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RESPONSE OF MYELODYSPLASTIC SYNDROME BONE MARROW CELLS TO MULTIPLE CYTOKINE STIMULATION IN LIQUID CULTURES: AN IN SITU HYBRIDIZATION STUDY

PATRIZIA BOSSOLASCO, DAVIDE SOLIGO, FEDERICA SERVIDA, LORENZA ROMITTI, LORENZA CANEVA, GIORGIO LAMBERTENGHI DELILIERS

Fondazione Matarelli, Centro Trapianti di Midollo Ospedale Maggiore IRCCS, and Laboratorio di Citogenetica, Istituti Clinici di Perfezionamento, Milan, Italy

ABSTRACT

Background and Objective. Myelodysplastic syndrome progenitor cells can be grown and expanded in long term bone marrow liquid cultures in the presence of multiple cytokines. In this study we investigated the pattern of differentiation and response to growth factors in six cases of myelodysplastic syndrome (MDS) with well-defined cytogenetic abnormalities by means of conventional cytogenetics and fluorescence *in situ* hybridization (FISH).

Methods. Bone marrow cells were grown in stroma-free liquid cultures in the presence of SCF, IL-3, IL-6 and GM-CSF.

Results. In three cases a CFU-GM expansion comparable to normal controls was observed, together with a decrease or increase of cells with abnormal karyotype. Two cases showed no response to growth factor stimulation, morphological signs of terminal myeloid differentiation and increase (one case) or decrease (one case) in the percentage of abnormal FISH signals along the cultures. In one additional case, while CFU-C expansion was present, clearcut leukemic transformation was observed in the culture, together with a sharp decrease in the percentage of abnormal FISH signals, indicating a leukemic transformation of MDS progenitor cells with a normal karyotype.

Interpretation and Conclusions. Our data indicate that FISH analysis is generally a poor indicator of clonality in MDS; nevertheless, determining the kinetics of cytogenetically abnormal clones in liquid bone marrow cultures may provide insight as to the growth abnormalities of MDS progenitor cells and may be useful prior to *in vivo* growth factor administration.

Key words: hematopoiesis, bone marrow cultures, cytogenetics, in situ hybridization, myelodysplastic syndromes

Defective *in vitro* growth of MDS bone marrow progenitor cells has been extensively described both with single cytokines[1] and combinations of cytokines.[2-5] Stroma-free cultures have been described in which BM progenitor cells can be maintained by repeated supplementation with growth factors.[6,7] These cultures are capable of expanding peripheral blood and bone marrow progenitor cells[8-11] while substaining multilineage hematopoiesis.[6] Other authors and our own previous studies have shown that expansion and differentiation can be achieved with this long-term bone marrow culture system in a subset of MDS patients.[12,13] To further elucidate some mechanisms of growth factor responsiveness and progression to leukemia, we collected data on a small cohort of patients with well-defined chromosomal abnormalities, which can be analyzed with either cytogenetics or FISH analysis during the culture. The results show that MDS progenitor cells can respond *in vitro* to growth factor stimulation, but also reveal a great deal of heterogeneity in both cytokine response and clonal karyotypic evolution.

Materials and Methods

Establishment of long-term marrow cultures

Marrow aspirates were obtained from 6 MDS patients (as defined by the FAB Cooperative Group): 1 patient with refractory anemia (RA), 3 patients with refractory anemia with excess of blasts (RAEB), 2 patients with acute transformation of MDS (LT-RAEBt). All patients showed hypercellular bone marrow and were selected for the presence of an abormal karyotype at diagnosis (Table 1).

In brief, 6.5×10^5 low density bone marrow cells/mL were seeded in 25 cm^2 tissue culture flasks

Correspondence: Dr. Patrizia Bossolasco, Fondazione Matarelli, c/o Ospedale Fatebenefratelli, corso di Porta Nuova 23, 20121 Milan, Italy. Fax international +39.2.29011564.

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Table 1. Characteristics of the patients.

