network have all been reported [15-17]. An association between increased apoptosis and proinflammatory cytokines has been best established by the demonstration that in vivo neutralization of TNF-α activity is accompanied by improvement in cytopenias in some MDS patients [18-20]. It would, therefore, be important to define the pretherapy cytokine profile and incidence of apoptosis in MDS marrows and to determine whether these measurements can be used to identify subgroups of patients with unique prognostic or biological characteristics.

In the present study, BM biopsy specimens of 145 MDS patients belonging to all morphologic categories were examined for the presence of 2 pro-apoptotic cytokines, TNF-α and TGF-β. The biopsy specimens were further correlated with the level of apoptosis, proliferation, monocyte/macrophage number, and clinical parameters, such as the level of cytopenias, percentage blasts in the BM aspirate, and BM biopsy specimen cellularity. Results show that TNF-α and TGF-β, monocyte/macrophage number, and apoptosis tend to be proportionately increased in MDS patients. In a multivariate logistic regression model controlling for the effects of TNF-α, apoptosis, and number of monocytes/macrophages, TGF-β remained a biological parameter associated with disease severity, being significantly higher in patients who presented with an excess of blasts. This study not only confirms the role of TNF-α and TGF-β in mediating the excessive apoptosis in hematopoietic cells of MDS patients, but also suggests an exceedingly important part played by TGF-β in the pathogenesis and progression of this disease.

2. Materials and Methods

A total of 145 MDS patients are the subjects of this report. Every individual received a 1-hour infusion of iododeoxyuridine (IUdR) and/or bromodeoxyuridine (BrdU) at 100 mg/m^2 intravenously on protocol MDS 90-02 for the purpose of in vivo cell cycle analysis. Informed consent for the infusion protocol was obtained from every patient. The protocol was reviewed and approved by the Institutional Review Board of the Rush-Presbyterian-St Luke's Medical Center, the National Cancer Institute (NCI), and the US Food and Drug Administration. The IUdR and BrdU for these studies were supplied by the NCI. Immediately upon completion of the infusion, peripheral blood, BM aspirate, and BM biopsy specimens were obtained from the patient and sent for routine studies including pathology, cytogenetics, and surface markers, while part of each sample was also transported on ice to A.R.'s laboratory for biological studies. The following studies were performed on these tissues. All the data in the study were acquired in samples obtained from patients prior to starting therapy.

2.1. Detection of Cytokines in the Microenvironment

Semiquantitative immunohistochemical methods were used to determine the levels of 2 cytokines, TNF-α and TGF-β, in the BM biopsy specimens, as described previously [8].

2.2. Detection of Monocyte/Macrophage Number

The presence of monocytes/macrophages was detected immunohistochemically by the method described previously [8]. The monoclonal antibody used was CD68 (Dakopatts, Glostrup, Denmark).

2.3. Detection of S-Phase Cells

The in situ detection of the 2 thymidine analogs, IUdR and BrdU, administered via intravenous infusions, was carried out by using monoclonal antibodies and immunohistochemical methods described previously [5,6]. After processing and mounting the cover slips with fluoromount, at least 2000 positively labeled S-phase myeloid cells were counted to determine the labeling index (LI). Erythroid and megakaryocytic cells were excluded.

2.4. Measurement of Apoptosis Using In Situ End Labeling of Fragmented DNA

Apoptotic cells were detected using the in situ end labeling (ISEL) technique on plastic-embedded BM biopsy sections of MDS patients as described in earlier studies [5,6].

2.5. Interpretation of Slides

A subjective quantitative scale was formulated to determine the degree of positivity of the different cytokines (TGF-β, TNF-α), ISEL staining and the cellular component (macrophages) as follows: negative, low, intermediate, and high. Low was defined as less than one third of the biopsy specimen positive; intermediate, one third to two thirds of the specimen positive; and high, greater than two thirds of the specimen positive.

2.6. Statistical Analysis

The nonparametric Spearman test was used for comparison between 2 noncontinuous variables, and Kruskal-Wallis 1-way analysis of variance was used to compare different variables. One-tail *P*-values are reported throughout the paper. Logistic regression was used to examine the relationship between the 4 significantly related biological measurements, including apoptosis, TNF-α, TGF-β, and number of macrophages. Scoring of all 4 biological parameters was based on the intensity of staining, ranging from 0 to 8. These measurements were then categorized as no staining or low, intermediate, or high degree of staining. Disease severity was dichotomized as group I (refractory anemia + refractory anemia with ringed sideroblasts) and group II (refractory anemia with excess blasts ± transformation). The results are presented as the odds of a patient with a higher staining characteristic having more severe MDS compared with the odds of a patient with no staining. Of 145 patients studied, 112 had complete data for inclusion in this analysis.

