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The value of cell cultures for the diagnosis of mixed myelodysplastic/myeloproliferative disorders

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Abstract

Background and Objective. **Myelodysplastic syndromes (MDS) are a group of disorders characterized by dyshematopoiesis in bone marrow (BM) and peripheral blood (PB) cytopenias. In recent years particular attention has been paid to myeloproliferative disorders with dysplastic features or myelodysplastic syndromes that evolve into a myeloproliferative disorder. The present study was designed to analyze patients with MDS but with a normal or increased colony forming capacity, in order to see whether or not cell cultures could contribute to the diagnosis of intermediate MDS-MPD conditions.**

Design and Methods. **A total of 80 patients diagnosed as having MDS were included in the study. CFU-GM assay was performed by plating 1×10^5 mononuclear cells/mL in IMDM and 0.9% methylcellulose containing 10% PHA-LCM. In all cases cultures were run in parallel without PHA-LCM to assess autonomous growth. Cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO_2 and scored at day 14. Cytogenetic analysis was performed according to standard procedures. Short-term cultures of 24 and/or 48 hours were used.**

Results. **Twenty-two patients out of the 80 MDS cases included in the study showed a normal or increased cell growth pattern. Among these 22 patients, eight were diagnosed as suffering from chronic myelomonocytic leukemia (CMML) according to the FAB criteria and were excluded from the present analysis. The remaining 14 cases, which constitute the body of this study, displayed an increased number of clusters and/or colonies, with an altered cluster/colony ratio (anomalous growth) in 10 cases. Autonomous colony formation was present in five of these 14 cases and autonomous cluster growth was seen in all but three of them. In addition, one patient showed endogenous BFU-E growth. Morphological diagnoses were then revised due to this aberrant colony growth pattern: based on actual criteria, 3 patients could have been considered as having a-CML (atypical chronic myeloid leukemia). Another 6 cases evolved to a more proliferative disorder: 5 to CMML, and one to a-CML. Interestingly, in 3 of these 6 patients the evolution took place concomitantly with an infectious episode. In one additional patient the platelet count increased up to 1000×10^9/L and required treatment with hydroxyurea.**

Interpretation and Conclusions. **Our results show that intermediate MDS-MPD cases are relatively common and that *in vitro* characteristics, i.e. high clonogenic capacity with a high cluster/colony ratio and scanty autonomous growth, in patients showing myelodysplastic features could contribute to an early diagnosis in these cases. It is possible that in some cases an infectious episode, through higher cytokine secretion, contributes to the development of these disorders.**

Key words: cell cultures, atypical chronic myeloid leukemia, mixed myelodysplastic/myeloproliferative disorders

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Myelodysplastic syndromes (MDS) are a group of disorders characterized by dyshematopoiesis in the bone marrow (BM) and cytopenias in the peripheral blood (PB).[1,2] Although they were initially classified by the FAB cooperative group into five main subtypes,[3] there is an increasing number of reports of MDS cases that are difficult to assign to a particular FAB subtype.[4,5] Moreover, the inclusion of one of the FAB subtypes, chronic myelomonocytic leukemia (CMML),[6,7] within the MDS group remains controversial since, due to its proliferative characteristics, it has been postulated that it would be better to consider this as a myeloproliferative disorder (MPD).[8]

In recent years particular attention has been paid to myeloproliferative disorders with dysplastic features or myelodysplastic syndromes that evolve into myeloproliferative disorders.[9-11] Some of them have been termed atypical-chronic myeloid leukemia (a-CML), an entity recently recognized by the FAB group[12] and that some authors have related to CMML,[13] while others have preferred to use the term mixed MDS-MPD for these conditions.[14]

Cultures of bone marrow cells from MDS patients show decreased CFU-GM and BFU-E *in vitro* colony growth.[15] This is probably a reflection of both the imbalance between cell proliferation/differentiation in MDS as well as their increased cell apoptosis, which will translate into cell cytopenias in all MDS variants except CMML.

The present study was designed to analyze the characteristics of MDS patients with evident dysplastic features in BM but with normal or increased

colony forming capacity, in order to see whether or not cell cultures could contribute to the diagnosis of intermediate MDS-MPD conditions. In our experience the majority of patients who displayed this behavior *in vitro* already had or evolved into a *proliferative* disorder and, accordingly, a diagnosis of MDS based only on conventional criteria should be reviewed.

