Pergamon

Leukemia Research Vol. 20, No. 11/12, pp. 891–900, 1996.

Printed in Great Britain
0145–2126/96 $15.00 + 0.00

PII: S0145-2126(96)00008-2

MEASUREMENT OF APOPTOSIS, PROLIFERATION AND THREE CYTOKINES IN 46 PATIENTS WITH MYELODYSPLASTIC SYNDROMES

Vilasini Shetty*, Suneel Mundle*, Sairah Alvi*, Margaret Showel*, LaTanya Broady-Robinson*, Saleem Dar*, Raphael Borok*, John Showel*, Stephanie Gregory*, Shelby Rifkin†, Sefer Gezer*, Agapi Parcharidou*, Parameswaran Venugopal*, Rohit Shah‡, Beatrice Hernandez§, Mary Klein¶, Devena Alston§, Erwin Robin‖, Carlos Dominquez** and Azra Raza*

*Rush Cancer Institute and the Department of Pathology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois, U.S.A. and †Northwest Community Hospital, Arlington Heights, Illinois, U.S.A.; ‡Lake Forest Hospital, Deerfield, Illinois, U.S.A.; §LaPorte Cancer Treatment Center, LaPorte, Indiana, U.S.A.; ¶St. Anthony Hospital, Michigan City, Indiana, U.S.A.; ‖Ingalls Memorial Hospital, Harvey, Illinois, U.S.A.; and **The Mount Sinai Comprehensive Care Center, Miami Beach, Florida, U.S.A.

(*Received* 18 *October* 1995. *Revision accepted* 10 *January* 1996)

Abstract—Extensive apoptosis or programmed cell death (PCD) of both hematopoietic (erythroid, myeloid, megakaryocytic) and stromal cells in myelodysplastic syndromes (MDS) cancels the high birth-rate resulting in ineffective hematopoiesis and has been demonstrated as the probable basis for peripheral cytopenias in MDS by our group. It is proposed that factors present in the microenvironment are inducing apoptosis in all the cells whether stromal or parenchymal. To investigate this hypothesis further, bone marrow biopsies from 46 MDS patients and eight normal individuals were examined for the presence of three cytokines, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and granulocyte macrophage-colony stimulating factor (GM-CSF) and one cellular component, macrophages, by the use of monoclonal antibodies immunohistochemically. Results showed the presence of TNF-α and TGF-β in 41/46 and 40/46 cases of MDS respectively, while only 15 cases showed the presence of GM-CSF. Further a significant direct relationship was found between the degree of TNF-α and the incidence of PCD ($p = 0.0015$). Patients who showed high PCD also had an elevated TNF-α level. Thus, the expression of high amounts of TNF-α and TGF-β and low amounts of the viability factor GM-CSF may be responsible for the high incidence of PCD leading to ineffective hematopoiesis in MDS. Future studies will be directed at attempting to reverse the lesion in MDS by using anti-TNF-α drugs such as pentoxifylline. Copyright © 1996 Elsevier Science Ltd

Key words: Myelodysplastic syndrome, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), granulocyte macrophage-colony stimulating factor (GM-CSF), apoptosis and macrophages.

Introduction

Myelodysplastic syndromes (MDS) encompass a group of clinical, hematological disorders characterized by dysplastic maturation of precursor cells in the bone marrow and an approximately 30% incidence of eventual transformation into acute myeloid leukemia (AML) [1–4]. The paradox of this disease is that despite a hypercellular or normocellular bone marrow (BM), the patients generally present with peripheral cytopenias [5]. To address this paradox, we developed unique double labeling methods to simultaneously determine the rate of proliferation and apoptosis or programmed cell death (PCD) [6]. The studies revealed that MDS is an extremely proliferative disorder with all three lineages of hematopoietic cells synthesizing DNA. At the same, time, there was a high rate of intramedullary hematopoietic cell death by apoptosis. A large number of erythroid, myeloid and megakaryocytic cells were undergoing PCD in the BM biopsies. A variety of stromal cells such as the fat cells, endothelial cells and fibroblasts were also found to undergo PCD simultaneously in the microenvironment. We concluded that

Correspondence to: V. Shetty, Rush Cancer Institute, Rush-Presbyterian-St. Luke's Medical Center, 2242 West Harrison Street, Suite 108, Chicago, IL 60612, U.S.A. (Tel: 312 455 8474; Fax: 312 455 8479).

