THROMBOPOIETIN RESPONSIVENESS REFLECTS THE NUMBER
OF DOUBLINGS UNDERGONE BY MEGAKARYOCYTE PROGENITORS

SHORT TITLE : TPO RESPONSIVENESS OF MEGAKARYOCYTE PROGENITORS

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ABSTRACT

To assess the variation of thrombopoietin (TPO) responsiveness associated with megakaryocyte (MK) progenitor amplification, TPO dose-response curves were obtained for normal human, single-cell plated CD34⁺CD41⁺ cells. The number of MK per well was determined in situ and expressed as number of doublings (NbD). Dose-response curves of the mean frequency of clones of each size vs logTPO concentration showed highly significant differences in the TPO concentration needed for half-maximum generation of clones of different sizes (TPO₅₀): 1.89±0.51 pg/mL for 1 MK clones; 7.75±0.81 for 2-3 MK clones; 38.5±5.04 for 4-7 MK clones and 91.8±16.0 for 8-15 MK clones. These results were consistent with a prediction of the generation-age model, because the number of previous doublings in vivo was inversely correlated with the number of residual doublings in vitro. TPO responsiveness decreased in vitro by a factor of 3.5 per doubling, reflecting the recruitment of progressively more ancestral progenitors. In support of this hypothesis, the more mature CD34⁺CD41⁺CD42⁺ cell fraction had a lower TPO₅₀ (p<0.001), underwent lesser NbD (p<0.001) and expressed a 2.8-fold greater median Mpl receptor density (p<0.001) than the CD34⁺CD41⁺CD42⁻ fraction. Progenitors which have completed their proliferative program have maximum factor responsiveness and are preferentially induced to terminal differentiation.
INTRODUCTION

Studies of megakaryocyte colonies have provided a simple, robust model of the genesis of clonal size heterogeneity in hemopoietic colonies. Dividing megakaryocyte colony-forming units (CFU-MK) cultured in vitro leave the proliferative pool at a constant rate per doubling, so that the numbers of doublings (NbD) undergone by these progenitors are exponential distributions \(^{1,2}\). The rate of exit from the proliferative compartment is decreased by stimulators of megakaryocyte colony formation and is lower in mixed erythroid-megakaryocyte than in pure megakaryocyte colonies \(^{2}\).

It was first thought that the exponential functions were generated as a result of a single random event arresting proliferation and occurring somewhat synchronously within individual clones. Among several, non-mutually exclusive possibilities, the critical event may be geared to the assembling or disassembling of a protein-protein complex binding to a regulatory DNA element \(^{3}\), the TPO-induced change in a critical component of the cell cycle resulting in the abortive mitoses which induce polyploidy \(^{4-6}\), and/or the all-or-none, switch-like responses of the mitogen-activated protein kinase (MAPK) cascade \(^{7,8}\) which has been associated with induction of polyploidization in megakaryocyte cell lines \(^{9-11}\) and normal megakaryocytes \(^{10,11}\). However, the random occurrence of the critical event and the validity of a stochastic model based on this premise are refuted by several data. In fact, data demonstrate that CFU-MK determine in part the amplification of their progeny and therefore impart a degree of homogeneity to this progeny \(^{12}\). In the granulocyte-macrophage series, the squared correlation coefficient of approximately 0.50 in NbD achieved by each of paired CFU-GM daughter cells, which can be calculated from the data of Metcalf \(^{13}\), indicates that one half of the total
variance in NbD undergone by CFU-GM is related to preexisting, heritable characteristics of the progenitor cells. Thus, evidence of synchronization in whole clones implies that the commitment event is not random among the progeny of the colony-forming cell\(^2\). A second major difficulty with a model based on stochastic commitment to polyploidization is the existence within the proliferative continuum of intrinsic differences which are correlated with proliferative potential\(^2,14\). These intrinsic characteristics contradict the view that interclonal heterogeneity is generated by a homogeneous population of progenitors undergoing random cessation of proliferation. Therefore, it appears that clonogenic cells are themselves heterogeneous in parameters which predict, or even determine, their proliferative behavior. How this heterogeneity is created is not precisely understood. Among individual progenitors of the granulocytic\(^15\), erythrocytic\(^16\) and megakaryocytic\(^14,17,18\) series, intrinsic cytokine sensitivity varies inversely to proliferative potential. In megakaryocyte cultures, the progenitors of single megakaryocytes, of doublets and of CFU-MK which generate colonies with \(\geq 3\) megakaryocytes display progressively decreasing responsiveness to thrombopoietin, respectively\(^18\). Generation-age may therefore be a determinant of progenitor heterogeneity. However, intrinsic heterogeneity of colony progenitors does not necessarily reflect an age structure or differences in mitotic history since large variations in proliferative potential have been demonstrated between paired daughter progenitor cells born from hemopoietic stem cells\(^19-22\). In addition, there is evidence that some interlineage plasticity may persist during differentiation\(^23,24\) and induction of a program of megakaryocytic differentiation associated with transcription of the TPO receptor and acquisition of TPO responsiveness has been described\(^25\). Factors affecting progenitor sensitivity to growth regulators independently from generation-age may therefore be important. However, the precise relationship between age-dependent and age-independent variations in factor responsiveness remains unknown. A basic
question is whether the number of doublings (NbD) undergone by megakaryocyte progenitors in vitro depends on the NbD that previously occurred in vivo or whether they are set by some intrinsic properties, such as cytokine responsiveness, which would be totally or partially independent of progenitor past mitotic history.