Table 1.
Clinical and Biological Characteristics of Patients in the Study*

	Clinical					Biological				
	Hb, g/dL	WBC, /μL	Platelets, 1000/μL	BM Asp Blasts, %	BM BX Cellularity, %	LI, %	ISEL	TNF-α	TGF-β	CD68
Overall										
n	145	143	143	133	141	104	121	131	145	136
Median	9.3	3.4	81	2	70	25	1	3	3	4
FAB										
RA										
n	74	74	74	68	72	52	63	69	74	69
Median	9.6	3.45	87.5	1	60	25.81	1	2	2.5	2
RARS										
n	21	21	21	21	21	14	18	18	21	20
Median	9.2	4.15	191	1	80	27.85	1	2	3	5
RAEB										
n	36	34	34	31	34	28	31	33	36	35
Median	9.05	2.91	55.5	7	80	24.4	1	4	3	5
RAEB-t										
n	8	8	8	8	8	6	4	5	8	7
Median	9.15	1.365	31.5	18	70	21.5	1.5	5	3	5
CMMoL										
n	6	6	6	5	6	4	5	6	6	5
Median	9.6	7.84	55	5	80	19.55	0	0.5	3.5	4
P-values	.532	.012	.004	<.001	.014	.04	.878	.722	.143	.013

*Hb indicates hemoglobin; WBC, white blood cells; BM Asp blasts, bone marrow aspirate blasts; BM Bx Cellularity, bone marrow biopsy specimen cellularity; LI, labeling index; ISEL, in situ end labeling (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); CD68, macrophage marker (1+ to 8+); n, number of patients; FAB, French-American-British classification; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEB-t, RAEB in transformation; CMMoL, chronic myelomonocytic leukemia.

3. Results

Of the 145 MDS patients included in this study, there were 102 men and 43 women, with a median age of 68 years. According to the French-American-British (FAB) classification [21], 74 patients had refractory anemia (RA), 21 patients had RA with ringed sideroblasts (RARS), 36 patients had RA with excess blasts (RAEB), 8 patients had RAEB in transformation (RAEB-t), and 6 patients had chronic myelomonocytic leukemia (CMMoL). The International Prognostic Scoring System (IPSS) was also computed for 126 patients based on published criteria [22]. Fifty-four patients belonged to the low-risk group, 41 patients to intermediate 1 (Int 1), 20 patients to intermediate 2 (Int 2), and 11 patients to the high-risk group. Median values for clinical parameters including hemoglobin, white blood cell count, platelet count, BM aspirate blasts, and BM biopsy specimen cellularity are presented for all MDS patients as well as by FAB category in Table 1.

3.1. Cell Proliferation and Apoptosis

The median LI for all patients was 25% (n = 104). The median LI by FAB groups is presented in Table 1. As previously reported by ourselves and others, the LI tends to decrease with increasing disease severity such that patients with RAEB-t have a lower LI (21.5%) than do those with RA (25.8%) or RARS (27.8%). CMMoL patients have the lowest LI (19.5%, Table 1). These differences in LI between the various FAB groups were statistically significant (n = 104, $P = .04$). With respect to apoptosis, the overall median for the entire group was low or 1+ (n = 121, Table 1), and the median for ISEL in RAEB-t patients was slightly higher than in the other FAB groups (Table 1). It must be noted that the blasts in RAEB and RAEB-t patients were rarely found to be apoptotic; rather, apoptosis appeared confined to maturing cells. Apoptosis was not detected in any of the 5 CMMoL patients. Based on the intensity of staining, the ISEL-positive patient samples could be divided into 3 groups. Thirty-five samples stained with low intensity, 16 stained with an intermediate intensity, and 15 stained with high intensity (Table 2). Fifty-five MDS patients (46%) in this study did not have a detectable level of apoptosis. at least by the ISEL technique, in their BM biopsy specimens.

3.2. Cytokine Levels and Macrophage Number in BM Biopsy Specimens

TNF-α levels were evaluated in 131 MDS patients, and 40 (31%) of them had no positive staining (Table 2). Low-intensity TNF-α staining was seen in the BM biopsy specimens of 35 patients, whereas 19 patients showed intermediate staining and 37 patients showed high-intensity staining. The median TNF-α level in the CMMoL group was lowest among the 5 FAB groups (Table 1). Most of the TNF-α was found in interstitial areas of the biopsy specimens, although slightly higher amounts were also seen in monocytic/macrophage and megakaryocytic cells. TGF-β levels were evaluated in 145 MDS patients (Tables 1 and 2), and 37 (26%) patients showed no staining for this cytokine in their biopsy specimens. Forty-nine patients showed low TGF-β staining, 34 patients showed intermediate staining, and 25 patients

Table 2.
Pattern of Relative Staining in BM Biopsy Specimens*

	ISEL		TNF-α		TGF-β		CD68	
	n	%	n	%	n	%	n	%
Negative	55	46	40	31	37	26	31	23
Low	35	29	35	27	49	34	32	24
Intermediate	16	13	19	15	34	24	38	28
High	15	12	37	29	25	17	35	26

*BM indicates bone marrow; ISEL, in situ end labeling (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); CD68, macrophage marker (1+ to 8+); n, number of patients.

showed high-intensity staining. Neither TNF-α nor TGF-β was detected in BM biopsy specimens of 16 MDS patients. The number of macrophages was available in 136 MDS patients. Overall, the median CD68 staining intensity was intermediate in BM biopsy specimens of MDS patients (Table 1). The median CD68 number was statistically significant among the different FAB categories (n = 136, $P = .013$). Thirty-one (23%) patients had no detectable cells belonging to the monocyte/macrophage lineage (Table 2).