Pt #	*FAB*	*% Blasts*	*Karyotype (QFQ banding)*
1	RAEB	6.7	45,XY,-7 [37]/46,XY [5]
2	RAEB	15	47,XY,+8/46,XY
3	RAEB	10	45-46,X,del(X)(q12),del(5)(q13q33),-7, add(17)(p12),-18 [25]/46,XX [12]
4	RA	4	47,XY,+8 [7]/46,XY [21]
5	LT-RAEB-t	63	48,XY,t(1;3)(q24;p25),+6,+8 [68]/49, idem,+21 [2]
6	LT-RAEB-t	39	47,XY,+8 [2] /46,XY [35]

in IMDM supplemented with 10% fetal bovine serum (FBS), and incubated in a 100%-humidified atmosphere at 5% CO_2. On day 0, and every 48 hours, the cultures were fed with SCF (50 ng/mL); GM-CSF (100 ng/mL); G-CSF (100 ng/mL); IL-3 (100 ng/mL), and IL-6 (10 ng/mL). At weekly intervals for 4 weeks, one-half of the supernatant was removed and replaced with fresh media and cytokines. The cells in the collected media were counted, assessed for viability by means of trypan blue dye exclusion and assayed for progenitor cells. Aliquots of 5×10^4 cells removed from the cultures were cytocentrifuged, air-dried and stained with May-Grünwald-Giemsa for differential cell counts, or further processed for the karyotypic analyses described below.

Clonogenic assays

The CFU-GM, BFU-E and CFU-GEMM assays were carried out by plating 10^5 cells/mL in a methylcellulose culture medium (Iscove's Methylcellulose, Stem Cell Technologies Inc., Vancouver, Canada) containing 4 parts 2.2% methylcellulose, 3 parts pre-tested FCS, 1 part of 10% pre-tested BSA, 0.1 part 10^{-2} M mercaptoethanol and 1 part 3 U/mL human urinary erythropoietin. Triplicate dishes were incubated at 5% CO_2, 37°C, in a fully humidified atmosphere. Aggregates of ≥ 40 cells and those of < 40 cells were recorded respectively as colonies and clusters and counted after 14 days of culture.

The CFU-GM increase was calculated by dividing the absolute number of CFU-GM (CFU-GM incidence multiplied by the number of living nucleated cells) at each point in time during the culture by their absolute number at time 0 of the culture.

Cytogenetic and FISH analysis of the cultures

After culturing, cells were exposed to Colcemid overnight, incubated with a hypotonic solution (0.56% KCl) for 5 min and fixed twice in methanol and acetic acid with a proportion of 3:1 for 10 min. Cell suspensions were placed on glass slides for routine air drying preparation. The chromosomes were analyzed after Q banding, and identified and described according to the *International System for Human Cytogenetic Nomenclature* (ISCN) in all samples prior to culturing.

Interphase FISH was performed weekly on the cytospin preparations from the liquid cultures. Two α satellite DNA probes labelled with digoxygenin were utilized: the first, specific for chromosome 8 (D8Z2, Oncor Inc., Gaithersburg, MD, USA), was used in four cases and the second, specific for chromosome 7 (D7Z1, Oncor Inc.), was used in one case. The hybridization experiments were carried out according to Pinkel *et al.*[14] with modifications. Dry slides were pre-warmed at 37°C for 30 min in 2×SSC, and then for 10 min in 5 µg/100 mL proteinase K (Sigma Chemical Co.) in 20 mM Tris/HCl, 2 mM $CaCl_2$ pH7.4 at 37°C. The slides were washed once with proteinase K buffer and once in PBS/50 mM $MgCl_2$ for 10 min. They were post-fixed with 1% formaldehyde in PBS 50 mM $MgCl_2$ pH7 for 10 min at RT. After a PBS wash, the slides were dehydrated in 70%, 80% and 95% alcohol, treated for 2 min with 70% deionized formamide in 2×SSC at 70°C and then dehydrated again at 4°C. The hybridization was performed overnight at 37°C in a moist chamber. The post-hybridization washes consisted of 15 min with 50% formamide/2×SSC pH7 at 43°C, followed by 10 min in 2×SSC, 10min in 1×SSC and 10 min in 0.1×SSC at 43°C. The slides were rinsed three times for 2 min in 4×SSC/0.025% Triton X 100, incubated with rhodamine labelled anti-digoxygenin for 5 min at 37°C in a moist chamber, and then washed three times for 2 min in 4×SSC/0.025% Triton X 100. Finally, they were counterstained with DAPI/antifade (0.1 g/mL) (Oncor Inc.). A total of 200 nuclei in every sample and control (cytospin preparations of normal bone marrow donors grown in liquid cultures) were studied using a ZEISS fluorescence microscope.