In this study, we determined the thrombopoietin (TPO) dose-response curves of human CD34⁺CD41⁺ megakaryocyte progenitors which generated clones of various sizes. We found that the range of responsiveness of individual progenitors covered 4 orders of magnitude and that TPO responsiveness was inversely correlated to clone size, expressed as number of doublings (NbD). In order to interpret these data, we tested a prediction of the generation-age model, namely that under constant culture conditions, the percentage of progenitors undergoing a given number of residual doublings (NbDr) in vitro is determined by the number of previous doublings (NbDp) they had undergone in vivo. The data established that in the serum-deprived culture system used, 78 % of the variation in TPO responsiveness of megakaryocyte progenitors could be interpreted as the effect of a 3.5-fold increase in responsiveness per previous progenitor doubling in vivo.

**MATERIALS AND METHODS**

**Cell cultures.** Bone marrow was obtained from normal adult donors using femoral bone fragments discarded during hip surgery. CD34⁺CD41⁺ cells (average, 2% of the CD34⁺ cells) were sorted as single cells into 96-well Terasaki plates containing 20 µL of Iscove’s modified Dulbecco’s medium (Invitrogen, Cergy-Pontoise, France) which contained penicillin (250 U/ml), streptomycin (250 µg/ml), glutamine (2 mmol/L) (Invitrogen), 76 µM alphamonothioglycerol (Sigma), 1.5% deionized bovine serum albumin (BSA, Cohn fraction
V, Sigma), 10 µl insulin-transferrin-selenium (Invitrogen), and a mixture of sonicated lipids (20 µl/ml) prepared as previously reported 26. The serum-deprived medium was then supplemented with various concentrations of a truncated form of rhuTPO, kindly provided by Kirin Brewery, Tokyo, Japan. Four plates were used for each of 8-11 TPO concentrations ranging from 0-10³ pg/mL. Cells were cultured for 5 days at 37°C in a fully humidified atmosphere containing 5% CO₂ in air, fixed, and then counted in situ at 100 x magnification by a single microscopist. Previous studies have shown that only CD41⁺ cells develop under these conditions 27,28.

For each experiment, at least 8 TPO concentrations were used and 4, 96-well Terasaki plates were used for each concentration of TPO for a total of at least 32 plates (total = 3072 wells per experiment).

**Fractionation of CD34⁺CD41⁺ progenitors.** In 3 additional experiments, CD34⁺CD41⁺CD42a⁺ and CD34⁺CD41⁺CD42a⁻ progenitors were separated by cell sorting, using antiCD34 conjugated with phycoerythrin-cyanin 5 (PC5; Beckman Coulter, Villepinte, France), antiCD41-allophycocyanin (APC) and antiCD42a-FITC (Becton-Dickinson, Le Pont de Claix, France). Cell sorting was done as described previously 27. In one further experiment, Mpl expression on CD42a⁺ and CD42a⁻ progenitors was compared. Mononucleated cells were treated with anti-Mpl monoclonal antibody (Becton-Dickinson). Following treatment with antimouse IgG-phycoerythrin (PE), cells were incubated with excess mouse serum, and further labeled with conjugated antiCD34, antiCD41 and antiCD42a as above.

**Regression and statistical analyses.** Dose-response curves are hyperbola when response is plotted versus dose on a linear scale and S-shaped (sigmoid) curves with an inflection point ("transition") when plotted on a logscale. Concentrations of TPO ranged from 0.1 to 1,000 pg/mL; in addition, data obtained from culture wells to which no TPO had been
added were plotted at the concentration of 0.01 pg/mL. Use of the logscale in all graphs and of logTPO concentrations in all calculations was justified by the 10^4-fold range in TPO concentrations required and by the fact that the distributions of logTPO threshold for megakaryocyte progenitor stimulation obeyed the gaussian law or the very similar sigmoid model (see below). Quadruplicate counts of megakaryocyte number per clone were determined for each TPO dose. The relative weight of each replicate was calculated as the inverse of its variance, so that the mean counts with the highest precision were given the greatest weight. The weighted means vs logTPO concentration were then fitted to the 29 built-in transition regression equations offered by Tablecurve 2Dv5 software (SPSS, Chicago, IL). Both the cumulative logdose-response curves and their analytical derivatives, the bell-shaped frequency distributions of TPO thresholds, were obtained. These analyses yielded the TPO50, defined as the TPO concentration corresponding to the logTPO concentration at half-plateau number of clones. They also provided a measure of progenitor responsiveness, defined as the inverse of TPO50 (1/TPO50).