high cell death and high cell birth cancel each other resulting in ineffective hematopoiesis [7]. Apoptosis frequently observed in the stromal cells of the bone marrow microenvironment raised questions regarding the role of the microenvironment in generating this paradox. One hypothesis was that since MDS is a clonal disease, perhaps the abnormal clone is producing factors which are poisoning the microenvironment so that all cells whether stromal or parenchymal are forced to undergo PCD. A number of cytokines have been identified that inhibit or stimulate the progenitor cells either by a direct action on these cells or by modifying or modulating the production or actions of other molecules. The cytokines measured in this study were two inhibitory proteins namely, tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) and one viability factor, granulocyte macrophage-colony stimulating factor (GM-CSF), as well as a cellular stromal component, the macrophages. It has been demonstrated that TNF-α exerts a variety of activities on hematopoietic cells which include inhibition of hematopoietic progenitors in *in vitro* clonogenic assay [8–11], induction of the release of various growth factors by stromal cells [12–14] and also has the ability to initiate the cascade of cytokines and other factors associated with the inflammatory response. GM-CSF on the other hand is a pleiotropic cytokine that can stimulate the proliferation, maturation and function of hematopoietic cells. It is produced by a variety of cell types including T-cells, B-cells, macrophages, mast cells, endothelial cells and fibroblasts in response to cytokine or immune and inflammatory stimuli [15–17]. The other cytokine of interest in this study was transforming growth factor-beta. TGF-β are a group of growth and differentiation modulation proteins of diverse function that induce a variety of biological responses on different tissues [18]. While the common effects of TGF-β are both growth stimulatory [19] as well as growth inhibitory [20], the latter effect is more pronounced upon hematopoietic progenitors. This study was undertaken to better understand the biology by examining bone marrow biopsies of MDS patients for relative amounts of these cytokines using semi-quantitative methods, the hypothesis being that either the presence of excessive amounts of TNF-α or TGF-β and/or the relative absence of GM-CSF may account for the ineffective hematopoiesis observed in these disorders.

Materials and Methods

The study was carried out on 46 MDS patients, 32 being males and 14 females. Morphological classification as per the French–American–British (FAB) proposal [21] was available on all patients. These details are provided in Table 1. Briefly, there were 18 patients in the refractory anemia (RA) category, six in refractory anemia with ringed sideroblasts (RARS), 14 in refractory anemia with excess of blasts (RAEB), seven in refractory anemia with excess of blasts in transformation (RAEB-t) and one in chronic myelomonocytic leukemia (CMMOL). Similar studies were also carried out on eight bone marrow biopsies obtained from patients with non-Hodgkin's lymphoma (NHL). These biopsies did not show any tumor involvement and were confirmed as being normal by two histopathologists. All the above patients and the normal biopsies from eight patients with NHL received a 1 h infusion of thymidine analogs bromodeoxyuridine and/or iododeoxyuridine at 100 mg/m^2 intravenously. Informed consent was taken by all patients before the infusion. The infusion protocols were reviewed and approved by the Investigative Review Board (IRB) of the Rush-Presbyterian-St. Luke's Medical Center, National Cancer Institute (NCI) and the Food and Drug Administration (FDA). The IUdR and BrdU for these studies were supplied by NCI. The bone marrow biopsies on these patients have been analyzed for studies on cell cycle kinetics and apoptosis.

Detection of cytokines in the microenvironment

Levels of three cytokines TNF-α, TGF-β and GM-CSF were determined semiquantitatively 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 labeled for each cytokine using the respective monoclonal antibodies as follows: after the tissues were rehydrated 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, La Jolla, CA, U.S.A.) 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-β2/β3 (1:50) antibody (Oncogene Science Inc., Manhasset, NY, U.S.A.) or anti GM-CSF (1:80) (Genzyme, Cambridge, MA, U.S.A.) or anti TNF-α (1:180) (Promega, Madison, WI, U.S.A.) diluted in 0.5 M 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 0.5 M 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, U.S.A.). 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.

Detection of macrophages

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

Detection of S-phase cells

The *in situ* detection of the two thymidine analogs IUdR and BrdU administered via intravenous infusions was carried out using the protocols and immunohistochemical methods described previously [22]. After processing and mounting the coverslips with fluoromount, at least 2000 positively S-phase labeled myeloid cells were counted to determine the labeling index (LI). Erythroid and megakaryocytic cells were excluded.