3.3. Relationship Between TGF-β, TNF-α, Monocyte/Macrophage Number, LI and Apoptosis

The nonparametric Spearman test was used to determine the relationship between the clinical and biological parameters studied, and a summary of the statistically significant findings is provided in Table 3. LI was not related to any of the other biological measurements, but there was an inverse relationship between LI and number of blasts in the BM aspirate ($P = .048$). Degree of apoptosis showed a direct relationship to the number of monocytes/macrophages ($P = .048$, Table 3) and was inversely related to the BM biopsy specimen cellularity ($P = .041$, Table 3). The 2 pro-apoptotic cytokines TNF-α and TGF-β were directly correlated with each other ($r = 0.311$, $P < .001$). In addition, TNF-α was also directly related to the number of macrophages ($P = .003$) and to the percentage of BM aspirate blasts ($P = .028$). TGF-β was also directly related to the number of macrophages ($r = 0.181$, $P = .018$) and BM aspirate blasts ($r = 0.198$, $P = .011$). The number of macrophages in the BM biopsy specimens positively correlated with TNF-α levels ($P = .003$) and apoptosis ($P = .048$). The BM biopsy cellularity inversely correlated with apoptosis ($r = –0.161$, $P = .041$) and positively correlated with the percentage of blasts in the BM aspirates ($r = 0.251$, $P = .002$).

3.4. Clinical and Biological Characteristics Versus IPSS and FAB Groups

Table 4 shows the biological measurements versus the IPSS classification. The higher-risk groups had lower LIs ($P = .011$, Table 4). Table 1 shows the relationship of various clin-

Table 3.
Overall Correlation Between Biological/Clinical Parameters*

Parameter 1	Parameter 2	n	*r*	*P*
LI	BM Asp blasts†	98	–0.169	.048
ISEL	CD68	118	0.154	.048
	BM Bx cellularity†	117	–0.161	.041
TNF-α	TGF-β	131	0.311	<.001
	CD68	128	0.246	.003
	BM Asp blasts	121	0.174	.028
TGF-β	TNF-α	131	0.311	<.001
	CD68	136	0.181	.018
	BM Asp blasts	133	0.198	.011
CD68	ISEL	118	0.154	.048
	TNF-α	128	0.246	.003
	TGF-β	136	0.181	.018
	BM Bx Cellularity	132	0.158	.035
BM Asp blasts	TNF-α	121	0.174	.028
	TGF-β	133	0.198	.011
	BM Bx Cellularity	129	0.251	.002
	LI†	98	–0.169	.048
BM Bx Cellularity	ISEL†	117	–0.161	.041
	CD68	132	0.158	.035
	BM Asp blasts	129	0.251	.002

*n indicates number of patients; LI, labeling index; BM Asp blasts, bone marrow aspirate blasts; ISEL, in situ end labeling (1+ to 8+); CD68, macrophage marker (1+ to 8+);BM Bx Cellularity, Bone marrow biopsy specimen cellularity; TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+).

†Inverse relationship.

Table 4.
Correlation Between IPSS Score and Biological Parameters*

Parameter	n	*r*	*P*
LI†	96	–0.234	.011
ISEL†	108	–0.088	.183
TNF-α	117	0.032	.366
TGF-β	130	0.11	.107
CD68	123	0.071	.217
BM Asp blasts	129	0.632	<.001
BM Bx cellularity	126	0.203	.011

*IPSS indicates International Prognostic Scoring System; LI, labeling index; ISEL, in situ end labeling (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); CD68, macrophage marker (1+ to 8+);BM Asp blasts, bone marrow aspirate blast; BM Bx cellularity, bone marrow biopsy specimen cellularity.

†Inverse relationship.

ical and biological parameters to FAB categories. Although the median BM biopsy specimen cellularity for the 141 MDS patients was 70%, a significant difference was seen in the cellularity between the FAB groups ($P = .033$), being lowest for RA patients (median = 60%, Table 1). There were also several important biological differences between the various FAB categories. The LI was significantly lower for the RAEB-t patients compared to that of the other groups, and CMMoL patients had no evidence of apoptosis in their biopsy specimens. The significant *P*-values are provided in Table 1.