Characterization of clone size classes. As described previously, the term "megakaryocyte clone" was used as a generic name for all megakaryocyte entities growing from single sorted cells. Such entities included colonies, generally defined as clones with ≥ 3 megakaryocytes, as well as single megakaryocyte clones or two-megakaryocyte clones. Clone size was expressed as number of doublings (NbD), which were determined as \( \log_2 \) of megakaryocyte count per well (i.e., \( \log_{10} \) count x 3.3219). \( \log_2 \) was chosen because it provides a suitable basis for expressing the number of doublings, although a log of any base could have been utilized and would have generated the same results. The overall curve was resolved into class-size dependent curves by fitting dose-response regression equations to the
frequencies of clones with 1 (0 doubling; db), 2-3 (1-1.58 db), 4-7 (2-2.8 db), or 8-15 (3-3.9 db) megakaryocytes.

In some experiments, the aim was to determine the previous number of doublings, NbD_p, undergone in vivo by progenitors which undergo in vitro the residual number of doublings NbD_r. For determination of NbD_r, megakaryocyte counts per well were used. For determination of NbD_p a generation-age structure was assumed, in which the progeny of the earliest TPO-responsive MK progenitor undergo a maturation process involving a nearly constant total NbD before mitotic arrest. For the size class NbD_r, the corresponding NbD_p was obtained from the plateau number of clones N yielded by the dose-response curve. N is proportional to 2^{NbD_r} (for example, if NbD_r is 3, then N is proportional to 2^3 = 8) so that N = 2^{NbD_r} k, where k, a proportional factor accounting for sample size may be omitted if the NbD_r of the earliest TPO-responsive MK progenitor is set at zero doubling. Then, \log_2 N = NbD_p and provides an estimate of the number of previous doublings elapsed since the earliest progenitor.

**RESULTS**

**Overall TPO dose-response curves.** A logdose-response curve for all clones (≥ 1 megakaryocyte) is shown in Fig 1, top panel. In 3 independent experiments, the mean plateau cloning efficiency (percent wells containing at least one megakaryocyte) was 53.5 ± 19.9 %. In each experiment, the gaussian cumulative model provided the best fit to data points when compared to 29 transition regression equations (r^2>0.99; p< 0.001 in all cases). The inflection point, i.e., the TPO concentration (TPO_{50}) corresponding to the logTPO at half-plateau number of total clones, was 5.91 ± 0.72 pg/mL (mean±1 SEM; n = 3). The 95% confidence intervals of the estimates are given in Table 1. Also shown in Fig 1 (bottom panel) is the
Fig 1. Cumulative (top panel) and derivative (bottom panel) dose-response curves for the overall sample of clones derived from CD34+CD41+ progenitors. Sorted progenitors were individually cultured for 5 days in serum-deprived medium with the indicated concentrations of TPO. Clones contained 1-16 megakaryocytes, corresponding to 0-4 progenitor doublings. Data points are the mean ± 1 SEM of quadruplicate determinations of the number of clones at day 5 per 96-well Terasaki plate. A gaussian cumulative equation gave the best fit to data among 29 functions based on transitional models (r²=0.99; p<0.001). The bell-shaped bottom curve, the gaussian analytical derivative of the top curve, is the frequency distribution of TPO thresholds of individual progenitors. Counts obtained for wells with no TPO were assigned a concentration of 10⁻² pg/mL to preclude entering the log of zero values in regressions. In this experiment, the mean absolute number of clones per 96-well plate which survived in absence of TPO was 4.44 ± 0.82 (i.e., 6.05 % of the total clones). The dotted vertical drop lines indicate TPO₅₀, i.e., the TPO dose at which 50% of the plateau number of clones were counted. Results are from experiment #1 and are representative of 3 experiments.

derivative curve, i.e., the bell-shaped frequency distribution of TPO thresholds, the concentrations required to recruit individual progenitors. Note that the peak of this curve corresponds to the median TPO threshold for all clones, which is also the TPO₅₀. The fact that this curve fitted a gaussian model indicated that the distribution of TPO thresholds was lognormal. Because counting showed that single megakaryocytes were present in some wells to which no TPO had been added, curves with an intercept parameter were preferred for both
the gaussian and sigmoid models. The intercept on the ordinate of the dose-response curves for 1 MK and 1-15 MK. indicated that 7.62 ± 0.57 % (mean±1 SEM; n = 6) of CD34^+CD41^+ cells plated were not dependent on TPO for survival in the conditions used.

