

## **Invited Review**

# **Human megakaryocyte ploidy**

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**Summary.** We reviewed the literature concerning the history of determination of the ploidy of human megakaryocytes and its relationship with diseases. The ploidy of rabbit megakaryocytes was analyzed by microspectrophotometry in 1964, and the analysis of the ploidy in human megakaryocytes was first performed in 1968. Presently, microphotometry and flow cytometry are the primary methods for the evaluation of the ploidy, but they have their merits and demerits. In the ploidy of human megakaryocytes, a peak has often been reported at 16N in healthy individuals, and the next peaks have been observed at 32N and 8N. The results of ploidy analyses have been reported by many investigators to be comparable between patients with idiopathic thrombocytopenic purpura and normal subjects, but various shifts of the peaks have also been documented. The ploidy is often reported to shift to a larger ploidy class in polycythemia vera and essential thrombocythemia, but it has invariably been reported to shift to a smaller class in chronic myelogenous leukemia. In reactive thrombocytosis, the ploidy pattern was reported to be the same as that in normal individuals by some investigators but to shift to a larger ploidy by others. These differences are considered to be due to heterogeneity of the subjects. In myelodysplastic syndrome, the ploidy shifts mostly to a smaller class, but it may show various patterns. We also reviewed the ploidy in other rare hematological disorders, the relationships of the ploidy with diabetes mellitus and atherosclerotic disorders, and its changes in the ontogeny. Details of the mechanism of polyploidization and its biological significance remain unknown, and further advances in the studies of these topics are anticipated.

**Key words:** Megakaryocyte ploidy, Microfluorometry, Thrombocytopenia, Thrombocytosis

### **Introduction**

Megakaryocytes, named by Howell in 1890, are unique and mysterious cells that are physiologically polyploidy. However, there have not been many studies of qualitative differences of individual megakaryocytes, because of their low frequency in bone marrow cells and the wide variation in their shape and size. The ploidy of megakaryocytes was first analyzed in 1943 by Japa using the nuclear lobe counting method, but direct measurement of DNA content of megakaryocytes is considered to have been first made by Garcia in rabbits by microspectrophotometry using the two-wavelength method in 1964 (a,b). The measurement of megakaryocyte ploidy was first applied to humans by de Leval in 1968. There have since been many studies on the ploidy of megakaryocytes. In this article, our data and the reports to date concerning human megakaryocyte ploidy are reviewed.

### **Methods for ploidy analysis**

Although there are several methods for ploidy analysis, they may be generally categorized as microphotometry and flow cytometry (FCM).

FCM, by which a large number of cells can be counted instantaneously, is naturally a very useful technique. However, the counting of megakaryocytes, which account for only a small percent of bone marrow cells, have large sizes and vary in shape, is difficult, and approaches such as the enrichment of megakaryocytes and/or the exclusive marking of megakaryocytes are necessary. Nakeff et al. (1979) were the first investigators to use FCM for the measurement of DNA in mouse and rat megakaryocytes. In that study, they observed cells after enriching megakaryocytes by centrifugal elutriation and staining them with Hoechst 33342. In 1984, Jackson et al. and Worthington et al. labeled rat megakaryocytes with fluorescein isothiocyanate (FITC) using an antiplatelet antibody and measured the DNA content by propidium iodide (PI) staining. Probably because human megakaryocytes are larger than those of rats and since it is difficult to acquire

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a great enough sample volume to obtain results, the clinical analysis of the ploidy in human megakaryocytes using two-color FCM was not successful until 1988, when Tomer et al. labeled glycoprotein IIb/IIIa and measured the DNA content by PI staining, although Bessman in 1982 applied FCM using a single color. After that, many investigators applied FCM using two colors (Tomer et al., 1989; Conklin et al., 1990; Kanz et al., 1990; Rabellino and Bussel, 1990; Hegyi et al., 1991; Bath et al., 1994; Jacobsson et al., 1996; Ma et al., 1996; Brown et al., 1997).

By microphotometry, analysis is possible even when the frequency of target cells is low or the sample volume is small. Additionally, microphotometry has a major advantage of allowing comparison with the morphology. It is therefore useful for the evaluation of the significance of the ploidy by means of comparing the ploidy with the size or area of a cell or nucleus, or with the morphology or the degree of maturity of individual megakaryocytes (Odell et al., 1965; Odell and Jackson, 1968; de Leval, 1968; Queisser et al., 1971; Queisser et al., 1974, 1976; Mayer et al., 1978; Nomura et al., 1983; Renner et al., 1987; Nagasawa and Nakazawa, 1988; Renner and Queisser, 1988; Woods et al., 1990; Ridell et al., 1990; Kobayashi et al., 1991, 1995; Maruo et al., 1992; Hancock et al., 1993; de Alarcon and Graeve, 1996). In particular, we reported that micromegakaryocytes observed in myelodysplastic syndrome (MDS) are mature but have defects in polyploidization (Kobayashi et al., 1995). These findings would not have been obtained without microphotometry. However, its precision is generally inferior to that of FCM because of the limitation in the number of cells analyzed, and it is more time- and labor-consuming.