Measurement of apoptosis using in situ *end labeling (ISEL) of fragmented DNA*

ISEL was carried out on all the bone marrow biopsies of the 46 patients as mentioned in our earlier studies [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, La Jolla, CA, U.S.A.) were incubated with a mixture of dATP, dCTP, dGTP (0.01 M, Promega, Madison, WI, U.S.A.), bio-dUTP (0.001 M, 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, U.S.A.) and diamino benzidine tetrachloride. Thus, cells labeled positively for ISEL showed brown staining in their nuclei under the light microscope.

Simultaneous detection of apoptosis by in situ *end labeling and TNF-α*

All the cases were further double-labeled to simultaneously detect apoptosis and TNF-α. The sections were first treated as described above (ISEL) to label the apoptotic cells. After staining with DAB, they were thoroughly rinsed in distilled water followed by rinsing with 0.5 M Tris buffered saline (TBS), pH 7.5 [0.5 M NaCl in 0.05 M Tris buffer]. After rinsing with 0.5 M TBS, the specimens were sequentially treated with 4 N HCl for 15 min, the anti TNF-α monoclonal antibody diluted 1:180 in 0.5 M TBS containing 0.25% Tween 20 for 60 min at room temperature, rabbit anti-IgG (Dako, Carpenteria, CA, U.S.A.) diluted 1:20 in 0.5 M TBS, for 30 min and with mouse alkaline phosphatase-anti-alkaline phosphatase antibody complex (APAAP; Dako) diluted 1:40 in 0.5 M TBS for 30 min. The sections were thoroughly rinsed with 0.5 M TBS after each of the above-mentioned incubations. Tissues were then immersed in the solution prepared as follows—naphthol AS-MX phosphate (20 mg) was dissolved in 2 ml of *N,n*-dimethylformamide and this was added to 100 ml 0.1 M Tris buffer, pH 8.2 at 20°C. Next, 0.1 ml of 1.0 M levamisole was added to the solution to inhibit endogenous alkaline phosphatase, followed by 100 mg of Fast Blue BB salt. This mixture was stirred for 2 min and filtered before the sections were immersed. Color development took 8–10 min. The specimens were then washed in distilled water and mounted in fluoromount. The TNF-α stained blue under the light microscope, while ISEL positive cells stained brown.

Interpretation of slides

All the slides were observed on a televised screen by three different investigators (A.R. being one of them every time). A subjective quantitative scale was formulated to determine the degree of positivity of the different cytokines (TGF-β, TNF-α and GM-CSF), ISEL staining and the cellular component (macrophages) as follows: negative, low, intermediate and high.

Low:	less than 1/3 of the biopsy positive
Intermediate:	1/3 to 2/3 of the biopsy positive
High:	greater than 2/3 of the biopsy positive.

Statistical analysis

The non-parametric Mann–Whitney *U*-test was used for comparison between two parameters.

Results

Table 1 describes in detail the FAB type, white blood cell count (WBC), labeling index (LI), apoptosis and the presence of cytokines and macrophages. Results will be described under the following sub-headings.

Apoptosis and proliferation

A high degree of proliferation (Fig. 1a) and apoptosis (Fig. 1b) were seen in this group of patients as reported previously [7]. Briefly, of the 46 patients studied, apoptosis was detected in 42 patients, while three cases showed little or no apoptosis and one biopsy was unevaluable. Twenty-five patients (54.3%) showed a high degree of apoptosis where more than 75% of the cells demonstrated ISEL positivity, seven (15.2%) showed intermediate and 10 (21.7%) showed low

Table 1. Studies on cell cycle kinetics, programmed cell death and microenvironment on bone marrow biopsies of patients with myelodysplastic syndromes