Materials and Methods

Patients

A total of 80 patients with MDS were included at diagnosis in the study. Case distribution according to FAB criteria was as follows: RA (21 cases), RARS (8 cases), RAEB (26 cases), RAEB-t (15 cases), CMML (9 cases) and abnormal chromatin clumping syndrome (ACCS) in one patient. A full blood count, differential white cell count, BM aspirate and chromosome analysis were performed in each case at presentation.

Cell cultures

Bone marrow samples were collected in sterile, preservative-free heparin tubes and separated by Ficoll-Hypaque (d=1070) gradient density centrifugation. The CFU-GM assay was performed using the method described by Iscove *et al*.[16] Briefly, 1×10^5 mononuclear cells/mL in Iscove's modified Dulbecco's medium (IMDM) were plated on 35 mm Petri dishes in 0.9% methylcellulose containing 10% PHA-leucocyte conditioned medium (PHA-LCM),[17] 10% bovine serum albumin and 10% human AB serum. Cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO_2 and scored at day 14 under an inverted microscope. In addition, in all cases cultures were run in parallel without PHA-LCM to assess autonomous growth. A colony was defined as a cell aggregate with more than 40 cells, while clusters consisted of 4 to 40 cells.

In order to avoid the possibility of leukemic colony growth, only fourteen-day colony growth was analyzed. Based on the values of CFU-GM obtained from normal bone marrows, we defined three patterns of *in vitro* growth as previously described:[18]

1. *Normal* (N): normal number of CFU-GM and a cluster/colony ratio < 2 (normal values in our lab were: 142±60 CFU-GM per 10^5 cells plated and cluster/colony ratio 1.22±0.18).
2. *Hypoplastic* (H): low number of CFU-GM (below 80 colonies/10^5 cells plated) and cluster/colony ratio < 2.
3. *Anomalous* (A): cluster/colony ratio > 2.

Increased (I) growth was defined as more than 200 colonies or more than 250 clusters. The values found in our lab for MDS patients were 29±40 colonies and 75±75 clusters (Table 1).

Table 1. *In vitro* growth assays in study patients, MDS patients and normal controls.

	Controls	*MDS patients*	*Study patients*
Clusters	200±41	75±75	293±394
Colonies	140±60	29±40	571±491

Cytogenetic analysis

Cytogenetic analysis was performed according to standard procedures.[19] Short-term cultures of 24 and/or 48 hours were used. At least 25 metaphases were analyzed in each case. Karyotypes are presented according to ISCN nomenclature.[20]

Results

Twenty-two patients out of the 80 MDS cases included in the analysis showed a normal or increased cell growth pattern based on cluster and/or colony numbers. Out from these 22 patients, eight were diagnosed according to FAB criteria as suffering from CMML,[3] an entity in which the presence of increased clonogenic growth is well established,[21] and therefore they were excluded from the present study. Of the remaining 14 patients, 4 cases were diagnosed as refractory anemia (RA), 3 as refractory anemia with excess of blast cells (RAEB), 6 as refractory anemia with excess of blasts in transformation (RAEB-t); one patient had an abnormal chromatin clumping syndrome.[22]

Clinical data on these 14 patients are shown in Table 3. Although the median age was 70 years, interestingly, 3 patients (21.4%) were under 50. The male/female ratio was 9/5. All cases but two had hemoglobin levels < 9 g/dL, but only 2 patients presented WBC counts below 2×10^9/L. Moreover, five displayed >10×10^9/L WBC. In 11 cases out of 14 the platelet counts were above 50×10^9/L. In all cases monocyte counts were below 1 $\times10^9$/L when the cultures were performed.

Chromosome findings

Clonal cytogenetic abnormalities were observed in 9 out of the 14 cases (64%). The most frequent chromosomal abnormalities were trisomy 8 (4 cases), 5q- and abnormalities involving chromosome 7 (2 cases each). Two out of the four patients with RA and the case with ACCS showed normal karyotypes (Table 2).

Cell cultures

All patients demonstrated an increased number of clusters and/or colonies, with an altered cluster/colony ratio (anomalous growth) in 10 out 14 cases (Table 2). Autonomous colony formation

Table 2. Clinical characteristics of the study patients.