### Table 1. Characteristics of TPO dose-response curves of CD34^+CD41^+ progenitors

<table>
<thead>
<tr>
<th>Exper No.</th>
<th>Clone size</th>
<th>Clone size Mean NbD (range)</th>
<th>Plateau no.of clones*</th>
<th>% of total (95% CI)</th>
<th>TPO_{50} (pg/mL) (95% CI)</th>
<th>CV$^{§}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>1</td>
<td>0 (0)</td>
<td>55.1 (51.9 – 58.3)</td>
<td>1.26 (0.62 – 2.55)</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>1.16 (1-1.58)</td>
<td>20.2 (17.3 – 23.0)</td>
<td>8.09 (4.87 – 13.4)</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-7</td>
<td>2.28 (2-2.80)</td>
<td>18.4 (17.9 – 19.0)</td>
<td>41.1 (37.3 – 45.3)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8-15</td>
<td>3.20 (3-3.90)</td>
<td>6.23 (6.23 - 6.23)</td>
<td>64.7 (64.4 – 64.9)</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1-15</td>
<td>0.50 (0-3.90)</td>
<td>102 (84.0 –117)$</td>
<td>4.92 (2.68 – 9.04)</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>1</td>
<td>0 (0)</td>
<td>50.6 (41.6 – 59.5)</td>
<td>1.52 (0.64 – 3.62)</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>1.24 (1-1.58)</td>
<td>19.8 (15.9 – 23.6)</td>
<td>6.22 (2.51 – 15.5)</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-7</td>
<td>2.29 (2-2.80)</td>
<td>21.8 (19.7 – 24.0)</td>
<td>45.7 (34.7 – 60.2)</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8-15</td>
<td>3.21 (3-3.90)</td>
<td>7.80 (7.80 – 7.80)</td>
<td>90.7 (50.0 – 165)</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1-15</td>
<td>0.73 (0-3.90)</td>
<td>96.8 (88.5 –105)$</td>
<td>5.50 (3.64 – 8.00)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>1</td>
<td>0(0)</td>
<td>43.2 (33.0 – 53.1)</td>
<td>2.89 (1.48 – 5.65)</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>1.22 (1-1.58)</td>
<td>25.6 (21.6 – 29.6)</td>
<td>8.95 (7.31 – 11.0)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-7</td>
<td>2.30 (2-2.80)</td>
<td>21.8 (21.1 – 22.5)</td>
<td>28.8 (27.7 – 30.0)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8-15</td>
<td>3.30 (3-3.90)</td>
<td>9.42 (7.83 – 11.0)</td>
<td>120 (88.0 – 165)</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1-15</td>
<td>0.83 (0-3.90)</td>
<td>100 (90.5 –110)</td>
<td>7.31 (5.53 – 9.66)</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>

*The mean ± 1 SD absolute number of each MK clone size per 96-well Terasaki plate (based on 3 experiments with quadruplicate determinations for each of 8-10 TPO concentrations) was: 1 MK, 26.1 ± 12.6; 2-3 MK, 10.9 ± 3.20; 4-7 MK, 10.4 ± 2.89; 8-15 MK, 3.92 ± 0.63; 1-15 MK, 51.3 ± 20.1. Among 1 MK and 1-15 MK clones, the mean absolute number of clones which survived in absence of TPO was 3.83 ± 0.65. In each experiment, 4, 96-well Terasaki plates were used for each concentration of TPO (total of at least 32 plates).

§ Total percent plateau no. of clones does not equal 100% because the total dose-response curve has been fitted independently of the individual size classes.

§ CV refers to the coefficient of variation (SD/mean) of TPO thresholds.
Responsiveness to TPO and number of residual doublings (NbD_r) are negatively correlated. The overall curve was then resolved into size-class dependent curves by fitting regression equations to the frequencies of clones with 1 (0 doubling; db), 2-3 (1-1.58 db), 4-7 (2-2.8 db), or 8-15 (3-3.9 db) megakaryocytes (Table 1, Fig 2). Again, in each experiment, the

