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. Micromegakaryocytes, multi-separated-nuclear megakaryocytes and mononuclear megakaryocytes are considered pathologic. Refractory anaemia with an excess of blasts (RAEB) and refractory anaemia (RA) with 5q-anomaly 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 $4.4 \times 10^3$/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 30mg/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 D3 (3ug/day) for three months. Hb value reached 13.5 g/dl, neutrophil count 2.5X $10^9/l$ 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% CO2 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 Immunocytochemical Method

Candidate colonies of unique morphology suggestive of megakaryocytic lineage were individually aspirated by a finely drawn out capillary tube and mixed with 10ul of Iscove’s MDM placed on a poly-L-lysine-coated slide. After 15 min standing overlayer 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, Marselle) for platelet glycoprotein (GP) IIb/IIIa (CDw41) and incubated for 60 minutes at from 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 (streptoadvidin-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
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

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Colony type</th>
<th>Colony No./3×10⁵ MNC</th>
<th>GPIIb/IIIa positive colony (concordance %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td>Candidate Meg-colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>on relapse</td>
<td>I</td>
<td>37</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>24</td>
<td>8/10 (80)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>40</td>
<td>9/10 (80)</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>15</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>7</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Candidate Meg-colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>on remission</td>
<td>I</td>
<td>5</td>
<td>n.e.</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>25</td>
<td>n.e.</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>22</td>
<td>n.e.</td>
</tr>
</tbody>
</table>

n.e. not examined

Table 3. Morphological Analysis of Megakaryocyte Colony Composing Cells

<table>
<thead>
<tr>
<th>Case</th>
<th>Colony type</th>
<th>Colony No. examined</th>
<th>Colony No. with nuclear ploidy No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colony No. examined</td>
<td>1</td>
</tr>
<tr>
<td>RAEB</td>
<td>I</td>
<td>7</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>8</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>9</td>
<td>125</td>
</tr>
<tr>
<td>Normal Subject</td>
<td>No.1</td>
<td>I</td>
<td>10</td>
</tr>
</tbody>
</table>

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

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

positive for magakaryocyte-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 erythropoieton 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 50μ) 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 (15-30μ) 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 thrombopoiesisstimulating factor (TSF) according to Hoffman et al\(^6\), 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 uniformaly 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\(^6\). These factors are known to be increased in concentration in vivo when megakaryocyte number or platelet count is reduced. Arriga et al recently suggested recipocal interrelationship between mitosis and endoreduplication by megakaryocyte progenitors\(^6\). 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 significane of micromegakaryocyte formation in MDS was suggested by Varela et al in respect to later leukemic change\(^7\). 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\(^8\). 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') immu-
nocytochemical 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.

REFERENCES