Patients were also grouped according to whether they had <5% blasts in the marrow (group I) or had excess blasts (group II), and their clinical and biological parameters are presented in Table 5. Group I had 95 patients with RA + RARS, whereas group II had 42 RAEB + RAEB-t patients. CMMoL patients were excluded from this analysis. A comparison of the relative staining for ISEL, TNF-α, TGF-β, and $CD68^+$ cells is shown in Figure 1. Group I had a median LI of 26%, whereas group II had a median LI of 24% (Table 5). No differences were seen in the groups as far as apoptosis was concerned. Samples from group II patients stained with a higher intensity for TNF-α than did those from group I patients (4+ versus 2+, Table 5), but this was not statistically significant. As far as TGF-β is concerned, groups I and II both had a median staining intensity of 3 (Table 5), but the mean was statistically significantly different ($P = .01$). Statistically significant correlations between the different biological characteristics within each group of patients are presented in Table 6. In group I (RA + RARS) patients, significant positive correlations were seen between LI and CD68 staining ($P = .027$), between TNF-α and CD68 ($P = .006$), between TNF-α and TGF-β ($P = .001$), and between TGF-β and macrophage number ($P = .023$). In group II patients, inverse relationships were found between LI and BM aspirate blasts ($P = .013$), between apoptosis and BM biopsy specimen cellularity ($P = .009$), and between apoptosis and TGF-β ($P = .048$). An interesting observation is that in the earlier stages of the disease, TGF-β had no relationship to apoptosis (Table 6, group I), but in the later stages TGF-β was inversely related to apoptosis ($P = .048$) (Table 6, group II). This suggests a more complicated role for TGF-β in the pathogenesis of MDS than merely serving as a pro-apoptotic cytokine.

3.5. Logistic Regression Model

Logistic regression was used to examine the relationship between apoptosis, TNF-α, TGF-β, and macrophage number in 112 MDS patients in whom all measurements were simultaneously available. First, each of the 4 parameters was examined in a univariate analysis. Only TGF-β staining intensity was found to be significantly associated with disease severity (group I versus group II) as indicated by an increase in blast number (Wald $X^2 = 10.21$, $df = 3$, $P = .02$). Table 7 presents the multivariate regression results. When controlling for the effects of TNF-α, apoptosis, and macrophage number, TGF-β remained the only biological parameter significantly associated with disease severity (Wald $X^2 = 10.53$, $df = 3$, $P = .01$). Also, the estimated odds of a patient with low or high TGF-β staining having RAEB or RAEB-t was higher than the estimated odds of a patient with no TGF-β staining (Table 7).

4. Discussion

The purpose of this study was to identify subsets of MDS patients recognizable on the basis of distinct biological characteristics within the known morphologic (FAB) and prog-

Table 5.
Clinical and Biological Characteristics in the 2 Groups of Patients*

	Clinical					Biological				
	Hb, g/dL	WBC, /μL	Platelets, 1000/μL	BM BX Cellularity, %	BM Asp Blasts, %	LI, %	ISEL	TNF-α	TGF-β	CD68
Group I										
n	95	95	95	93	89	66	81	87	95	89
Median	9.5	3.6	113	70	1	26.4	1	2	3	3
Group II										
n	42	42	42	42	39	34	35	38	44	42
Median	9.05	2.62	50	80	8	24.25	1	4	3	5
P-values	.109	.006	.002	.018	<.001	.224	.752	.167	.01	.05

* Hb indicates hemoglobin; WBC, white blood cells; BM Bx Cellularity, bone marrow biopsy specimen cellularity; BM Asp Blasts, bone marrow aspirate blasts; LI, labeling index; ISEL, in situ end labeling (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); CD68, macrophage marker (1+ to 8+); n, number of patients. Group I includes RA + RARS patients. Group II includes RAEB + RAEB-t patients.