Pt	Sex/ age	Diag.	WBC x10⁹/L	Hb g/dL	Plat. x10⁹/L	BM blasts %	Cytogenetics
1	M/65	RA	4.4	6.5	160	1	46,XX
2*	M/73	RA	32	8.7	1200	1	47XY,t(8;12) (q14;p13) +der(8) t(8;12) (q14;p13)
3	M/73	RAEB-t	4.1	6	400	25	46,XY, 45,XY-7
4	M/43	RAEB	7.6	5.9	52		46XY
5	F/86	RAEB-t	4.3	6	122	21	46,XX; 46XX del(5) (q14q32), 46XX del(5) (q14q32) del (9) (p12p23)
6*	M/84	RAEB-t	18	5.1	142	8	46XY; 47XY+8
7	F/75	RA	11.2	8	501	4	46XX; 46XX, del(5) (q13q31)
8	F/69	RA	2	11	66	1	46XX
9	F/68	ACCS	53	8.5	9	2	46XX
10*	F/16	RAEB-t	20	8.1	27	15	46XX; 45X -X
11	M/61	RAEB	3.7	7.1	18	7	46XY; 47XY+8
12	M/28	RAEB-t	1.5	11.5	95	25	46XY; 46XY del(7) (q21q31);46XY del(7)(q21q31)+13
13	M/76	RAEB-t	6.8	5.4	67	25	46XY
14	M/71	RAEB	6.7	5.4	227	7	47XY +8

Diag: diagnosis; Chrom: Chromosomes; M: male; F: female; RA: refractory anemia; RAEB: RA with excess of blasts; RAEB-t: RAEB in transformation; ACCS: abnormal chromatin clumping syndrome.
**With current criteria (12) they have been diagnosed as a-CML.*

was present in five cases (#1, 3, 7, 8 and 14), and autonomous cluster growth was seen in all but three cases. In addition, one patient (#2) showed endogenous BFU-E growth (197 BFU-E/10^5 cells plated). *In vitro* growth for CMML was 597±550 colonies/10^5 cells plated and 24±38 colonies for the remaining MDS patients.

Clinical evolution

Morphological diagnoses were then revised due to the colony growth patterns seen in these 14 patients and it was found that 3 (cases #2, 9 and 10) could have been considered, based on current criteria,[12] as a-CML. One patient received an allogeneic bone marrow transplantation (BMT) and achieved complete remission (CR). Out of the other 10 patients (Table 4), only four showed stable disease at 3 to 14 months after diagnosis. The remaining 6 cases (cases #1, 3, 4, 5, 11 and 14) developed a more *proliferative* disorder (Table 4): 5 CMML and one a-CML. Interestingly, in 3 of these 6 patients the evolution coincided with an infectious episode. In another patient (#7) the platelet number increased to 1000×10^9/L and required treatment

Table 3. *In vitro* growth of clonogenic cells in the study patients.

	Clonogenic growth				Autonomous growth	
Pt	Colonies*	Clusters*	Col/Clust ratio	Type of growth	Colonies*	Clusters*
1	39	500	12.3	I+A	26	350
2	112	293	2.6	I+A	0	0
3	201	303	1.5	I	95	291
4	36	277	7.7	I+A	0	2
5	75	188	2.5	I+A	1	63
6	340	930	2.7	I+A	0	315
7	104	1904	18.3	I+A	62	848
8	488	398	0.8	I	56	283
9	1498	360	0.24	I	0	74
10	60	251	3.5	I+A	0	0
11	448	1034	2.3	I+A	2	13
12	52	1042	20	I+A	2	66
13	68	372	5.4	I+A	0	2
14	587	143	0.24	I	31	61

**Number of colonies or clusters/10^5 cells plated.*
I: increased growth pattern. A: anomalous growth pattern (see Materials and Methods section).

with hydroxyurea. In addition to this proliferative progression, three patients eventually evolved to AML. Two had previously developed CMML (cases #4 and 5) and one (case #10) had a-CML. Eight patients have died 3 to 28 months after diagnosis.

Discussion

MDS and MPD constitute two groups of stem cell disorders that usually display clear hematological, cytogenetic and clinical differences. Nevertheless, in recent years there have been some reports about intermediate cases of MDS/MPD for which a new diagnostic category is needed. However, most of these descriptions correspond to case reports and for a correct definition of this entity the records of a higher number of patients would be of great value. The cell colony growth pattern has been shown to be markedly different in classical MDS and MPD and could represent an additional valuable tool for the characterization of these intermediate cases. The present study was designed to analyze the characteristics of a series of patients who, despite being mophologically diagnosed as MDS, showed a *proliferative* pattern of colony growth *in vitro*. Twenty-two out of 80 MDS patients in whom the colony growth pattern was assessed showed an increased number of clusters and/or colonies. Eight of them were diagnosed as CMML, a well-defined entity[6-8] that, although grouped among MDS, displays features of both MDS and MPD with a well established *proliferative* growth pat-

Table 4. Clinical evolution of the patients' diseases.