Fig 2. Dose-response curves for CD34+CD41+ progenitors of megakaryocyte clones of various sizes. In the five top panels, dose-response curves have been fitted to the absolute frequencies of clones with the indicated numbers of megakaryocytes. In the bottom panel, the curves have been normalized to a plateau frequency of 100% and superimposed in order to facilitate comparison. Data points are the mean ± 1 SEM of quadruplicate determinations of the number of clones at day 5 per 96-well Terasaki plate. R² values for the gaussian cumulative model were ≥ 0.99 (p<0.001) for all curves. Counts obtained for wells with no TPO were assigned a concentration of 10⁻² pg/mL to preclude entering the log of zero values in regressions. For 1 MK clones, the mean absolute number of clones per 96-well plate which survived in absence of TPO was 4.28 ± 0.72 (i.e., 5.92 % of the total clones). The dotted drop lines indicate TPO₅₀, i.e., the TPO dose at which 50% of the plateau number of clones were counted. Results are representative of 3 experiments.
gaussian cumulative model provided the best fit to the data points when compared to 29 transition regression equations ($r^2 \geq 0.99$; $p<0.001$ in all cases). The curves showed highly significant differences in TPO$_{50}$. Overall, in 3 experiments, TPO$_{50}$, expressed in pg/mL, was $1.89 \pm 0.51$ (mean $\pm$ 1 SEM) for 1 MK clones; $7.75 \pm 0.81$ for 2-3 MK clones; $38.5 \pm 5.04$ for 4-7 MK clones and $91.8 \pm 16.0$ for 8-15 MK clones ($p<0.001$). Figure 3 demonstrates the shift to decreasing responsiveness (i.e., increasing TPO$_{50}$) as the clone size increased.

Fig 3. Distributions of TPO thresholds for progenitors of 1, 2, 3 and 4-cell MK clones and the total population. The bell-shaped curves are the gaussian analytical derivatives of the cumulative dose-response curves for 4 clone sizes and the total population. The peak of each curve indicates the median TPO threshold for the indicated class and corresponds to the respective TPO$_{50}$ shown in Fig 2. The solid line represents the TPO threshold distribution for the total MK progenitor population. The 95% confidence intervals (95% CI) of TPO$_{50}$ were 2.68-9.04 (total MK), 0.62-2.55 (1 MK), 3.36-9.32 (2 MK), 13.9-21.7 (3 MK) and 32.3-35.7 (4 MK) pg/mL. Since the CI did not overlap, TPO$_{50}$ values differed significantly from each other at the 5% level. A representative experiment is shown.

In order to quantitatively estimate the association between TPO responsiveness ($1/TPO_{50}$) and clone size, a correlation analysis was performed. Separate dose-response curves for clones of 1 to 8 MK were used without grouping to benefit from maximum resolution of plateau and NbD values. When responsiveness to TPO was plotted versus NbD$_r$, there was a strong negative correlation between these variables (Fig 4, top panel; $p<0.001$).
Using the overall values of the three experiments, the slope of the regression line indicated that TPO responsiveness decreased by a factor of 3.5 (95% CI 2.9-4.1) per doubling.

Fig 4. Interrelationships between TPO responsiveness and NbD undergone by megakaryocyte progenitors in vivo and in vitro. Relative responsiveness was defined as the absolute responsiveness (1/TPO₅₀) at each data point divided by the responsiveness of the least responsive MK progenitor. NbD have been calculated as detailed in Methods. Inverse correlations were obtained between responsiveness and numbers of residual doublings undergone in vitro (NbDᵣ; top panel) and positive correlations were obtained between responsiveness and numbers of previous doublings undergone in vivo (NbDᵃ; middle panel). As a result, a reciprocal relationship was found between NbDᵣ and NbDᵃ, indicating that a high number of previous doublings was associated with a low number of residual doublings. The converse was also true (bottom panel). The p values for the correlation coefficients were < 0.005.
Responsiveness to TPO and number of previous doublings (NbD_p) are positively correlated. For each size class, the plateau number of clones per 96-well Terasaki plate reflected the relative number of progenitors of that class which were present in the sample of sorted CD34⁺CD41⁺ progenitors cultured. Therefore, under the generation-age assumption, the log₂ of the plateau percentages of each size class provided an estimate of the number of previous doublings (NbD_p) undergone in vivo by the progenitors of the various classes. Because a valid estimate of NbD_p depended on plateau estimates, separate dose-response curves for clones of 1 to 8 MK were used without grouping. There was a strong positive correlation between relative responsiveness and NbD_p (Fig 4, middle panel; p<0.005).

There is a reciprocal relationship between NbD_p and NbD_r. Since TPO responsiveness was negatively correlated with NbD_r and positively correlated with NbD_p, a reciprocal relationship between NbD_p and NbD_r was expected. Indeed, Fig 4 (bottom panel) demonstrates that those progenitors which had previously undergone many doublings in vivo (NbD_p) had the lowest proliferative potential in vitro (NbD_r), and vice versa (p<0.001). As a result of the reciprocal relationship, the total NbD, i.e., NbD_p + NbD_r, calculated as the sum of the abcissa and the ordinate at any point on the correlation slope in the bottom panel of Fig 4, tended to be constant with an approximate value of 3-5 doublings under the conditions of these experiments. Since these curves were based on clones of 1-8 MK rather than 1-15 MK, the best approximation for the total NbD would be 5-7 doublings.