Microphotometry can be classified into spectrophotometry and fluorometry. Spectrophotometry is liable to distributional errors and non-specific light loss, and this tendency is considered to be greater in cells with a large nucleus such as megakaryocytes (Penington and Olsen, 1970; Paulus et al., 1971; Fujita et al., 1972; Goldstein, 1981). Therefore, the two-wavelength method (Ornstein, 1952; Patau, 1952), scanning microspectrophotometry using an integrating microdensitometer (Deeley, 1955), the two-wavelength-scanning method (Fujita et al., 1972) and an image analysis system (Woods et al., 1990) designed to overcome these defects are employed for ploidy analyses. Using spectrophotometry including these methods, many investigators have measured human megakaryocyte ploidy (de Leval, 1968; Penington and Weste, 1971; Queisser et al., 1971; Lagerlof, 1972; Queisser et al., 1974, 1976; Mayer et al., 1978; Levine, 1980; Nomura et al., 1983; Winkelmann et al., 1984; Renner et al., 1987; Renner and Queisser, 1988; Ridell et al., 1990; Woods et al., 1990).

Fluorometry, which we use, is theoretically immune from distributional errors and is considered to accurately indicate the DNA level if proportionality errors are eliminated (Bohm and Sprenger, 1968; Paulus et al., 1971; Weste and Penington, 1972; Fujita, 1973). It has

been applied to the ploidy analysis of human megakaryocytes (Weste and Penington, 1972; Paulus et al., 1974; Penington et al., 1974; Haanen et al., 1975; Mazur et al., 1988; Nagasawa and Nakazawa, 1988; Kobayashi et al., 1988, 1991, 1995, 1997; Mori et al., 1991; Maruo et al., 1992; Hancock et al., 1993; Nagasawa, 1993; de Alarcon and Graeve, 1996) after Kinet-Denoel et al. and Penington and Weste first used it (in one normal subject) in 1971.

Methods for DNA staining are described in detail in the review by Paulus et al. (1990); our present review indicates that Feulgen staining is employed most frequently. However, Feulgen staining may induce proportionality errors depending on the staining process or post-irradiation conditions. Moreover, the cytoplasm of megakaryocytes may also be stained. For these reasons, we used the 4',6-diamidino-2-phenylindole (DAPI) staining technique instead of the Feulgen method for our evaluation, and found that DAPI staining is useful in that it provides stable fluorescence and specific staining of the nucleus by a very simple procedure (Kobayashi et al., 1988, 1991). In addition, since micromegakaryocytes and megakaryoblasts are often difficult to distinguish on the basis of morphology alone, we reported a method to examine the ploidy in cells stained by the DAPI method after labeling CD41b with FITC (Kobayashi et al., 1997).

A method for ploidy analysis using the morphology antibody chromosome (MAC) technique based on *in situ* hybridization with a chromosome probe has been reported (Larramendy et al., 1994) and has attracted attention. However, analyses by this method should be made carefully, because megakaryocytes with small ploidy classes are clearly more numerous.

### Shifts of the ploidy in hematological disorders

In this section, shifts of the ploidy distribution of human megakaryocytes depending on the disease are reviewed primarily on the basis of our data.

#### *I. Normal controls*

As for the normal ploidy distribution, most reports are in agreement in that the first peak is observed at 16N, with the exceptions of Levine (1980) and Bessman (1982, 1984), who reported a peak at 8N, and Mazur et al. (1988), who reported a peak at 32N, when studies using MAC technique (Larramendy et al., 1994) are excluded. The second peak has been reported by some investigators to be at 32N but by others at 8N, but this discrepancy appears to be due more to individual variation among subjects than to variation among investigators. In our study also, the second peak was observed at 32N in 9 out of 14 normal subjects and at 8N in the others. The minimum ploidy class is 2N in some cells, depending on the method used to identify megakaryocytes, but the morphological identification of these megakaryocytes is generally considered to be