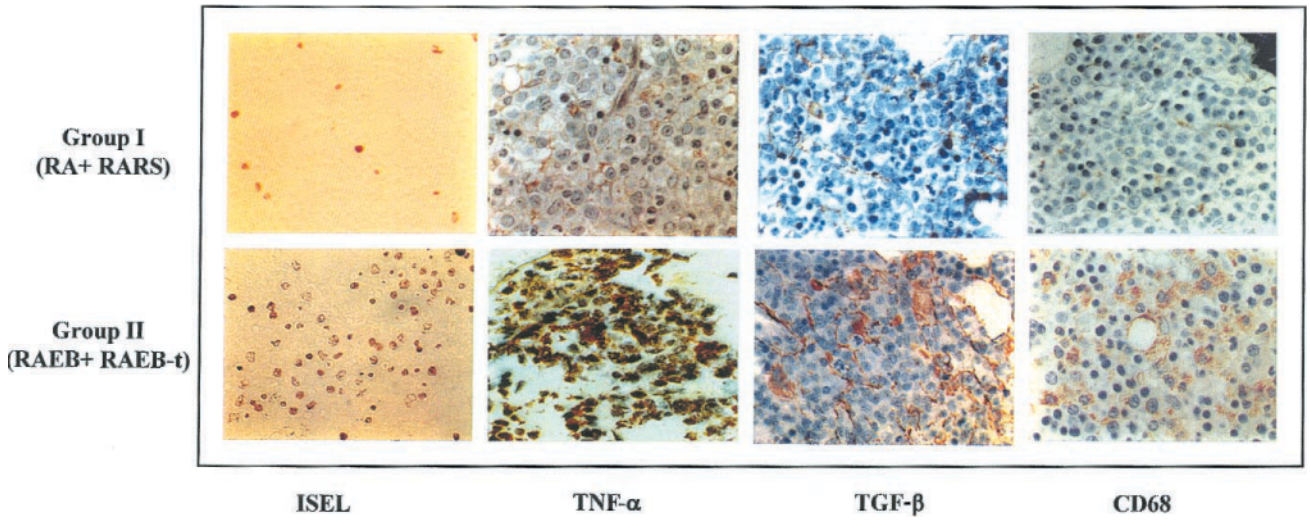

Figure 1. Bone marrow biopsy specimens from myelodysplastic syndrome (MDS) patients stained for in situ end labeling (ISEL), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and CD68 in group I and group II MDS patients. A significant difference was seen in staining intensity of TGF-β and in the number of monocyte/macrophage cells. RA indicates refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEB-t, RAEB in transformation.

nostic (IPSS) categories. The following important observations have been made in this study of 145 MDS patients:

• Overall, the rate of proliferation, as measured by labeling S-phase cells in BM biopsy specimens in vivo, slows significantly as the disease progresses from a stage with a normal percentage of BM blasts to one with excessive blasts.

Table 6.
Correlation Between Biological Parameters in Group I and Group II Patients

Parameter 1	Parameter 2	n	*r*	*P*
Group I				
LI	CD68	66	0.239	.027
TNF-α	CD68	85	0.271	.006
	TGF-β	87	0.329	.001
TGF-β	CD68	89	0.212	.023
	TNF-α	87	0.329	.001
CD68	LI	66	0.239	.027
	TNF-α	85	0.271	.006
	TGF-β	87	0.329	.001
Group II				
LI	BM Asp blasts†	32	–0.394	.013
ISEL	BM Bx cellularity†	33	–0.408	.009
	TGF-β†	35	–0.287	.048
TNF-α	CD68	38	0.301	.033
TGF-β	ISEL†	35	–0.287	.048
CD68	TNF-α	38	0.301	.033
BM Asp blasts	LI†	32	–0.394	.013
BM Bx cellularity	ISEL†	33	–0.408	.009

*n indicates number of patients; LI, labeling index; CD68, macrophage marker (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); BM Asp blasts, bone marrow aspirate blasts; ISEL, in situ end labeling (1+ to 8+); BM Bx cellularity, bone marrow biopsy specimen cellularity.

†Inverse relationship.

• Degree of apoptosis appears to be directly related to the number of macrophages and inversely to the cellularity of the BM biopsy specimen.

• TNF-α and TGF-β levels were highly correlated with each other and with the number of macrophages.

• BM specimen cellularity was directly related to the number of monocyte/macrophage cells and inversely related to the degree of apoptosis.

• BM aspirate blast percentage was directly related to both TNF-α and TGF-β and inversely related to LI.

• Both TGF-β and monocyte/macrophage number were statistically higher in patients with excess blasts as opposed to those with RA/RARS.

• LI decreased significantly with increasing IPSS risk categories, being lowest for the high-risk groups.

• In patients with RA/RARS, there was a strong relationship between TNF-α, TGF-β, and monocyte/macrophage number, but this relationship was completely lost in patients who presented with an excess of blasts, suggesting a loss of paracrine influence in the latter patients.

• The only significant biological correlations in patients with excess blasts related to an inverse relationship between LI and BM aspirate blasts and an inverse relationship between apoptosis and BM specimen cellularity.

• In a linear regression model, controlling for the effects of TNF-α, apoptosis, and monocyte/macrophage number, TGF-β emerged as the most significant biological parameter, which distinguishes patients who present with RA/RARS from those with an excess of blasts.

LI has always been found to be considerably higher in BM biopsy specimens compared to matched BM aspirates not only of patients with MDS [23] but also of those with acute myelogenous leukemia [24]. Because there are no S-phase cells in the peripheral blood, it was presumed that the higher

Table 7.
Logistic Regression Results*

Variable	Degree of Staining	Coefficient	SE	Wald χ^2	*df*	*P*	OR	95% CI
ISEL				0.62	3	.89		
TNF-α				2.69	3	.44		
TGF-β				10.53	3	.01		
	Low	1.62	0.67				5.07	1.36-18.9
	Intermediate	0.12	0.78				1.12	0.24-5.21
	High	1.74	0.79				5.7	1.21-26.78
CD68				3.15	3	.37		

*SE indicates standard error; OR, odds ratio; CI, confidence interval; ISEL, in situ end labeling (1+ to 8+); TNF-α, tumor necrosis factor-α (1+ to 8+); TGF-β, transforming growth factor-β (1+ to 8+); CD68, macrophage marker (1+ to 8+).