Patient #	FAB	WBC×10^9 /l	ISEL	LI %	TGF-β	TNF-α	GM-CSF	Macrophages
1	RA	4.50	high	39.60	negative	high	negative	negative
2	RA	8.40	high	26.00	intermediate	high	intermediate	low
3	RA	2.70	high	25.10	intermediate	high	negative	low
4	RA	3.90	high	15.00	low	high	low	high
5	RA	2.90	high	25.00	high	intermediate	intermediate	high
6	RA	3.10	high	24.00	negative	high	negative	intermediate
7	RA	2.50	high	31.50	low	high	low	high
8	RA	NA	intermediate	46.60	high	low	negative	low
9	RA	15.20	intermediate	39.00	intermediate	high	negative	negative
10	RA	1.00	low	33.10	intermediate	intermediate	negative	negative
11	RA	3.10	low	25.30	low	low	negative	high
12	RA	8.70	low	41.00	high	high	negative	low
13	RA	1.70	low	16.00	low	negative	negative	low
14	RA	3.40	low	35.00	intermediate	intermediate	high	intermediate
15	RA	3.70	low	NA	negative	high	negative	negative
16	RA	6.30	low	NA	low	low	negative	low
17	RA	NA	negative	22.90	low	negative	negative	intermediate
18	RA	4.00	bad bx	NA	negative	negative	negative	negative
19	RARS	4.50	high	37.00	intermediate	intermediate	negative	intermediate
20	RARS	7.50	high	30.60	low	high	negative	high
21	RARS	38.10	high	29.30	high	low	intermediate	low
22	RARS	13.00	high	13.00	high	intermediate	high	low
23	RARS	1.40	intermediate	49.10	intermediate	intermediate	high	intermediate
24	RARS	3.10	intermediate	32.30	intermediate	high	low	negative
25	RAEB	12.80	high	21.30	intermediate	high	negative	high
26	RAEB	0.70	high	24.00	intermediate	high	high	high
27	RAEB	3.70	high	26.00	low	high	negative	negative
28	RAEB	1.60	high	18.00	intermediate	intermediate	low	low
29	RAEB	14.60	high	33.60	high	low	negative	high
30	RAEB	1.50	high	18.00	intermediate	high	negative	low
31	RAEB	25.70	high	24.50	negative	high	negative	high
32	RAEB	1.60	high	23.90	intermediate	high	negative	high
33	RAEB	4.70	high	28.70	low	negative	negative	intermediate
34	RAEB	6.70	intermediate	29.00	high	intermediate	negative	low
35	RAEB	0.80	low	38.00	intermediate	low	negative	high
36	RAEB	6.30	low	NA	low	high	negative	low
37	RAEB	3.10	negative	14.00	negative	low	negative	high
38	RAEB	2.50	negative	24.00	intermediate	low	negative	high
39	RAEB-t	1.70	high	47.00	low	intermediate	low	intermediate
40	RAEB-t	2.50	high	32.00	intermediate	high	negative	high
41	RAEB-t	NA	high	31.00	high	high	low	high
42	RAEB-t	10.10	high	18.00	intermediate	intermediate	high	intermediate
43	RAEB-t	1.30	high	28.50	low	high	negative	high
44	RAEB-t	4.90	intermediate	39.30	low	high	high	intermediate
45	RAEB-t	4.00	intermediate	31.00	intermediate	intermediate	negative	high
46	CMMOL	11.80	low	19.10	low	negative	negative	high

FAB, French–American–British classification; ISEL, *in situ* end labeling; LI%, labeling index; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEB-t, refractory anemia with excess blasts in transformation; CMMOL, chronic myelomonocytic leukemia; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; GM-CSF, granulocyte macrophage-colony stimulating factor; NA, not available.

Table 2. Degree of positivity of the different parameters in MDS (n = 46)

Degree of positivity	ISEL	TGF-β	TNF-α	GM-CSF	Macrophages
High	25.00	8.00	22.00	6.00	18.00
Intermediate	7.00	18.00	11.00	3.00	9.00
Low	10.00	14.00	8.00	6.00	12.00