difficult in normal individuals (de Leval, 1968; Kinet-Denoel et al., 1971; Penington and Weste, 1971; Queisser et al., 1971, 1974, 1976; Weste and Penington, 1972; Lagerlof, 1972; Paulus et al., 1974; Penington et al., 1974; Haanen et al., 1975; Mayer et al., 1978; Levine, 1980; Nomura et al., 1983; Winkelmann et al., 1984; Kobayashi et al., 1988, 1991, 1995, 1997; Nagasawa and Nakazawa, 1988; Ridell et al., 1990; Woods et al., 1990; Mori et al., 1991; Maruo et al., 1992; de Alarcon and Graeve, 1996). There is no doubt that there are 2N cells among megakaryoblasts and precursors of megakaryocytes when cells are identified by the immunological method (Renner et al., 1987; Mazur et al., 1988; Renner and Queisser, 1988; Tomer et al., 1988, 1989; Conklin et al., 1990; Kanz et al., 1990; Rabellino and Bussel, 1990; Hegyi et al., 1991; Hancock et al., 1993; Jacobsson et al., 1996; Ma et al., 1996; Brown et al., 1997; Kobayashi et al., 1997). The maximum ploidy class is reported by many investigators to be 64N, but Mazur et al. (1988) have reported the presence of 128N cells. Although the effect of aging has not been directly evaluated, the ploidy pattern does not seem to change in the elderly, according to our evaluations (Kobayashi et al., 1991).

## 2. Chronic idiopathic thrombocytopenic purpura (ITP)

The ploidy of megakaryocytes has long been studied in ITP as a representative benign thrombocytopenic disorder, but findings regarding shifts of the ploidy pattern in this disorder have not been consistent. The ploidy pattern was not reported to be different compared with normal individuals by Queisser et al. (1971) in 3 patients, by Penington and Weste (1971) in 3 patients, and by Nomura et al. (1983) in 6 patients. We found no difference in 5 patients (Kobayashi et al., 1988) and subsequently also obtained the same results in 15 patients. Kinet-Denoel et al. (1971) noted a peak at 8N in 3 out of 5 patients and at 16N in the remaining 2 with an increase in 8N cells, indicating a shift to lower ploidy. Paulus et al. (1974) reported that 16 out of 20 patients had an increased percentage of 8N megakaryocytes. However, Haanen et al. (1975) reported that a peak of ploidy classes was observed at 32N in 11 patients and the mean ploidy values of those patients were significantly increased compared with 10 normal controls, Bessman (1984) reported increases in the ploidy in 4 patients, Mazur et al. (1988) found increases in the ploidy in 5 out of 7 patients, but no difference when compared with normal individuals in 2, and Tomer et al. (1989) reported that megakaryocytes with 32N or greater ploidy classes were increased in 5 patients compared with normal individuals. Conklin et al. (1990) reported increases in the ploidy in 5 patients.

Technical differences in the analytical method and sample preparation procedure as well as the heterogeneity of ITP patients appear to be major causes of the disagreement among the reports.

## 3. Thrombocytopenia due to liver cirrhosis (LC)

There are no reports of megakaryocyte ploidy at LC to our knowledge. According to our data, no difference was observed in 6 patients with thrombocytopenia due to LC compared with normal individuals. There is controversy as to whether thrombocytopenia due to LC is caused by a reduction in thrombopoietin (TPO) produced in the liver (Shimodaira et al., 1996; Martin et al., 1997; Peck-Radosavljevic et al., 1997). The elucidation of this mechanism and its relation to polyploidization are anticipated.

## 4. Chronic myeloproliferative disorder (CMPD)

### (i) Chronic myelogenous leukemia (CML)

Reports are in agreement in that the ploidy shifts to a lower class in CML. A peak was reported to have been observed at 8N by Lagerlof (1972) in 4 patients, by Renner and Queisser (1988) in 10 patients, by Kanz et al. (1990) in 10 patients and by Jacobsson et al. (1996) in 4 patients with CML; Nagasawa (1993) observed that the mean ploidy was smaller in 30 CML patients at 12.5N compared with 19.6N in 10 normal individuals. Paulus et al. (1974) reported that a peak was observed at 8N in 1 patient and at 2N in 2 patients. Penington et al. (1974) reported a shift to a smaller ploidy with a peak at 16N and an increase in 8N cells in 3 patients. The studies concerning CML are in complete agreement, whereas the ploidy pattern has often differed among investigators in other disorders. These findings, which suggest differences in the cell turnover of the megakaryocyte series and mark clear biological differences compared with CMPD such as polycythemia vera (PV) and essential thrombocythemia (ET), are worthy of attention.