Results

The characteristics of the patients are shown in Table 1, while morphological, CFU-GM expansion and cytogenetic data of 5 representative patients are summarized in Figure 1.

In particular, 3 patients responding to growth factor stimulation showed maximum CFU-GM expansions of 2.8 fold at week two of the culture (patient #1, Figure 1A); 1.7 fold at week 3 (#2; data not shown in Figure 1) and 3.8 fold at week 1 (#3, Figure 1B). In all cases morphological analysis showed a sharp decrease in the percentage of immature blasts and an increase in mature myeloid cells (Figure 1A,B and Figure 2a). However, in spite of homogeneous cultural and morphological data, a discordant cytogenetic pattern was observed dur-

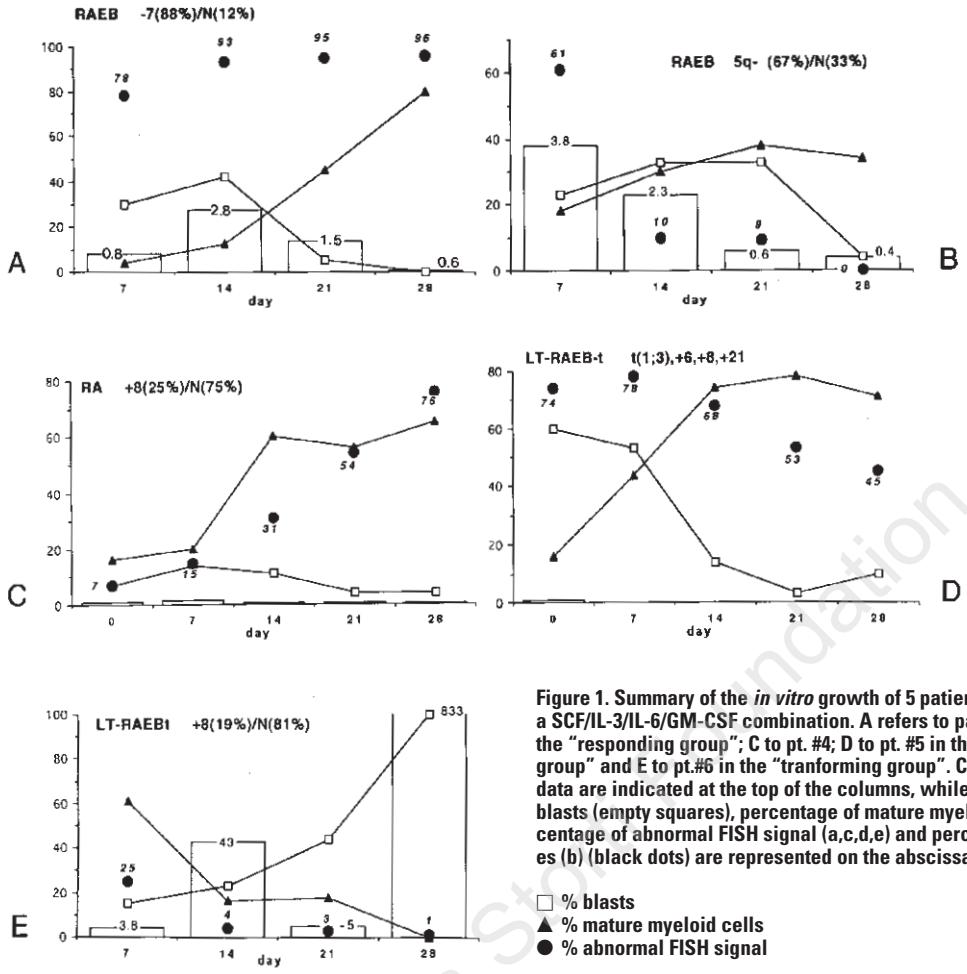

Figure 1. Summary of the *in vitro* growth of 5 patients in liquid culture with a SCF/IL-3/IL-6/GM-CSF combination. A refers to patient #1 and B to pt. #3 in the "responding group"; C to pt. #4; D to pt. #5 in the "non-responding group" and E to pt.#6 in the "tranforming group". CFU-GM fold expansion data are indicated at the top of the columns, while percentage of immature blasts (empty squares), percentage of mature myeloid cells (triangles), percentage of abnormal FISH signal (a,c,d,e) and percentage of 5q- metaphases (b) (black dots) are represented on the abscissa.

