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## **Loss of Endoreduplication as Morphogenesis of Micromegakaryocytes in Myelodysplastic Syndrome.**

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**Summary :** Morphogenesis of micromegakaryocytes in MDS was investigated in a case of refractory anemia with excess of blasts by analysing megakaryocyte colonies developed from peripheral blood mononuclear cells in a semi-solid culture containing aplastic anemia patient's plasma as source for megakaryocyte colony stimulating factor and thrombopoiesis stimulating factor. Candidate megakaryocyte colonies were individually stained for glycoprotein (GP)IIb/IIIa by immunocytochemical method. Three types of colonies were distinguished ; type I composed of 10-30 large megakaryocytes with high ploidy number, type II composed of 50-200 micromegakaryocytes with single nucleus, and type III composed of 20-50 micromegakaryocytes. Type I colonies were similar to megakaryocyte colonies obtained from three control subjects. Periodic in situ observation of type II and III colonies disclosed that large megakaryocytes never appeared during 21 days' culture, suggesting mitotic growth at every generation. These findings indicate that loss of endoreduplication is primary defect in micromegakaryocyte formation by MDS clone. Quantitative aspect of platelet formation by micromegakaryocytes could not be assessed in this study, but morphological observation in situ or on GPIIb/IIIa-stained preparation suggested reduced platelet production. Biological significance of diminished endoreduplication is yet to be determined in respect to leukemic predisposition.

### **INTRODUCTION**

Dysmegakaryocytopoiesis is one of the morphological dysplastic changes on which diagnosis of myelodysplastic syndromes (MDS) is based<sup>1)</sup>. Micromegakaryocytes, multi-separated-nuclear megakaryocytes and mononuclear megakaryocytes are considered pathologic. Refractory anaemia with an excess of blasts (RAEB)<sup>2)</sup> and refractory anaemia (RA) with 5q-anomaly<sup>3)</sup> characteristically manifest micromegakaryocytes.

Morphogenesis of micromegakaryocytes and

their pathologic significance in terms of leukemic predisposition are not well understood. The present study was conducted to obtain from a RAEB patient micromegakaryocyte colonies in semi-solid culture in order to analyse how micromegakaryocytes are produced in vitro.

### **MATERIALS AND METHODS**

#### *1. Patient and Control Subjects*

A 42-year-old male was admitted for further evaluation of pancytopenia. Hematological data on admission were as follows : Hb 9.2g/dl, WBC  $4.4 \times 10^9/l$ , band 6%, segment 5%, lymphocytes

86%, monocytes 1%, blasts 2%, platelets  $117 \times 10^9/l$ . Bone marrow was hypercellular with 10% blasts and myeloid/erythroid ratio of 0.86. Ringed sideroblasts were absent. Typical pseudo-Pelger-Huet anomaly, multi-nuclear erythroblasts, micromegakaryocytes and multi-separated nuclear megakaryocytes were abundant. A diagnosis of RAEB was established.

The patient was administered subcutaneously low dose of cytosine arabinoside (ara-C) for 14 days; 16mg/day for 8 days and 32mg/day for 6 days. The bone marrow became dry tap on aspiration during this treatment. Jamshidi's needle biopsy proved moderate myelofibrosis. Around three weeks after the cessation of ara-C peripheral blood hematopoiesis began to recover gradually. He was then treated with vitamin D<sub>3</sub> (3 $\mu$ g/day) for three months. Hb value reached 13.5 g/dl, neutrophil count  $2.5 \times 10^9/l$  and platelet  $280 \times 10^9/l$ . The bone marrow could be aspirated again. Cell morphology was still abnormal but less dysplastic than that of the initial marrow. Micromegakaryocytes were greatly decreased in number but multi-separated or mono-nuclear megakaryocytes remained. Initial megakaryocyte-colony formation was performed during this clinical remission.

After three months of almost full recovery in hematopoiesis, pancytopenia gradually developed and the bone marrow became dry tap again. Peripheral blood leukocyte analysis disclosed an increased number of blasts which were myeloperoxidase negative, 10% My 7 (CD 13) positive and 1% GPIIB/IIIa positive. The second megakaryocyte-colony formation was done on this peripheral blood mononuclear cells. In spite of the second course of low dose ara-C, leukemic manifestation became apparent with blast increase up to 40%. The patient died of cerebral hemorrhage.

For control experiments three normal subjects were studied. Bone marrow mononuclear cells were obtained from a 25-year-old male and peripheral blood mononuclear cells from 25-year-old and 23-year-old females.

All samples were obtained with informed consents.

