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Biologic characteristics of patients with hypocellular myelodysplastic syndromes

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Abstract

Rates of proliferation and apoptosis as well as expression of tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and the number of macrophages were measured in bone marrow (BM) biopsies of 33 patients who presented with hypocellular (cellularity < 30%) myelodysplastic syndromes (MDS). Results showed that 2/3 of the patients had high apoptosis, high cytokine levels and large number of macrophages in their biopsies while 1/3 did not. Apoptosis and TNF-α levels were directly related ($r = 0.583$, $P = 0.003$, $n = 24$) as was apoptosis and the degree of anemia ($P = 0.033$, $n = 18$). A subgroup of patients with abnormalities of chromosomes 5 or 7 had higher platelets ($P = 0.026$) and higher apoptosis ($P = 0.038$) when compared with the rest of the group. Eight patients had no evidence of apoptosis and almost no detectable TNF-α in their biopsies. We conclude that within the hypocellular variant of MDS, there may be two distinct sub-groups of patients, one who present with high cytokine-mediated intramedullary apoptosis and the other who may be better characterized as having a stem-cell failure defect since they showed no evidence of apoptosis.

Keywords: Hypocellular myelodysplastic syndromes; Apoptosis; Proliferation; TNF-α; TGF-β; Chromosomes 5 and/or 7

1. Introduction

The myelodysplastic syndromes (MDS) are a group of hematopoietic disorders that primarily originate in a pluripotential bone marrow (BM) stem cell and tend to predominate in the elderly [1]. The vast majority of patients present with a refractory anemia that may or may not be associated with additional cytopenias and supportive care continues to be the mainstay of therapy for these variable cytopenias [2]. Several recent advances have been achieved in understanding the biological basis for the paucity of circulating blood cells, the most significant being the recognition of excessive intramedullary apoptotic death of hematopoietic cells belonging to all three hematopoietic lineages [3–6]. This peculiar mode of suicidal cellular destruction appears to be cytokine mediated in a substantive majority of patients with tumor necrosis factor alpha (TNF-α) being the prominent pro-apoptotic cytokine involved [7–9]. In summary then, the biological hall-marks of MDS are rapid intramedullary proliferation of hematopoietic cells followed by an equally rapid and premature apoptotic death of the vast majority of these cells in the presence of increased levels of pro-inflammatory cytokines such as TNF-α. It is important to note that

Abbreviations: MDS, myelodysplastic syndromes; ISEL, in situ end labeling of fragmented DNA; IudR, lododeoxyuridine; BrdU, bromodeoxyuridine; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; LI, labeling index; BM, bone marrow.

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these biological characteristics may not be present universally in all MDS patients mainly because there are five different syndromes grouped under one umbrella. Each syndrome presents with its own unique natural history and prognosis and each has its own spectrum of clinical heterogeneity. Thus it is important to identify various subgroups within MDS and determine whether these patients have archetypal biologic characteristics which would distinguish them from other MDS patients, thereby allowing hematologists to design therapeutic strategies tailored to suit their individual needs. The present study is one such attempt.

While the general rule in MDS is that the bone marrow is hypercellular despite the presence of variable cytopenias, 10–30% cases present with a hypocellular BM [1,2]. In the absence of a recognizable karyotypic abnormality, such cases are often difficult to distinguish from classic aplastic anemias, although preponderance of hypocellularity in the therapy-related myelodysplasias and its subsequent association with pathognomic cytogenetic abnormalities are often helpful in negotiating such semantic matters in a small subsets of patients. In the present study, we have attempted to identify biologic characteristics which would be uniquely associated with hypocellular MDS cases. The most striking features of this study were related to findings of high intramedullary apoptosis of hematopoietic cells in association with high pro-apoptotic cytokines and presence of macrophages in approximately 2/3 of the cases studied, thereby suggesting a possible explanation for the hypocellularity at least in a sub-set of these patients. Hemoglobin was inversely related to the level of apoptosis in this group with patients showing high apoptosis being more anemic. Finally, the subset of patients with hypocellular MDS who presented with abnormalities of chromosomes 5 and/or 7 tended to have a higher platelet count than the rest of the group as well as a higher level of apoptosis.