LI in biopsy specimens could be explained on the basis of hemodilution of the aspirate sample by peripheral blood. However, even following corrections for this technical error, biopsy-specimen LI remained almost 3 times higher in the majority of samples, suggesting that this difference is real and reflects a true increase in proliferative activity of hematopoietic cells in the BM biopsy compartment. The same has been true for apoptosis rates, which were found to be strikingly higher in the biopsy specimens of MDS patients, whereas matched BM aspirate mononuclear cells showed almost no apoptotic cells [25-31]. We employed several methods for detecting putative but masked apoptotic cells in MDS aspirates. First, by examining the BM aspirate mononuclear cells following short-term cultures in complete medium for 2 to 4 hours, we found distinct DNA laddering patterns on agarose gels [5]. Second, by carefully examining both high- and low-density fractions of BM aspirate cells separated on a density gradient, we found a higher apoptotic index in the maturing high-density compartment, which had previously been missed in studies conducted solely on the low-density fractions [32]. Finally, we used pulse field gel electrophoresis to detect high-molecular-weight DNA fragments in BM aspirate cells of those MDS patients who showed no evidence of apoptosis by conventional ISEL techniques or DNA laddering, and we demonstrated the presence of apoptosis in the majority of these cases [33]. Once again, however, the apoptotic index of the BM biopsy specimen continues to be disproportionately higher compared to that of the aspirates, despite short-term in vitro cultures of the latter as well as inclusion of high-molecular-weight DNA fragments and high-density apoptotic cells. The most logical explanation for this remarkable increase in both proliferation and death of hematopoietic cells in this compartment is that the concentration of cells and proteins, which control these activities, is highest in the biopsy specimens.

Several additional observations were made regarding proliferative activity in MDS marrows. S-phase cells are often simultaneously apoptotic in these patients and may account for the ineffective hematopoiesis. Percentage of S-phase cells consistently dropped as MDS progressed toward acquisition of excess blasts. Within both FAB and IPSS categories, there was an inverse relationship between LI and disease severity. Apoptosis, on the other hand, was more consistently related to the number of monocyte/macrophage cells in the vicinity and was highest in hypocellular marrows. Patients with excess blasts showed more apoptosis in maturing cells, but almost none in the immature blasts. These observations again indicate the importance of stromal cells such as monocytes/macrophages as the primary source of proliferative and pro-apoptotic signals controlling the activities of hematopoietic cells. Not surprisingly, therefore, a strong relationship was found between both cytokines TNF-α and TGF-β and the number of monocytes/macrophages in the biopsy specimens.

The important observation that percentage of blasts in BM aspirates was directly related to both TNF-α and TGF-β suggests several possible biological effects of these cytokines. These cytokines may be driving the proliferation of transformed blasts. Alternatively, the blasts may themselves be immune to their effects but may be responsible for enhanced cytokine production via stimulation of stromal cells. This effect would suppress the proliferation of normal hematopoietic cells, which are still sensitive to the inhibitory effects of TNF-α and TGF-β. Excessive production of TGF-β by BM stromal cells can have 2 serious adverse effects. First, it causes a failure of early hematopoietic stem cells as shown in human chronic idiopathic neutropenia [34]. Second, by stimulating human type VII collagen gene, TGF-β promotes collagen synthesis in fibroblasts and its deposition in the marrow, leading to progressive myelofibrosis [35]. Another observation that supports the excessive production of cytokines by stromal cells under the influence of transformed MDS cells is that TNF-α, TGF-β, and monocyte/macrophage cells are highly interrelated in patients without excess blasts but lose this relationship in those who present with excess blasts. This observation suggests a more autonomous activity of blasts, which can nonetheless affect normal hematopoietic cells still responsive to paracrine signals. A final possibility is that the cytokines exert a dual effect in that they stimulate proliferation of dividing cells and induce apoptosis in maturing ones. In vivo suppression of pro-apoptotic cytokines using TNF blocking agents and the resulting improvement in cytopenias of some MDS patients lend support to this possibility [18-20]. In a linear regression model, TGF-β was found to be the most significant biological difference between patients with and without excess of blasts, being considerably higher in the former.

An equally important observation is the absence of detectable apoptosis (46%), TNF-α (31%), TGF-β (26%), and monocyte/macrophage cells (23%) in a substantial number of MDS patients studied (Table 2). Two explanations among several possibilities are either that the assays used were not sensitive enough to detect the desired property or

that the genesis of myelodysplasia in these individuals is mechanistically different. It is possible that these biological differences among patients will distinguish between individuals who will respond to anticytokine therapies (patients with evidence of cytokine-mediated apoptosis) versus those who will not (patients without detectable apoptosis, TNF-α, or TGF-β). In the past, we have examined MDS patients who presented with very high versus no apoptosis in their marrows and have shown that the former group had a higher LI, higher TNF-α level, and more monocyte/macrophage cells in their biopsy specimens than the latter [36]. Correlations of these biological parameters with the natural history of the disease, total survival, and response to therapy need to be undertaken in the future to understand the clinical significance of these biological findings.