## 2. Megakaryocyte-Colony Formation

Mononuclear cells (MNC) were separated from

peripheral blood or bone marrow by Ficoll-Hypaque gradient density centrifugation.  $2$  to  $3 \times 10^5$  MNC were plated in 1ml of Iscove's modified Dulbecco's medium (MDM) containing 0.88% methylcellulose, 30% aplastic anaemia patients's plasma and  $5 \times 10^{-5}$  M 2-mercaptoethanol and cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. Wells were observed for cluster or colony formation every three days during 21 days' culture.

## 3. Glycoprotein IIb/IIIa Staining of Individual Colonies by Immuno-cytochemical Method

Candidate colonies of unique morphology suggestive of megakaryocytic lineage were individually aspirated by a finely drawn out capillary tube and mixed with 10  $\mu$ l of Iscove's MDM placed on a poly-L-lysine-coated slide. After 15 min standing overlay medium was discarded by absorbant paper. The slide was air-dried rapidly and fixed for 90 min 4°C with 10% formalin-80% acetone mixture.

After washing with 0.1M phosphate buffered saline (PBS) cell fixed area of the slide (approximately 3 mm in diameter) was covered with optimally diluted monoclonal antibody (P2, Immunotech, Marseille) for platelet glycoprotein (GP) IIb/IIIa (CDw41) and incubated for 60 minutes at room temperature in a wet chamber. The slide was then thoroughly washed with PBS for three minutes and covered with link-antibody (biotinylated anti-mouse immunoglobulin antibody; Biotin-Streptoavidin Stravigen Immunostaining Kit, Biogenex Lab., California), followed by incubation at 37°C in a wet chamber for 15 min. The slide was then washed with PBS for three minutes and covered with enzyme-conjugate (streptoavidin-alkaline phosphatase) and incubated at 37°C in a wet chamber for 15 min. After final washing with PBS for three minutes the slide was subjected to alkaline phosphatase stain by azo-dye method. The slide was mounted with glycerine gelatin (Merck).

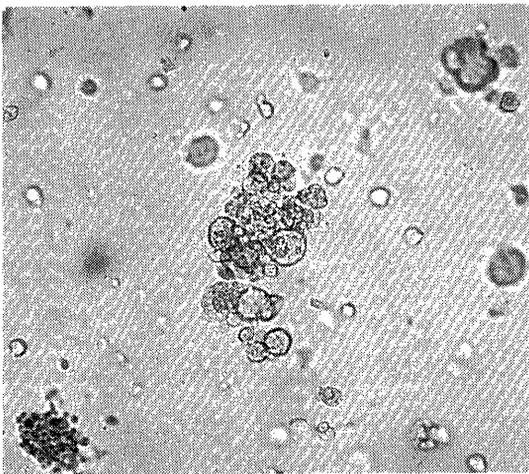
## RESULTS

### 1. Megakaryocyte-Colony Formation in Normal Subjects

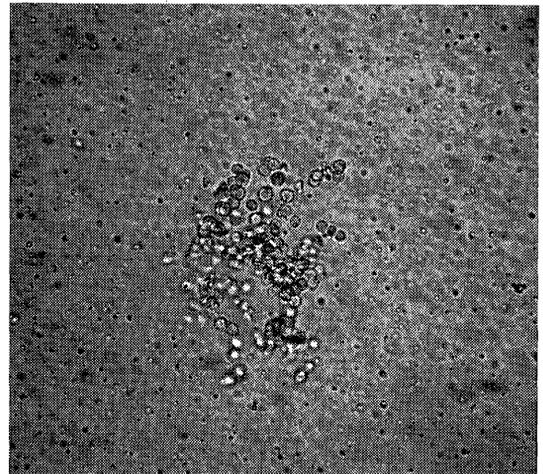
As shown in Table 1 all samples from three

**Table 1.** Megakaryocyte Colony Formation in Normal Subjects

Subject No.	Cell source	Candidate Meg-Colony		GPIIb/IIIa positive colony colony examined	(concordance %)
		type	No./ $2 \times 10^5$ MNC		
1.	bone marrow	I	$24 \pm 2$	10/10 (100)	
		II	0		
		III	0		
2.	peripheral blood	I	$20 \pm 2$	10/10 (100)	
		II	0		
		III	0		
3.	peripheral blood	I	$2 \pm 1$	5/5 (100)	
		II	0		
		III	0		



**Fig. 1.** A megakaryocyte colony of type I developed from normal subject's peripheral blood mononuclear cells in methylcellulose culture containing aplastic anemia patient's plasma. Colony composing cells have generally large size, translucent cytoplasm and abundant particulate materials on their cell margin ( $\times 100$ ).