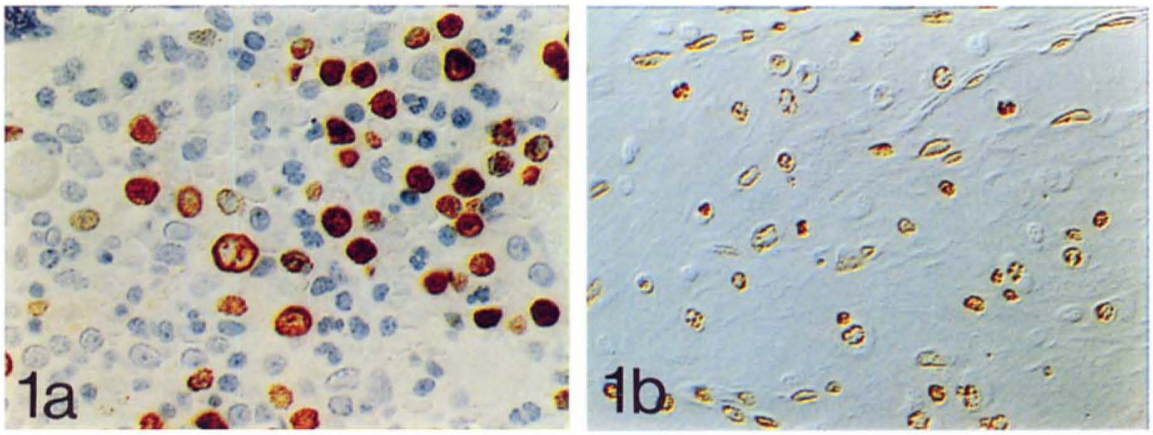

Fig. 1. (a) Bone marrow biopsy from an MDS patient showing high percentage of S-phase cells (brown staining in the nucleus) detected by virtue of incorporation of IUdR/BrdU in their DNA using specific monoclonal antibody. Original magnification ×400. (b) Extremely high incidence of apoptotic cell death involving all types of stromal and hematopoietic cells in the bone marrow of MDS patients, detected using ISEL (brown staining). Original magnification ×400.

positivity. The normals showed low (6/8) and intermediate (2/8) ISEL positivity. As for the rate of proliferation, labeling indices (LI) from bone marrow biopsies were available in 41 patients. The median LI was 28.7% with a range of 13.0–49.1%. When the LI and apoptosis was correlated with the WBC, no significant relationship was seen.

Localization and degree of positivity of the different cytokines in MDS

TGF-β and TNF-α were expressed in varied amounts in the bone marrow biopsies (Table 1). Briefly, TGF-β was expressed in 40 patients of the 46 patients studied, TNF-α in 41 patients and GM-CSF in 15 patients. Table 2 summarizes the extent of positivity seen for each of the above cytokines. TGF-β was seen in the megakaryocytes and blood vessels, primarily along the endothelial lining as well as in the interstitial areas (Fig. 2). TNF-α on the other hand was demonstrated in monocytes and few immature progenitor cells (Fig. 3). GM-CSF was localized in the interstitium mainly around the cells in S-phase (Fig. 4.).

Detection of macrophages

The macrophages were detected using a specific monoclonal antibody (EBM-11) (Fig. 5a). The degree of positivity is depicted in Table 2. Presence of macrophages were also revealed by ISEL staining. The cytoplasm of the macrophages were filled with ISEL positive apoptotic bodies, while the nuclei of these macrophages were still intact (Fig. 5b). The present phenomena clearly demonstrates the scavenging activity of the macrophages present.

Patterns of different parameters studied in different FAB categories

All parameters studied were further analyzed for common trends or predominant patterns with respect to FAB classification (Table 3). The majority of patients in the group of RA category (55.6%) revealed a lower degree of apoptosis as compared to the other three, most noteworthy being RAEB-t. The most striking observation seen was that all seven patients with RAEB-t showed intermediate to high expression of both TNF-α along with high apoptosis. GM-CSF ratings for RAEB patients were more likely to be negative than were those for RAEB-t patients ($P = 0.0486$).

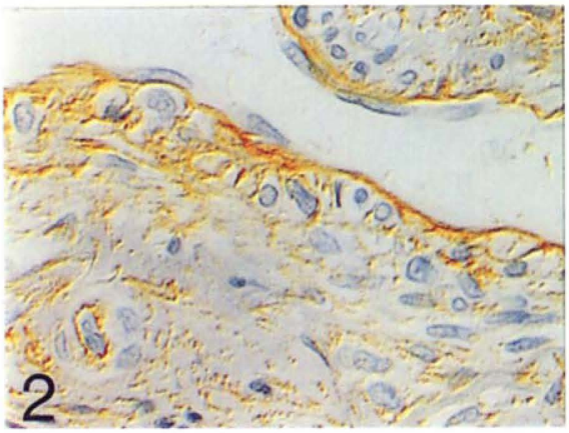

Fig. 2. Bone marrow biopsy from an MDS patient showing high expression of TGF-β (brown) in the interstitium and blood vessels, primarily along the endothelial lining. Original magnification ×400.

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