Variations in the number of doublings account for most of the heterogeneity in progenitor responsiveness. In the above analyses, the logTPO₅₀ represented the average response threshold for a given clone size class but did not contain any information about the heterogeneity in response threshold within that class. In order to introduce the missing information into the correlation analysis, four samples of 500 progenitors undergoing 0, 1,
1.58 and 2 db (corresponding to clones with 1, 2, 3 and 4 MK, respectively) were simulated using a random number generator so that their TPO$_{50}$ and the coefficient of variation of their TPO thresholds were those in Table 1. The 2000 simulated values and the corresponding NbD$_r$ were then entered into the computation of a Pearson product moment correlation (Fig 5). The $R^2$ value was 0.78 ($p<0.001$), indicating that an estimated 78% of the total variation in NbD$_r$ and logTPO was explained by their mutual association and that the remaining 22% reflected the effect of factors which influenced clone size and TPO responsiveness independently of the other.

**Fractions of CD34$^+$CD41$^+$ megakaryocyte progenitors differ in responsiveness and NbD$_r$.** In view of the association between high responsiveness and loss of proliferative potential, a comparison was made of the CD34$^+$CD41$^+$CD42a$^+$ and CD34$^+$CD41$^+$CD42a$^-$
fractions separated by cell sorting from the CD34<sup>+</sup>CD41<sup>+</sup> megakaryocyte progenitor population. Fig 6 (top panel) shows that compared with the CD42a<sup>-</sup> fraction, the CD42a<sup>+</sup> fraction was shifted toward the generation of smaller sized clones (chi-square =108, p<0.001).
and produced twice as many single megakaryocyte clones (64 vs 33%). Correspondingly, the CD42a⁺ fraction had a significantly lower TPO₅₀ (1.13 pg/mL; 95% CI 0.427-1.82) than the CD42a⁻ fraction (2.75 pg/mL; 95% CI 1.91-3.96) (Fig 6, middle panel; p<0.001). Again, progenitors which responded best to TPO underwent fewer NbDᵣ.

In one experiment using quadruple labeling and sorting of megakaryocyte progenitors, the expression of Mpl by CD34⁺CD41⁺CD42a⁺ and CD34⁺CD41⁺CD42a⁻ fractions was compared. The expression of Mpl by the total CD34⁺CD41⁺ population, expressed in arbitrary fluorescence units, covered a 3-log range with a median of 25.5 units. As shown in Fig 6 (bottom panel), the most responsive CD42a⁺ cells had a 2.8-fold greater median expression of Mpl (32.5; 95% CI 22.6-42.4) than their 42a⁻ counterparts (11.6; 95% CI 7.7-15.5; p<0.001), suggesting that TPO receptor expression is a significant factor in megakaryocyte progenitor responsiveness to TPO.

**NbDᵣ distributions are derived from TPO dose-response curves.** In the previous sections, the overall TPO dose-response curve was resolved into curves for clones having undergone 0 - 3 doublings. We wondered whether there was any relationship between these dose-response curves and the exponential NbDᵣ distributions referred to in the Introduction. Therefore, dose-response curves were plotted on semi-log coordinates for the number of clones which had undergone ≥0, ≥1, ≥2, or ≥3 doublings as a function of TPO concentration (Fig 7, left panel). Using the plateau values of these curves, the frequencies (on logscale) of the clones which had undergone these NbDᵣ were plotted (Fig 7, right panel). The resulting straight line documented an exponential NbDᵣ distribution, which was derived from the set of size-class dose-response curves.
Fig 7. Relationship between dose-response curves and clone size distributions expressed as NbD. Left panel presents dose-response curves for clones with ≥0, ≥1, ≥2 or ≥3 doublings. Right panel plots the normalized plateau values versus the corresponding NbDr.

DISCUSSION

In this investigation, TPO dose-response curves have been determined for normal human CD34⁺CD41⁺ progenitors sorted into 96-well Terasaki plates and cultured for 5 days in serum-deprived medium supplemented with TPO at a concentration range of 0.1-10³ pg/mL. Only CD41⁺ megakaryocyte clones (defined as entities containing at least one megakaryocyte) are present at day 5 under these conditions 27,28. TPO acts preferentially on late, CD41⁺ megakaryocyte progenitors 29 by binding to the Mpl receptor, which is expressed at low levels in CD34⁺ cells and at high levels during the late stages of human megakaryocyte differentiation 30. The CD42a antigen (glycoprotein Ibα) appears later than CD41 (glycoprotein IIb) 31,32, and was found in this study to be associated with the expression of greater number of Mpl receptors (Fig 6). It had been established that GATA and cis-acting sequences coregulate the megakaryocyte progenitor expression of Mpl 33, CD41 34 and CD42a 35.