2. Materials and methods

Thirty-three MDS patients are the subject of this report. These MDS patients were studied prior to starting a treatment protocol, but had been off all therapies including growth factors and vitamins for at least 4 weeks. Every individual received a 1 h infusion of IUdR (or in case of an iodine allergy, BrdU) at 100 mg/M^2 intravenously prior to undergoing a bone marrow (BM) examination. Informed consent for the infusion protocol (MDS 90-02) was obtained from every individual. The infusion protocol was reviewed and approved by the Institutional Review Board (IRB) of the Rush-Presbyterian-St. Luke's Medical Center, the National Cancer Institute (NCI) and the Food and Drug Administration (FDA). The IUdR and BrdU for these studies were supplied by the NCI. Immediately upon completion of the infusion, peripheral blood, BM aspirate and BM biopsies were obtained from the patient and transported on ice to Dr Raza's laboratory. The following studies were performed on the tissues.

2.1. Detection of cytokines in the microenvironment

Levels of two cytokines TNF-α, and transforming growth factor beta (TGF-β) were determined semi-quantitatively in the bone marrow biopsies immunohistochemically. All tissues were fixed in Bouin's solution and embedded in glycol methacrylate. Two to three micron thick sections were obtained and placed on alcian blue coated coverslips. The sections were then individually labelled for each cytokine using the respective monoclonal antibodies as follows. After the tissues were dehydrated in distilled water for 10 min they were incubated with freshly diluted 3% H_2O_2 for 30 min and then with pronase 1 mg/ml (Calbiochem, LaJolla, CA) for 45 min. Specimens were rinsed carefully with 0.15 M phosphate buffered saline (PBS) [0.15 M sodium chloride in 0.1 M phosphate buffer, pH 7.5] after each incubation. Following the last 0.15 M PBS rinse they were placed in 0.5 M PBS [0.5 M sodium chloride in 0.1 M phosphate buffer, pH 7.5] for 15 min. The sections were treated with 0.5 M PBS containing 1.5% horse serum for 60 min to block non-specificity. Subsequently the sections were incubated with the respective monoclonal anti TGF-$\beta_2\beta_3$ (1:50) antibody (Oncogene Science, Manhasset, NY) or anti TNF-α (1:180) (Promega, Madison, WI) antibody diluted in 0.5M PBS containing 1.5% horse serum for 60 min. This was followed by incubating the sections with biotinylated anti-mouse IgG [diluted (1:200) in 0.5M PBS with 1.5% horse serum] for 30 min and with the avidin-biotin complex or ABC reagent. The horse serum, biotinylated anti-IgG and ABC complex were reagents in the Vectastain Elite ABC kit (Vector, Burlingham, CA). After each of the above incubations, specimens were rinsed in 0.5 M PBS. The color reaction was then developed using 0.025%, 3,3′ diamino benzidine tetrachloride (DAB) diluted in 100 ml of 0.5 M Tris buffer, pH 7.5, with 0.01 ml 30% H_2O_2 for 10 min and rinsed with distilled water. After processing, the coverslips were mounted on glass slides and examined by light microscopy.