ing the cultures. One RAEB patient (#1) with monosomy 7 showed an increase from 78 to 96% in the percentage of cells with one signal at interphase FISH at the end of the culture (Figure 1A); a second RAEB patient (#2) with trisomy 8 revealed an increase from 13 to 58% in cells with 3 signals (Figure 2b); the third *responder* patient with RAEB (#3) showed a complete disappearance of 5q- metaphases at the fourth week of culture (Figure 1B).

In two patients (#4 and #5; Figure 1C and 1D) with trisomy 8 there was a complete unresponsiveness to growth factor stimulation. One patient with RA (#4) showed an up to 70% increase in the percentage of mature myeloid cells at day 28 of the culture (Figure 1C), but a progressive increase in the percentage of cells with trisomy 8 as indicated by the presence of 3 signals at FISH analysis. Conversely, in the other non-responding patient (#5), with a leukemic transformation of a RAEBt and showing multiple karyotypic abnormalities (including trisomy 8 and morphological signs of myeloid differentiation), were present together with a decrease in the percentage of cells with three signals at FISH analysis (Figure 1D and Figure 2c,d).

One patient with a leukemic transformation of RAEBt (#6) who also carried the trisomy 8 abnormality, showed a clearcut leukemic growth, a complete morphological conversion with 100% immature blasts at the end of the culture, but a complete disappearance of cells with 3 signals at FISH analysis (Figure 1E and Figure 2e,f).

Discussion

In this study we analyzed the bone marrow stroma-free liquid cultures of six MDS patients by conventional cytogenetics and FISH analysis in order to determine the response of karyotypically abnormal cells to growth factor stimulation. Since MDS is the result of a multistep process in which cytogenetic changes occur late during the course of the disease,[15-17] karyotypic abnormalities may only indicate the presence of an unstable subclone mixed with karyotypically normal cells (either normal or myelodysplastic),[18] but are poor indicators of clon-