TPO responsiveness was recorded both as distributions of TPO thresholds (i.e., the TPO concentrations necessary and sufficient to ensure the survival of megakaryocyte
progenitors, either as single cells or as colonies) and as the medians of these distributions, termed \(\text{TPO}_{50}\) (i.e., the TPO concentration corresponding to the half-plateau number of clones). TPO responsiveness was defined as \(1/\text{TPO}_{50}\). Whereas TPO thresholds had a lognormal distribution, \(\text{TPO}_{50}\) fitted exponential lines when plotted vs \(\text{NbD}_r\). The significance of these distributions is discussed in the following three sections.

The range of responsiveness of individual progenitors covers 4 orders of magnitude. Responsiveness to stimulators depends on the dynamics of ligand-receptor interaction, the combined efficiency of the signal transduction pathways activated by these stimulators, and the pattern of gene expression. The diversity in megakaryocyte progenitor responsiveness is well shown by the bell-shaped frequency distribution of \(\log\text{TPO}\) thresholds which covers a 4-log range. This distribution was recorded, not only for the overall megakaryocyte progenitor population (Fig 1, bottom panel), but also for clones of different sizes (Fig 3). This distribution, which is the derivative of the dose-response curve (Fig 1), obeyed the gaussian or the very similar sigmoid model. Lognormality of progenitor TPO thresholds has straightforward implications for the process which generates diversity of responsiveness, because the genesis of lognormal distributions is well understood. Briefly, a variable (for example, the activity of a TPO-responsive signaling pathway) tends to assume a lognormal distribution when its value at each step of a process is a random proportion (i.e., a random multiplier) of its value at the preceding step. In the case of TPO responsiveness, multiplicative variations (i.e., variations whose effects multiply each other) must originate in the chain of amplifying signaling events triggered by TPO receptor binding. TPO receptor expression itself may be a significant factor in \(\text{CD34}^+\text{CD41}^+\) progenitor responsiveness to TPO since it covered a 3-log range in membrane density and since its median value was 2.8-fold greater in the most responsive \(\text{CD42a}^+\) fraction than in the \(42a^-\) fraction (Fig 6, bottom)).
Lognormality arises because random *multiplicative* variations in the multistep signaling process translate as random *additive* variations of the log of this variable; therefore, logTPO thresholds, rather than TPO thresholds, have a gaussian distribution. Heterogeneity in the number of cycles undergone in vivo before sampling may contribute another mechanism generating lognormality, insofar as these additional variations are also multiplicative.

**Interrelationships between TPO responsiveness and NbD undergone by megakaryocyte progenitors in vivo and in vitro are compatible with a generation-age structure.** When clones were grouped as a function of the size they achieve in vitro, expressed as number of doublings (NbDₐ), it was found that logTPO concentrations at half plateau values (logTPO₅₀) increased, and accordingly that TPO responsiveness, defined as 1/TPO₅₀, decreased with clone size (Table 1, Figs 2, 3). Furthermore, when TPO responsiveness was plotted versus NbDₐ, there was a strong negative correlation between these variables (Fig 4, top panel) and 78% of the variance in TPO responsiveness of individual progenitors was accounted for by its association with NbDₐ (Fig 5). (The remaining 22% appear to be generated by factors which influence TPO responsiveness and NbDₐ independently of each other. Because in each size class, the distribution of TPO thresholds was lognormal (Fig 2, 3), the diversification processes inherent in the cell signaling system discussed above provide the most likely mechanism for the NbD-independent variance in these TPO thresholds).

Comparisons of the 42a⁺ and 42a⁻ fractions of CD34⁺CD41⁺ progenitors, separated by cell sorting, confirmed the inverse relationship between TPO responsiveness and proliferative potential (Fig 6). Compared to the CD42a⁻ fraction, the corresponding CD42a⁺ fraction had lower TPO₅₀ (p<0.001) and underwent lesser NbDₐ (p<0.001). Furthermore, the 42a⁺ fraction expressed a 2.8-fold greater median Mpl receptor density than the 42a⁻ fraction (p<0.001).
These data therefore established a link between TPO responsiveness and Mpl receptor density. A similar relationship between factor responsiveness and receptor density had been found in other systems.  

Two main hypotheses can account for the negative correlation between TPO responsiveness and NbD, depending on which of these two variables is seen as determining the other. In the first, differences in TPO responsiveness, generated by variations in the TPO signaling systems, determine the number of cycles undergone by megakaryocyte progenitors before arrest of mitosis and onset of polyploidization. In that view, the differentiative effects of TPO are dominant over its mitogenic action, so that only the less sensitive progenitors undergo several divisions in culture. In the other view, those megakaryocyte progenitors which accomplish no or few divisions in vitro are those which had undergone several cell cycles in vivo and have thus exhausted all or most of their proliferative capacity. Progenitors of 1 MK clones are therefore very responsive to TPO because they have previously completed a significant part of their proliferative program and because the latter entails a stepwise, generation-linked increase in responsiveness. The latter presumably leads to polyploidization and cytoplasmic maturation.