2.2. Detection of macrophages

The presence of macrophages was detected immunohistochemically by the method described above. The monoclonal antibody used was EBM-11 (Dakopatts, Denmark)

2.3. Detection of S-phase cells

The in-situ detection of the two thymidine analogues IUdR and BrdU administered via intravenous infusions was carried out by using the protocols and immunohistochemical methods described before [5,6]. After processing and mounting the coverslips 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 (ISEL) of fragmented DNA

ISEL was carried out on all the bone marrow biopsies of the 33 patients as described in earlier studies [5,6]. Briefly, the sections following pre-treatment with sodium chloride sodium citrate (SSC) solution at 80°C and with 1% Pronase (1 mg/ml in 0.15 M PBS; Calbiochem, LaJolla, CA) were incubated with a mixture of dATP, dCTP, dGTP (0.01M, Promega, Madison, WI), bio-dUTP (0.001M, Sigma) and DNA Polymerase I (20 U/ml, Promega) at 18°C. Incorporation of bio-dUTP was finally visualized using avidin-biotin-peroxidase conjugate (Vectastain Elite ABC Kit, Vector, Burlingham, CA) and diamino benzidine tetrachloride. Thus cells labeled positively for ISEL showed brown staining in their nuclei under the light microscope.

2.5. Interpretation of slides

All the slides were observed on a televised screen by several investigators. 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. The intensity of staining/cell was not recorded. The percentage of cells were not calculated. Data on reproducibility of the results have been provided in the past [5,8].

Low	1–3+
Intermediate	4–5+
High	6–8+

2.6. Statistical analysis

The nonparametric Spearman test was used to determine correlations between groups

3. Results

A total of 33 MDS patients are the subject of this report. According to the French–American–British (FAB) classification [10], 22 patients had refractory anemia (RA), one had RA with ringed sideroblasts (RARS), seven had RA with excess blasts (RAEB), two had RAEB in transformation (RAEB–t) and one had chronic myelomonocytic leukemia (CMMoL). There were 20 males and 13 females with a median age of 60 years for the entire group. All patients had primary de-novo MDS.

3.1. Biologic characteristics

The cellularity was assessed from BM biopsies and ranged between 5–30% (median = 20%). Table 1 shows the details of all the clinical/biological parameters measured. The median white blood cell (WBC) count at presentation was 3.0×10^9/l and the median hemoglobin was 9.4 Gm/dl (Table 1). The median labeling index (LI) for the entire goup was 22% ($n = 16$, range = 11–37%), median ISEL was 2 ($n = 26$, range = 0–8), median TNF-α level assessed in the BM biopsy was 3 ($n = 29$, range = 0–8), median TGF-β was 4 ($n = 28$, range = 0–8) and the number of macrophages as measured by EBM-11 antibody were a median of 2 ($n = 30$, range = 0–8).

3.2. Apoptosis and cytokine studies

Among the 33 hypocellular MDS cases, ISEL studies were available in 26 cases and 8/26 cases did not have any evidence of ISEL positivity in the entire biopsy. Ten of the 18 patients who had evidence of apoptosis showed greater than 4^+ positivity. A highly significant relationship was found between ISEL positivity and TNF-α levels ($r = 0.583$, $P = 0.003$, $n = 24$). Futhermore, in patients who were ISEL-positive versus those that were ISEL-negative, there was a significant difference in the level of TNF-α ($P = 0.005$). The median TNF-α level was 0 for the ISEL-negative patients ($n = 7$) and the median TNF-α was four for the ISEL positive cases ($n = 17$). Finally, level of apoptosis was also inversely related to the level of hemoglobin in these patients ($r = -0.505$, $P = 0.033$, $n = 18$).

TNFα levels were available in a total of 29/33 patients with a median value of 3^+ (Table 1). Nine of these 29 patients showed no detectable TNF-α at the protein level in the BM biopsies. A significant difference in the biological characteristics of patients with any detectable TNFα levels was noted in comparison with patients who were entirely negative. The most significant difference was related to the level of apoptosis as already mentioned, TNF levels being high when ISEL was high ($n = 18$, $P = 0.013$) and low when ISEL

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ical/biological characteristics of patients with hypocellular myelodysplastic syndromes