**Fig. 2.** A micromegakaryocyte colony of type II developed from peripheral blood mononuclear cells of RAEB case. Colony composing cells have generally small size equivalent to those in granulocyte-macrophage colonies, translucent cytoplasm but no particulate materials on their cell margin ( $\times 100$ ).

controls developed type I megakaryocyte-colonies. Colony number was counted on day 21. Most of them were composed of 10 to 30 cells. Two third of them showed extremely large sizes and translucent cytoplasm with many particulate materials on their cell margin (**Fig. 1**). Individual colonies were aspirated and stained for GPIIb/IIIa. 100% concordance was noted between in-situ determined megakaryocyte-colonies and GPIIb/IIIa positive colonies.

## 2. Megakaryocyte-Colony Formation in RAEB

### Case

#### (a) From Peripheral Blood MNC on Relapse

As shown in Table 2, there formed three types of in situ determined megakaryocyte-colonies. Type I is very similar to normal megakaryocyte-colonies composed of 10-30 cells containing large cells. GPIIb/IIIa stain revealed that type I colonies examined were all megakaryocytic. Type II was composed of 50-200 cells most of which were small cells (**Fig. 2**). Particulate materials were never formed on their cellular margin. GPIIb/IIIa stain showed that 80% of these colonies examined were

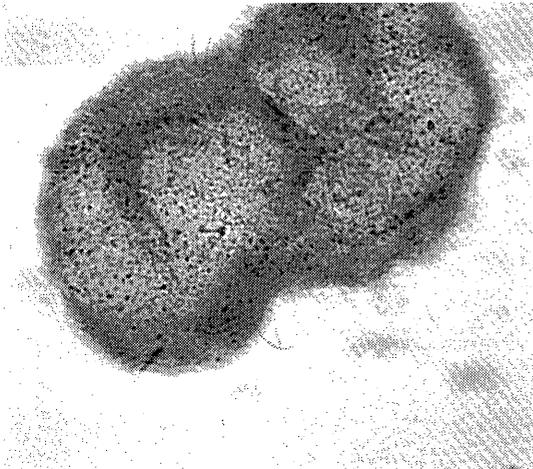
**Table 2.** Megakaryocyte and Other Haematopoietic Colony Formation in RAEB Case

Cell source	Colony type	Colony No./ $3 \times 10^5$ MNC	GPIIb/IIIa positive colony (concordance %)	
			colony examined	
Peripheral blood on relapse	Candidate			
	Meg-colony			
	I	37	7/7	(100)
	II	24	8/10	( 80)
	III	40	9/10	( 80)
	BFU-E	15	0/5	( 0)
Bone marrow on remission	CFU-GM	7	0/4	( 0)
	Candidate			
	Meg-colony			
	I	5	n. e.	
	II	0		
	III	0		
	BFU-E	25	n. e.	
	CFU-GM	22	n. e.	

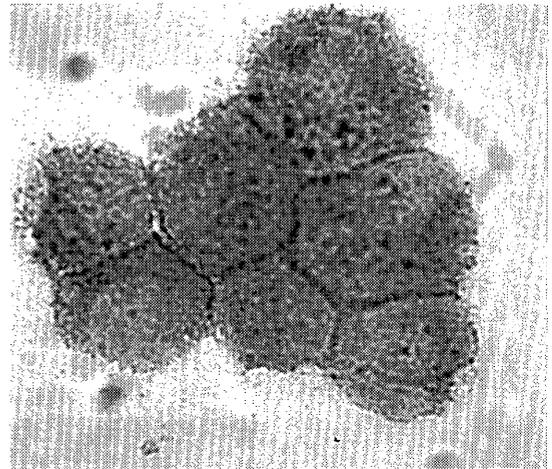
n. e. not examined

**Table 3.** Morphological Analysis of Megakaryocyte Colony Composing Cells

Case	Colony type	Colony No. examined	Colony No. examined	Colony No. with nuclear ploidy No.		
				1	2	2<
RAEB	I	7	75	0	0	75
	II	8	420	411	9	0
	III	9	125	120	5	0
Normal Subject No.1	I	10	68	0	0	68



**Fig. 3.** GPIIb/IIIa-stained megakaryocytes of type I (Fig. 1) showing diffuse cytoplasmic positivity and high nuclear ploidy number ( $\times 1000$ ).