In order to discriminate between the two models, additional data were required concerning the proliferative past of 1 MK – 8 MK clones. Determination of the number of previous doublings (NbDₚ) approximated this requirement. Assuming that the percentages of 1 MK, 2 MK, …8 MK clones at plateau TPO concentration reflects the number of doublings their ancestral progenitors had previously accomplished in vivo, it was possible to correlate first, TPO responsiveness with NbDₚ (Fig 4, middle panel) and second, NbDᵣ with NbDₚ (Fig 4, bottom panel). A highly significant positive relationship was obtained between TPO responsiveness and NbDₚ and a highly significant reciprocal relationship was shown between
NbD_p and NbD_r. A posteriori, the high correlations validate the estimation of NbD_p from the log2 percentages of plateau values. The results provided evidence that: (1) The number of residual doublings (NbD_r) achieved in vitro is determined by the past mitotic history of the progenitor so that those progenitors which had previously undergone many doublings in vivo (NbD_p) had the lowest proliferative potential in vitro (NbD_r), and vice versa; (2) With each doubling in vitro, TPO responsiveness decreases by a factor of 3.5 per doubling as a result of the recruitment of progressively more ancestral progenitors. Although the value of 3.5 depends on the culture conditions used and cannot be extrapolated to the doublings undergone in vivo, the data of Fig 4 (middle panel) confirm that responsiveness increases significantly at each doubling in vivo. Increase in factor responsiveness together with a decrease in proliferative potential has been shown previously for hematopoietic progenitors during in vivo lineage maturation 14-18,40. In megakaryocyte cultures, the progenitors of CFU-MK colonies with $\geq$ 3 megakaryocytes, of two megakaryocyte clones, and of single megakaryocytes display progressively increasing responsiveness to thrombopoietin 18. Similarly, in oligodendrocyte precursor cells, maturation is associated with an increasing number of thyroid hormone receptors and increasing sensitivity to the hormone 39. Increase in receptor level associated with megakaryocyte progenitor maturation was also demonstrated in the present study (Fig 6, bottom panel). Of interest, the expression level of the PLZF gene progressively increases through megakaryocytic development, and when it is transduced into the erythro-megakaryocytic TF1 cell line, its overexpression induces Mpl and upmodulates megakaryocytic specific glycoproteins as well as platelet factor 4 (PF4) 41. The observation that 7.62% of single megakaryocyte clones survived in the absence of added TPO may even suggest that at least some post-mitotic thrombopoietic cells are TPO independent. However,
this observation may also be explained by the effects of contaminants which have been demonstrated in Cohn's fraction V, and the insulin and transferrin present in the medium 42.

**Exponential distribution of NbD_r also favors the generation-age structure model.** Ascribing the heterogeneity in proliferative potential to the differences in individual progenitor responsiveness (as reflected in the lognormal distribution of TPO thresholds) does not suggest an immediate explanation for the exponential NbD_r distribution. However, the exponential NbD_r distribution naturally arises from the age structure model. At each doubling in vitro, the number of remaining progenitors capable of additional doublings will decrease according to the reciprocal of the in vivo geometric distribution, with the slope of the exponential distribution of NbD_r modulated by the culture conditions used 2. This simplified reasoning can account for the exponential distribution of NbD_r and for the reciprocal relationship between NbD_r and NbD_p. Variations in cell cycle times would not necessarily alter the exponential nature of the doubling distribution.

**Recruitment as a mechanism for increased production.** The effects of stimulators of hematopoiesis are mediated by suppression of apoptosis and promotion of survival 43-48, shortening of the cell cycle 13,49,50 and retardation of differentiation 50. The results described in the present investigation suggest that recruitment provides an additional mechanism of response to stimulators. In Mpl-transduced BaF-3 cells, TPO promotes survival in cells expressing low levels of the receptor but triggers proliferation in those expressing high levels of Mpl 47. This mechanism ensures that when low stimulator concentration and/or receptor density support survival but not proliferation of the least responsive, most ancestral progenitors, other progenitors with a longer proliferative history and greater factor responsiveness will be preferentially induced to proliferation. The more responsive progenitors may also be able to produce the all-or-none, switch-like responses of the MAPK
cascade\textsuperscript{7,8} which is required for the induction of polyploidization\textsuperscript{9-11}. Furthermore, under increasing stimulator concentration (e.g., high concentrations of TPO which have resulted from decreased numbers of megakaryocytes and platelets), progenitors which are progressively less responsive and less mature, but endowed with greater proliferative potential, will be triggered to proliferate. Subsequently, their more responsive progeny will be driven to terminal maturation\textsuperscript{15}. Such a pattern of regulation, based on the strong link between past proliferation and factor responsiveness, provides a coherent framework for the interpretation of the generation-age model of progenitor differentiation.
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Thrombopoietin responsiveness reflects the number of doublings undergone by megakaryocyte progenitors

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