Sex	Age	FAB	Cellularity	BM-Bx-blasts*	BM-Asp-blasts	WBC	HGB	Platelet	LI	ISEL	TNF-α	TGF-β	EBM-11
M	55	RA	20	3	0	2.1	13.1	143	Ne	4	8	2	6
M	55	RA	20	5	0	2.4	9.6	34	11	0	0	3	0
M	50	RA	20	5	0	3.9	7.7	31	Ne	8	8	4	0
M	62	RA	Hypo	5	5	5.5	9.0	131	Ne	Na	Ne	Ne	Ne
M	70	RA	10-20	5	5	4.5	8.5	18	Ne	Na	3	Na	4
M	59	RA	Hypo	5	5	1.6	9.2	225	26	8	4	1	1
M	69	RA	20	5	5	3.0	10.7	397	Ne	Na	5	7	6
M	75	RA	20	5	5	1.9	9.5	20	Ne	2	2	4	0
M	58	RA	Hypo	5	3	4.0	10.9	54	27	1	2	5	3
M	59	RA	10-20	5	1	4.4	11.2	237	Na	4	1	1	1
F	77	RA	5	5	5	3.9	9.1	131	Ne	0	0	Ne	0
F	53	RA	30	1	2	3.7	8.9	856	Na	4	8	8	2
F	49	RA	10	5	0	5.4	9.2	292	21	2	3	4	4
F	76	RA	Hypo	5	0	1.1	10.1	14	Ne	0	0	0	Ne
F	77	RAEB-t	Hypo	25	5	2.4	9.9	224	18	8	4	2	0
M	46	RAEB	10	16	31	1.7	13.3	225	28	0	1	1	4
F	79	RAEB	20	8	1	5.0	10.7	309	37	0	0	5	5
F	47	CMMoL	10	10	22	0.9	7.4	10	24	8	8	4	6
M	58	RA	10	5	0	4.2	10.4	10	11	0	0	3	0
M	66	RA	5	2	2	1.0	7.9	33	19	Na	0	0	0
M	81	RA	Hypo	5	0	3.3	9.0	197	14	8	8	2	7
M	83	RA	10	5	5	5.9	9.7	72	23	1	6	3	0
F	55	RA	20	5	0	3.1	12.6	225	24	1	3	4	3
F	53	RA	30	5	2	2.4	7.9	10	Ne	0	Na	Ne	0
M	67	RAEB	10-20	35	6	1.6	9.4	32	Na	2	Ne	0	4
M	50	RAEB	10-20	25	5	2.9	12.8	193	Ne	1	5	8	0
M	66	RAEB	20	9	5	8.6	8.1	628	29	0	7	0	4
M	57	RAEB	20	5	10	1.7	7.8	12	12	2	3	7	0
F	16	RAEB	Hypo	5	5	0.8	9.0	33	11	5	2	3	3
M	60	RAEB-t	20	33	30	1.1	10.0	33	Na	5	0	0	0
F	74	RA	20	0	0	4.4	9.1	77	Na	Na	Na	Na	Na
F	72	RA	5	1	1	2.4	9.2	39	Na	Na	0	Ne	0
F	77	RARS	20	5	5	4.2	10.5	280	Na	Na	0	1	3

BM-Bx-blasts, % blasts in bone marrow biopsy; Ne, not evaluable; BM-Asp-blasts, % blasts in bone marrow aspirate; LI, labeling index (% S-phase cells); WBC, cells $\times 10^9$/l; TNF-α: tumor osis factor alpha; Hgb, hemoglobin in gm/dl; TGF-β, transforming growth factor beta; Platelets, platelets $\times 10^9$/l; EBM-11, surface antigen for macrophage; Na, not available for evaluation.