In summary, therefore, we report on the highly complex relationship between the proliferation and apoptotic activities of hematopoietic cells and their regulation by monocyte/macrophage cells belonging to the marrow stromal compartment via the actions of 2 important cytokines, TNF-α and TGF-β, in a large number of MDS patients. Significant associations have been established between the various biological parameters in this preliminary study and have identified areas of future research in MDS. Along with continued emphasis on TNF-α–mediated apoptosis of hematopoietic cells in MDS, a better definition of the precise role of TGF-β in initiation and perpetuation of this disease is warranted and will likely identify novel preventive and therapeutic strategies.

Acknowledgments

This work was supported by a grant from the National Cancer Institute (PO1CA 75606), The Markey Charitable Trust (94-8), and the Dr. Roy Ringo Grant for basic research in MDS. The authors wish to thank Ms. Sandra L. Howery and Ms. Lakshmi Venugopal for excellent secretarial and administrative assistance.

REFERENCES

1. Iverson PO. Blood flow to the hematopoietic bone marrow. *Acta Physiol Scand.* 1997;159:269-276.
2. Wickramasinghe SN. *Blood and Bone Marrow*. 3rd ed. London, England: Churchill Livingstone; 1986.
3. San Miguel JF, Sanz GF, Vellaspi T, Canizo MC, Sanz MA. Myelodysplastic syndromes. *Crit Rev Oncol Hematol.* 1996;23: 57-93.
4. Resegotti L. The nature and natural history of myelodysplasia. *Haematologica.* 1993;5:191-204.
5. Raza A, Gezer S, Mundle S, Gao XZ, et al. Apoptosis in bone marrow biopsy samples involving stromal and hematopoietic cells in 50 patients with myelodysplastic syndromes. *Blood.* 1995; 86:268-276.
6. Raza A, Gregory SA, Preisler HD. The myelodysplastic syndromes in 1996: complex stem cell disorders confounded by dual actions of cytokines. *Leuk Res.* 1996;20:881-890.
7. Molnar L, Berki T, Hussain A, et al. Detection of TNF alpha expression in the bone marrow and determination of TNF alpha production of peripheral blood mononuclear cells in myelodysplastic syndrome. *Pathol Oncol Res.* 2000;6:18-23.
8. Shetty V, Mundle S, Alvi S, et al. Measurement of apoptosis, proliferation and three cytokines in 46 patients with myelodysplastic syndromes. *Leuk Res.* 1996;20:891-900.
9. Mundle SD, Venugopal P, Pandav DV, et al. Indication of an involvement of interleukin-1β converting enzyme (ICE)-like protease in intramedullary apoptotic cell death in the bone marrows of patients with myelodysplastic syndromes (MDS). *Blood.* 1996;88: 2640-2647.
10. Allampallam K, Shetty V, Hussaini S, et al. Measurement of mRNA expression for a variety of cytokines and its receptors in bone marrows of patients with myelodysplastic syndromes. *Anticancer Res.* 1999;19:5323-5328.
11. Peddie CM, Wolf CR, McLellan LI, Collins AR, Bowen DT. Oxidative DNA damage in $CD34^-$ myelodysplastic cells is associated with intracellular redox changes and elevated plasma tumour necrosis factor-α concentration. *Br J Haematol.* 1997;99:625-631.
12. Zoumbos N, Symeonidis A, Kourakli A, et al. Increased levels of soluble interleukin-2 receptors and tumor necrosis factor in serum of patients with myelodysplastic syndromes. *Blood.* 1991;77: 413-414.
13. Herold M, Schmalzl F, Zwierzina H. Increased serum interleukin 6 levels in patients with myelodysplastic syndromes. *Leuk Res.* 1992; 16:585-588.
14. Mareni C, Sessarego M, Montera M, et al. Expression and genomic configuration of GM-CSF, IL-3 M-CSF receptor (C-FMS), early growth response gene-1 (EGR-1) and M-CSF genes in primary myelodysplastic syndromes. *Leuk Lymphoma.* 1994;15:135-141.
15. Gersuk GM, Beckham C, Loken MR, et al. A role for tumour necrosis factor-α, FAS and FAS-Ligand in marrow failure associated with myelodysplastic syndrome. *Br J Haematol.* 1998;103: 176-188.
16. Kitagawa M, Yamaguchi S, Takahashi M, Tanizawa T, Hirokawa K, Kamiyama R. Localization of Fas and Fas ligand in bone marrow cells demonstrating myelodysplasia. *Leukemia.* 1998;12:486-492.
17. Mundle S, Mativi BY, Bagai K, et al. Spontaneous down-regulation of Fas-associated phosphatase-1 (Fap-1) may contribute to the excessive apoptosis in myelodysplastic marrows. *Int J Hematol.* 1999;70:83-90.
18. Raza A, Venugopal P, Gezer S, et al. Pilot study of pentoxifylline and Ciprofloxacin with or without dexamethasone produces encouraging results in myelodysplastic syndromes. In: Hiddemann W, Buchner T, Wormann B, Ritter J, Creutzig U, Keating M, Plunkett W, eds. *Acute Leukemias VII: Experimental Approaches and Novel Therapies*. New York, NY: Springer-Verlag; 1998:42-51.
19. Raza A, Qawi H, Andric T, et al. Pentoxifylline, Ciprofloxacin and dexamethasone improve the ineffective hematopoiesis in myelodysplastic syndrome patients. *Hematology.* 2000;5:275-284.
20. Raza A. Anti-TNF therapies in rheumatoid arthritis, Crohn's disease, sepsis, and myelodysplastic syndromes. *Microsc Res Tech.* 2000;50:229-235.
21. Bennett JM, Catovsky D, Flandrin DMT, Galton DGAD, Gralnick HR, Sultan C. Proposal for the classification of myelodysplastic syndromes. *Br J Haematol.* 1982;51:189-199.
22. Greenberg PL, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood.* 1997;89:2079-2088.
23. Raza A, Alvi S, Broady-Robinson L, et al. Cell cycle kinetic studies in 68 patients with myelodysplastic syndromes following intravenous iodo- and/or bromodeoxyuride. *Exp Hematol.* 1997;25: 530-535.
24. Raza A, Yousuf N, Bokhari SAJ, et al. Cell cycle characteristics: alterable determinants of remission duration in a study of 179 standard risk newly diagnosed patients with acute myeloid leukemia. *Int J Oncol.* 1993;2:301-307.
25. Raza A, Mundle S, Iftikhar A, et al. Simultaneous assessment of cell kinetics and programmed cell death in bone marrow biopsies of myelodysplastics reveals extensive apoptosis as the probable basis for ineffective hematopoiesis. *Am J Hematol.* 1995;48: 143-154.
26. Raza A, Mundle S, Shetty V, et al. A paradigm shift in myelodysplastic syndromes. *Leukemia.* 1996;10:1648-1652.
27. Rajapaksa R, Ginzton N, Rott LS, Greenberg PL. Altered onco-