**Fig. 4.** GPIIb/IIIa-stained micromegakaryocytes of type II colony (Fig. 2) showing diffuse positivity and mononuclear or rarely binuclear configuration ( $\times 1000$ ).

positive for megakaryocyte-specific antigen. Type III was smaller than type II, containing 20-50 small cells. 90% of these colonies examined

were GPIIb/IIIa positive. Erythroid bursts and granulocyte/macrophage colonies were also formed in the same dish. Some of these colonies

were examined for GPIIb/IIIa; all being negative.

(b) From Bone Marrow MNC on Clinical Remission

GM-colonies and erythroid bursts were formed fairly well with recombinant GM-CSF and erythropoietin respectively. A few megakaryocyte-colonies of type I were formed when stimulated by aplastic anaemia plasma. Type II and III colonies were absent.

### 3. Morphological Analysis of Colony-Composing Cells on GPIIb/IIIa-Stained Slides

As shown in Table 3, normal colonies were composed of large (over  $50\mu$ ) cells with multilobulated nuclei (Fig. 3). Type I colonies from RAEB case showed a similar pattern. Type II and III colonies were composed of small ( $15-30\mu$ ) cells with mostly single nucleus or rarely two separate nuclei (Fig. 4). All megakaryocytes, regardless of colony types, showed strong diffuse cytoplasmic stain for GPIIb/IIIa.

## DISCUSSION

By employing semi-solid culture stimulated by aplastic anemia plasma which is supposed to contain megakaryocyte-colony stimulating factor (Meg-CSF) and thrombopoiesis stimulating factor (TSF) according to Hoffman *et al*<sup>4</sup>, we could observe atypical megakaryocyte colonies composed of apparent GPIIb/IIIa positive micromegakaryocytes. Most of them possessed single nucleus or rarely two nuclei. Periodic in situ observation during 21 days' culture disclosed that large cells equivalent to those appearing around day 10 in normal megakaryocyte colonies or type I colonies of RAEB never appeared among uniformly small-sized cells of type II and III colonies. Maximum number of colony (type II) composing cells reached 200 cells, indicating more than seven mitotic generations. This means that micromegakaryocytes were produced in vitro only by mitosis of precursor cells at every generation. Therefore, it is conceivable that loss of physiological endoreduplication is a primary defect in micromegakaryocyte formation by RAEB clone.

Although the mechanism of endoreduplication is not yet fully understood, experimental evidences suggest that it is controlled by soluble factors such as Meg-CEF or TSF<sup>5</sup>. These factors are known to be increased in concentration in vivo when megakaryocyte number or platelet count is reduced. Arriaga *et al* recently suggested reciprocal interrelationship between mitosis and endoreduplication by megakaryocyte progenitors<sup>6</sup>. In their experiment using aplastic canine serum as a source Meg-CSF, its lower concentration enhanced both mitosis and endoreduplication while higher concentration increased mitosis further but markedly reduced megakaryocyte ploidy number. In our culture condition employing high concentration of aplastic anaemia plasma, most of the normal megakaryocyte colonies were apparently composed of large cells with considerably high ploidy number. Therefore, the production of micromegakaryocytes with low ploidy number is not the result of high concentration of the plasma added, but most likely represents the pathologic megakaryocytopoiesis of RAEB clone. It may be interesting to further investigate the mechanism of such dissociation between mitosis and endoreduplication.

Our study could not estimate magnitude of thrombopoiesis by single micromegakaryocytes. However, morphological observation in situ or on GPIIb/IIIa-stained preparations showed abundant platelets shedding from the cytoplasmic rim of large megakaryocytes with high ploidy number, but rare shedding from micromegakaryocytes in spite of cytoplasmic maturation showing strong diffuse staining for GPIIb/IIIa. This observation suggests reduced platelet forming capacity by the latter cells.

Biological significance of micromegakaryocyte formation in MDS was suggested by Varela *et al* in respect to later leukemic change<sup>7</sup>. However, cases of RA with 5q-anomaly which characteristically has abundant micromegakaryocytes is regarded to have a lower probability of leukemic change compared with ordinary RA cases<sup>8</sup>. Our preliminary analysis on cell size showed that micromegakaryocytes of 5q-anomaly are much larger than those of RAEB. The size of micromegakaryocytes seems

another function of megakaryocyte pathology. As shown in our study and others<sup>9)</sup> immunocytochemical staining of platelet specific glycoproteins on routine peripheral blood and bone marrow preparations of MDS certainly facilitates analysis of megakaryocytes ranging in size from lymphocyte-equivalent to giant. Such analyses will lead to more detailed understanding of megakaryocyte pathology in AML and MDS.

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