### (ii) Polycythemia vera (PV) and essential thrombocythemia (ET)

Differences in megakaryocytes in disorders such as PV and ET are of interest, and the ploidy in these conditions has been evaluated. The ploidy is often reported to be greater in PV and ET than normal: Penington et al. (1974) in 6 myeloproliferative syndrome; Queisser et al. (1976) in 5 PV patients; Mazur et al. (1988) in 4 out of 5 ET patients; Tomer et al. (1989) in 2 PV patients and 2 ET patients; Kanz et al. (1990) in 6 PV and 8 ET patients; Ridell et al. (1990) in 4 PV and 7 ET patients; Woods et al. (1990) in 6 ET patients; Conklin et al. (1990) in 1 ET and 2 out of 3 PV patients; Nagasawa (1993) in 15 PV and 15 ET patients and Jacobsson et al. (1996) in 8 PV and 17 ET patients. Moreover, Mazur et al. (1988) detected megakaryocytes with a ploidy class of 256N or greater in 4 out of 5 ET patients. Woods et al. (1990) mentioned the presence of 128N megakaryocytes, which are not observed in reactive thrombocytosis, as a characteristic of ET and

PV. Between ET and PV, Tomer et al. (1989) and Jacobsson et al. (1996) showed that the ploidy tends to be greater in ET, and Nagasawa (1993) observed a larger ploidy in PV. Kanz et al. (1990) reported no significant difference between the two disorders.

Lagerlof (1972) found that the ploidy was the same as that in normal individuals in 2 out of 5 PV patients, shifted to a smaller class in 1, and shifted to a larger class in 2. Conklin et al. (1990) reported that only 1 of 3 PV patients had a shift to smaller ploidy.

### (iii) Chronic neutrophilic leukemia (CNL)

We observed no shift in the ploidy distribution in 1 patient with CNL. Similar results were obtained by Mori et al. (1991) in 1 patient and Nagasawa (1993) in 2 patients.

## 5. Reactive thrombocytosis

Most studies of reactive thrombocytosis have revealed that the ploidy is shifted to a larger class or is not different from the normal ploidy. Mazur et al. (1988) reported a shift to a larger ploidy in 1 patient. Kanz et al. (1990) found that the ploidy was greater than normal but smaller than that in PV or ET in 23 patients. Woods et al. (1990) noted that the ploidy was greater than that in normal individuals, with a peak being observed at 32N in 3 patients. Lagerlof (1972) observed that the ploidy was shifted to a larger class with a peak occurring at 32N in 1 of 2 patients but that there was no difference compared with normal individuals in the other patient. Penington et al. (1974) reported that the normal pattern or some shift to a larger ploidy was apparent in 6 out of 7 patients; only 1 patient showed a shift to a smaller ploidy. Tomer et al. (1989) also reported that the ploidy was not different compared with normal individuals in 6 out of 10 patients and was shifted to a larger class in the remaining 4 patients. Conklin et al. (1990) reported a shift to a larger ploidy in 17 out of 22 patients, no shift in 1 and a shift to a smaller ploidy in 4. Ridell et al. (1990) and Jacobsson et al. (1996) found that the ploidy was not different compared with normal individuals in all 9 and 7 patients studied, respectively. Bessman (1984), however, noted a shift to a smaller ploidy in 3 patients.

Concerning reactive thrombocytosis, the actual ploidy pattern may have some diversity depending on the underlying disorder and the platelet count, in addition to apparent differences depending on the analytical method.

## 6. Myelodysplastic syndrome (MDS)

Since the concept of MDS was presented in 1982 (Bennett et al., 1982), there have been few reports regarding the ploidy in MDS. Queisser et al. (1974) reported a shift to a smaller ploidy in 3 out of 6 patients and to a larger ploidy in the remaining 3 as a

preleukemic state. Paulus et al. (1974) reported a shift to a smaller ploidy in 3 out of 4 patients with refractory anemia and no shift in the other patient. Tomer et al. (1989) reported that two patients showed a unique pattern with an increase in both cells which were smaller than 8N and cells which were larger than 32N. Conklin et al. (1990) reported that 5 out of 7 patients had a shift to a smaller ploidy, 1 patient had no shift and 1 patient had a bipolar shift. We found a shift to a smaller ploidy in 12 patients with MDS (Kobayashi et al., 1991). Our subsequent evaluation in 23 patients using a method of morphological identification of megakaryocytes indicated a shift to a smaller ploidy in 91.3% of the patients, i.e., the peak was observed at 8N in 15 and at 4N in 6, but peaks at 16N and 32N were observed in 1 patient each. These findings are considered to represent heterogeneity of MDS, but polyploidization is thought to be impaired in most MDS patients. No direct relationship was observed between the degree of the shift in the ploidy and the platelet count, clinical course, or prognosis.