protein expression and apoptosis in myelodysplastic syndrome marrow cells. *Blood.* 1996;88:4275-4287.
28. Parcharidou A, Raza A, Economopoulos T, et al. Extensive apoptosis of bone marrow cells as evaluated by the in-situ end labeling (ISEL) technique may be the basis for ineffective hematopoiesis in patients with myelodysplastic syndromes. *Eur J Haematol.* 1999;62: 19-26.
29. Shimazaki K, Ohshima K, Suzumiya J, Kawasaki C, Kijuchi M. Evaluation of apoptosis as a prognostic factor in myelodysplastic syndromes. *Br J Hematol.* 2000;110:584-590.
30. Van de Loosdrecht AA, Vellenga E. Myelodysplasia and apoptosis: new insights into ineffective erythropoiesis. *Med Oncol.* 2000;17: 16-21.
31. Parker JE, Mufti GJ, Rasool F, Mijovic A, Devereux S, Pagliuca A. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood.* 2000;96:3932-3938.
32. Shetty V, Hussaini S, Broady-Robinson L, Allampallam K, et al. Intramedullary apoptosis of hematopoietic cells in myelodysplastic syndrome patients can be massive: apoptotic cells recovered from high density fraction of bone marrow aspirates. *Blood.* 2000;96: 1388-1392.
33. Alvi S, Borok R, Showel M, et al. Formation of large molecular weight DNA fragments may be a committed step of apoptosis in myelodysplastic syndromes vs acute myeloid leukemia [abstract]. *Proc Am Assoc Cancer Res.* 1996;37:Abstract 185.
34. Papadaki HA, Giouremou K, Eliopoulos GD. Low frequency of myeloid progenitor cells in chronic idiopathic neutropenia of adults may be related to increased production of TGF-β by bone marrow stromal cells. *Eur J Haematol.* 1999;63:154-162.
35. Vindevoghel L, Lechleider RJ, Kon A, et al. SMADk3/4–dependent transcriptional activation of the human type VII collagen gene (COL7A1) promoter by transforming growth factor β. *Proc Natl Acad Sci U S A.* 1998;95:14769-14774.
36. Dar S, Mundle S, Andric T, et al. Biological characteristics of myelodysplastic syndrome patients who demonstrated high versus no intramedullary apoptosis. *Eur J Haematol.* 1999;62:90-94.