Pts.	Diag.	Stable yes/no	Evolution to disease	Time to evolution (months)	Alive yes/no	Survival (months)
1	RA	no	CMML	12	yes	34
2	RA	no	a-CML + thrombopenia	24	no	28
3	RAEB-t	no	CMML (after infection)	1	yes	18
4	RAEB	no	CMML (after infection)	2		
			AML	5.5	yes	6
5	RAEB-t	no	CMML (after infection)	2		
			AML	10	no	11
6	RAEB-t	yes	–	–	no	6
7	RA	no	Plts >1000×10^9/L	6	no	30
8	RA	yes	–	–	yes	30
9	ACCS	yes	–	–	no	14
10	RAEB-t	yes	a-CML AML	1	no	3
11	RAEB	no	CMML (after GM-CSF treat.)	3	yes	7
12	RAEB-t	no	CR (allo-BMT)	2	yes	18
13	RAEB-t	yes	–	–	yes	3
14	RAEB	no	a-CML	5	no	14

RA: refractory anemia; RAEB: RA with excess of blasts; RAEB-t: RAEB in transformation; ACCS: abnormal chromatin clumping syndrome; a-CML: atypical chronic myeloid leukemia.

tern.[21] In the remaining 14 patients, who represent the body of the present study, a diagnosis of CMML was excluded because they did not show monocytosis (> 1×10^9/L),[3] but rather a more proliferative pattern of *in vitro* growth than that usually observed in MDS,[15, 23] and a high cluster/colony ratio. In one patient, endogenous BFU-E formation was even detected, a feature commonly seen in PV patients,[24] but in our case a marked anemia was present.

Most patients displayed chromosomal abnormalities, with chromosomes 8, 7 and 5 being most frequently involved. Trisomy of chromosome 8 is a frequent event in both MDS and MPD, as well as in AML;[25, 26] however, the incidence in this cohort of 14 patients (28%) is higher than that observed in conventional MDS series.[2] Whether amplification of chromosome 8 could play a role in the development of the disease remains to be seen.[27]

Regarding the clinical data, although the median age was similar to that of conventional MDS, it should be noted that three patients were under 50 years of age. Clinical evolution showed progression to a *proliferative* disease in the majority of our patients. One case was an ACCS, a myelodysplastic syndrome that shows proliferative features[22, 28] and has sometimes been included among a-CML. Five cases developed CMML and three could have been diagnosed as a-CML. In three cases the clinical evolution took place concomitantly with an infection and in another one after GM-CSF treatment. Several mechanisms have been postulated to explain the pathogenesis of these disorders.[14] Based on our results, it is conceivable that the process could be viewed as a stem cell disorder in which there is an inappropiate secretion of cytokines that in some cases induces low cell production. It is also possible that in some patients an infectious episode could contribute, through a higher cytokine secretion, to the *in vivo* stimulation of cell proliferation in a way similar to what was observed in the *in vitro* growth analysis. Likewise, HGF delivery could contribute to higher cell proliferation.

An additional finding from the cell gowth study is that all the patients who evolved to AML previously displayed not only increased cell growth but also an anomalous growth pattern. This is in accordance with our previous observations on minimal residual disease detection in AML, in which an anomalous growth pattern was generally associated with hidden residual leukemic hemopoiesis.[18] The poor clinical outcome of these patients, with the exception of the 5q- syndrome,[29] together with the relatively young age of some of them, could favor arguments for the use of intensive treatment with the aim of eradicating the proliferating clone, or for new therapeutic approaches in older patients.[30] Interestingly, one of our patients underwent allogeneic BMT and remains in complete hematological and cytogenetic remission 16 months after BMT.

To sum up, our results show that intermediate MDS-MPD cases are relatively common and that *in vitro* characteristics, i.e. high clonogenic capacity with a high cluster/colony ratio and scanty autonomous growth, in patients showing myelodysplastic features could contribute to the identification of these patients and to differentiating them from the classical MDS. This may be particularly important in young patients with an otherwise poor prognosis who could benefit from intensive therapy. These patients should be checked more frequently to allow a better diagnosis of these disorders. Other subentities of MDS have also been proposed.[31]

Contributions and Acknowledgments

MC Del Cañizo was responsible for the conception of the study, its design and direct supervision. JF San Miguel reviewed the study. A Mota, N. Lopez and ME Fernandez carried out the cell cultures. JM Hernandez and JL García carried out the cytogenetic studies. MC del Cañizo, C Vallejo and JF San Miguel were the clinicians involved in the follow-up of patients. All authors contributed to the analysis and writing of the paper.

Disclosures

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