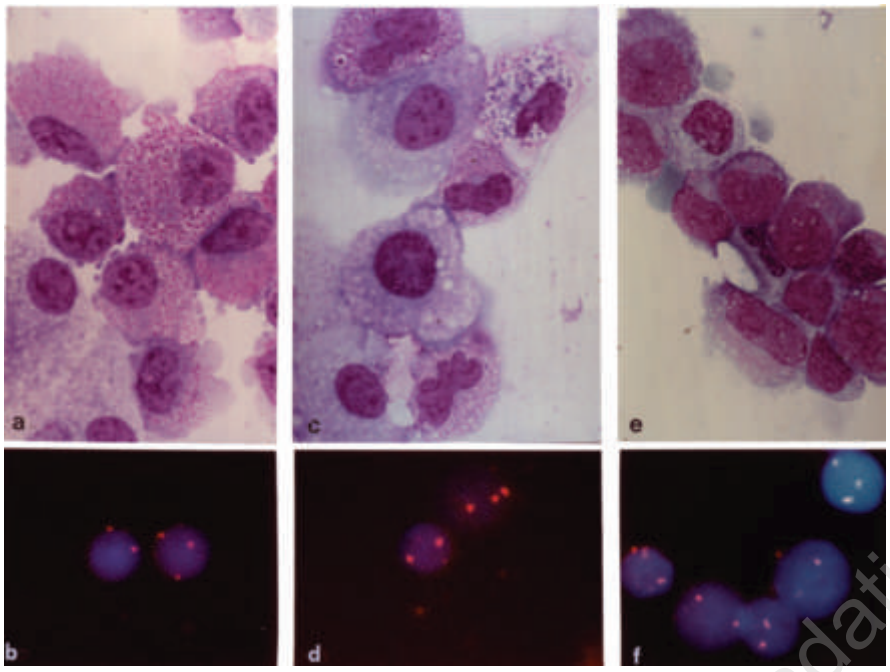

Figure 2. Morphology and interphase FISH.
a, b) RAEB case (pt. #2) with trisomy 8 at week 3 of culture, showing a predominance of hypergranulated myeloid cells at different stages of maturation and the presence of cells with 2 and 3 signals.
c, d) LT-RAEBt case with complex karyotypic abnormalities, including trisomy 8 at week 3 of culture (pt. #5). Both end stage myeloid cells and macrophages can be seen together with cells with 2 and 3 signals.
e,f) LT-RAEBt case with trisomy 8 (pt. #6). At week 4 of culture there is a predominance of immature blasts, but the presence of numerous cells with two FISH signals still remains.

ality. This was confirmed by our study, in which we observed MDS cases with more than 30% of blasts, but with a minority of karyotypically abnormal cells at both cytogenetic and FISH analysis. At the same time, the growth pattern of the expanded cultures evaluated by FISH analysis was apparently independent of karyotypic evolution.

Numerous studies have shown that growth factor administration may reduce granulocytopenia without an increased risk of leukemic transformation.[15,19] Indeed, it has been shown that non-clonal hemopoietic progenitor cells are present in a substantial number of patients, and that oligoclonal myelopoiesis may persist *in vitro* after stimulation with SCF.[20] Furthermore, stimulation of non clonal hemopoiesis has been demonstrated in patients stimulated *in vivo* with G- and GM-CSF.[21,22] One of our patients (#3), showed signs of myeloid differentiation, a normal response to growth factor stimulation and a progressive disappearance of the karyotypically abnormal clone during the liquid culture.

All these data suggest that residual normal hematopoietic progenitor cells could be expanded, although stimulation of karyotypically normal and myelodysplastic progenitor cells can not be ruled out. It is therefore evident that more accurate clonality studies must be carried out, such as those based on the inactivation of the X chromosome,[23] a method which is still not very sensitive and thus not applicable. These studies would allow, although at the single cell level, to clearly demonstrate the emergence of polyclonality along the cultures. Conversely, in the other two patients responding *in vitro* to growth factor stimulation, the progressive increase of cells with abnormal FISH signals clearly indicated that proliferation and differentiation of MDS progenitor cells had taken place. Karyotypic analysis of expansion cultures, especially when coupled with other clonality assays, may therefore be a useful tool in predicting restoration of normal hemopoiesis in MDS patients responsive to growth factor stimulation.

The two patients, both carrying the trisomy 8 abnormality and not responding to growth factor stimulation, indicate that MDS cells could not proliferate in response to growth factor stimulation but still retain some differentiation capability. Terminal differentiation was substained either by karyotypically abnormal or normal cells in three cases. It was interesting to observe the behavior in culture of the LT-RAEBt patients not responding to growth factor stimulation but showing a decrease of immature cells and of trisomy 8 signal, which would suggest a growth advantage of karyotypically normal cells.

One patient showed a complete leukemic transformation during the culture, but showed a sharp decrease in trisomy 8 signal, thus indicating stimulation of a leukemic clone not carrying the karyotypic abnormality. This confirms a previous report suggesting that karyotypically abnormal cells are not necessarily responsible for the disease progression after growth factor stimulation.[24]

Overall, our data suggest that cytogenetics and FISH analysis are poor predictors of the clonal evolution of bone marrow cultures after growth factor stimulation. Ideally, this would be more accurately evaluated with cytogenetics and PCR for sex chro-

mosome inactivation pattern[23] in the plucked colonies grown in semisolid media from sorted primitive cells (e.g. $CD34^+/DR^-$); unfortunately, these techniques are complicated and time consuming. Expansion of karyotypically abnormal cells *in vitro* may be difficult to predict regarding both the percentage of blasts and the percentage of cells with cytogenetic abnormalities at the onset of the cultures. Expansion cultures, especially when coupled with karyotypic analysis, may therefore prove to have a much wider clinical application in the diagnosis and follow up of patients with MDS. In addition, these cultures, when applied in larger series, would allow us to predict *in vivo* response to growth factor administration. However, since many patients respond to cytokines *in vitro* and possibly *in vivo*, karyotypic analysis of cultures may provide further evidence for the expansion of non-leukemic clones.

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