Table 2
Biologic correlates of TNF-α in patients with hypocellular myelodysplastic syndromes

	TNF-negative		TNF-positive		*P*
	Median	*n*	Median	*n*	
ISEL*	0	6	3	18	0.013
TGF-β*	1	7	4	19	0.06
EBM-11*	0	8	3	20	0.06

* ISEL, in situ end labeling of fragmented DNA; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; EBM-11, surface antigen for macrophage 11.

was negative ($P = 0.013$, $n = 6$). TGF-β was similarly high (median = 4, $n = 19$) in the TNF-positive group and low (median = 1, $n = 7$) in the TNF-negative group ($P = 0.06$). Macrophages were quite prominent in the high TNF-positive/TGF-β-positive group (median = 3, $n = 20$) and low in the TNF-negative/TGF-β negative group (median = 0, $n = 8$). These relationships were not statistically significant ($P = 0.06$). The summary of the data is shown in Table 2.

3.3. Cytogenetic studies

Karyotypes were determined in all 33 patients. Thirteen patients had normal chromosomes and 20 had an abnormal karyotype. Among these 20 patients, 13 had abnormalities of chromosomes 5 ($n = 9$) and 7 ($n = 4$), one had del (20)(q11 q13) and three had trisomy 8. The remaining three patients had variable anomalies listed in Table 3 (del 16, + 15, del 9, del 15, + 21 and marker chromosome). There were no statistically significant differences between individuals who had normal cytogenetics versus those who had any abnormal metaphases. However, when the subset of hypocellular MDS cases who presented with abnormalities of chromosomes 5 or 7 were compared to all other patients (normal as well as any other karyotypic abnormality), two statistically significant differences were noted.

Firstly, the platelet count was higher for patients with 5 or 7 abnormalities (median 247×10^9/l, $n = 13$ vs. 101×10^9/l, $n = 20$, $P = 0.026$) as shown in Table 4. Secondly, this subgroup of patients also had a statistically higher level of apoptosis in their bone marrow when compared to normal plus any chromosomal abnormality except that of 5 or 7 group (median ISEL = 4.0, $n = 10$ vs. median 1.0, $n = 16$, respectively, $P = 0.038$). These data are shown in Table 4.

4. Discussion

Hypocellular myelodysplastic syndromes have been recognized as a distinct variant of MDS however, no differences in the natural history, prognosis or response to treatment have been appreciated between hypocellular versus normo/hypercellular MDS [11–15]. The main source of confusion continues to be the difficulty of discriminating hypocellular MDS from aplastic anemia patients, in the absence of chromosomal abnormalities, however recent suggestions to treat these hypocellular MDS patients with immunosuppressive aplastic anemia-like therapies have resulted in modest successes thereby obviating the need to categorically make this distinction in every case [16]. The presentation of an identical clinical syndrome in the setting of such starkly contrasting bone marrow cellularity as seen in hypo versus hypercellular MDS however, poses an interesting biological challenge. The present study was therefore undertaken to determine whether the recently recognized biological characteristics commonly associated with hypercellular MDS are also a consistent feature in patients who present with the hypocellular variant. What we found in this study of 33 hypocellular MDS cases was in fact quite similar to what has already been described for the more frequently encountered hypercellular variety. In summary, 2/3 of these patients demonstrated the presence of intramedullary apoptosis accompanied by higher levels of TNF-α, TGF-β and macrophages when compared with 1/3 of the group that did not have apoptosis. Profundity of anemia was related to high apoptosis and patients with abnormalities of chromosomes 5 or 7 appeared to have more apoptotic cells in their marrows compared to patients who had a normal karyotype and cytogenetic abnormalities affecting chromosomes other than 5 and/or 7.

The paradox of variable cytopenias despite hypercellular BMs in MDS in general has been explained on the basis of excessive intramedullarly cytokine-induced death of hematopoietic cells [5–7,17]. Obviously, this cannot apply universally to all MDS patients since at least 1/3–1/2 of MDS cases do not show evidence of excessive apoptosis [17]. In those patients who have a hypercellular BM but no evidence of apoptosis, it is conceivable that the rapidly proliferating hematopoietic cells are being retained in the marrow for abnormally

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