## 7. Acute leukemia

Queisser et al. (1974) found that the ploidy was shifted to a smaller class in 5 patients with untreated acute leukemia and that it normalized with complete remission. Bessman (1982) serially measured megakaryocyte ploidy during 17 courses of chemotherapy for 10 patients (subsequently in 13 patients (1984)) with acute nonlymphocytic leukemia and noted that the mean ploidy class undergoing chemotherapy was smaller than that in normal individuals and that the changes in the ploidy occurred before changes in the platelet volume and platelet count. Mazur et al. (1988) also reported shifts of the ploidy to a smaller class in 2 patients. We have not studied the ploidy in acute leukemia, because megakaryocytes are rarely observed in acute leukemia before treatment, but we have confirmed normalization of the ploidy pattern on complete remission (unpublished).

## 8. Megaloblastic anemia

Queisser et al. (1971) reported that the ploidy was shifted to a smaller class in 3 patients with pernicious anemia, but Bessman (1984) noted no difference in 2 patients with megaloblastic anemia due to folate deficiency compared with normal individuals. Conklin et al. (1990) reported that 2 patients had a shift to a smaller ploidy.

## 9. May-Hegglin anomaly

Mayer et al. (1978) reported that the ploidy pattern was not different and the proportion of mature megakaryocytes was decreased in 3 patients with May-Hegglin anomaly compared with normal individuals, suggesting that the cytoplasmic maturation was

disturbed. These findings are in an interesting contrast to findings that megakaryocytes in CML (Renner and Queisser, 1988) and MDS (Queisser et al., 1971; Kobayashi et al., 1991, 1995) were mature and had impaired polyploidization.

#### 10. Aplastic anemia

Bessman (1984) reported that the ploidy was shifted to a smaller class in 3 aplastic anemia patients. Conklin et al. (1990) reported that 1 of 2 patients had a shift to a larger ploidy and 1 patient had a shift to a smaller ploidy with 64N cells increased to 3.2% compared with 0.5% in normal controls. They suspected that some toxic process prevented the polyploidization, while another subset that survived or escaped the toxic process was driven toward high ploidy classes.

#### Shifts of the ploidy in other disorders

Relationships of the ploidy and disorders such as atherosclerosis and diabetes mellitus have also been evaluated (Bath et al., 1994; Brown et al., 1997; van der Loo and Martin, 1997). Winkelmann et al. (1984) observed that patients with metastatic tumors had a peak of ploidy at 32N regardless of the presence or absence of paraneoplastic thrombosis and that their ploidy was greater than that of normal individuals.

#### Others

The relationship of the ploidy with the platelet volume is described in detail in the studies by Paulus et al. (1974) and Bessman (1982, 1984) and reviews by Corash (1989a,b) and Martin (1989). Hancock et al. (1993) evaluated the relationship between the ploidy and mRNA expression.

Concerning ontogeny, the ploidy has been reported to increase with the growth of the fetus (Hegyi et al., 1991; de Alarcon and Graeve, 1996; Ma et al., 1996).

#### Significance and mechanism of polyploidization

The polyploidy of megakaryocytes due to endomitosis is considered to be advantageous for platelet production. However, this has not been confirmed directly in any study to date. The mechanism of polyploidization also remains unknown, but some relevant information has accumulated.

Polyploidization and megakaryocyte maturation are considered to be regulated separately, and they appear to be related to different factors and genes (Odell et al., 1965; Odell and Jackson, 1968; Queisser et al., 1971; Queisser et al., 1974, 1976; Mayer et al., 1978; Renner and Queisser, 1988; Kobayashi et al., 1991, 1995; Maruo et al., 1992; Kikuchi et al., 1997). GATA-1 gene (Yoshino et al., 1996), the control of cyclins E and A, and cdc2 activation through the down-regulation of cdc25C protein phosphatase (Garcia and Cales, 1996),

cyclin D3 (Wang et al., 1995; Zimmet et al., 1997), cyclin B and Cdc2 kinase (Zhang et al., 1996, 1998; Yokoe et al., 1997), cyclin-dependent kinase inhibitor p21 (Kikuchi et al., 1997), and rho p21 (Takada et al., 1996) have been suggested to be involved in polyploidization.

#### Prospects for the future

The discovery of TPO is expected to lead to further increases in our understanding of megakaryocytes. The further accumulation of information concerning the platelet-producing ability of individual megakaryocytes and the biological significance of the polyploidy is anticipated.

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