A simple method for enrichment of polychromatic erythroblasts from rat bone marrow, and their proliferation and maturation in vitro

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ABSTRACT — To evaluate the effects of a variety of chemical, biological and physiological stimuli on erythropoiesis, in vitro assays using erythroid progenitor cells from humans or laboratory animals are well-known methods. On the other hand, little has been reported on in vitro assays using mature erythroblasts such as polychromatic erythroblasts. In the present study, we established a convenient method for enrichment of polychromatic erythroblasts from rat bone marrow and confirmed their development in vitro. To establish a method for the enrichment of polychromatic erythroblasts, bone marrow cells from 3- and 10-week-old rats were separated by discontinuous density gradient centrifugation using Percoll. As a result, polychromatic erythroblasts were most highly enriched in the bone marrow fraction from 3-week old rats at the density interface between 1.040 and 1.058 g/ml. The enriched polychromatic erythroblasts were then cultured in growth medium supplemented with 20% fetal bovine serum in the presence or absence of erythropoietin for 48 hr. During the culture period, cell proliferation and maturation to orthochromatic erythroblasts were observed, and intracellular heme contents were also increased. In particular, the culture in the presence of erythropoietin revealed higher proliferation of erythroid cells, and therefore might be more appropriate for in vitro experiments on the effects of various stimuli on late-stage erythropoiesis.

Key words: Polychromatic erythroblast, Discontinuous density gradient centrifugation, Culture, Rat

INTRODUCTION

To evaluate the effects of a variety of chemical, biological and physiological stimuli on erythropoiesis in vitro, colony-forming assays using erythroid progenitor cells from humans or laboratory animals such as dogs and mice, the burst and colony-forming unit-erythroid (BFU-E/CFU-E), are well-known methods (Gribaldo et al., 2000; Kreja et al., 1989; Merchav et al., 1988; Roda et al., 2010). Additionally, immature erythroblasts collected from bone marrow or fetal liver have been used for such in vitro investigations in laboratory animals such as rabbits and rats (Bonanou-Tzedaki et al., 1981; Mayeux et al., 1986). In the process of erythropoiesis, cells differentiate from erythroid progenitor cells through immature erythroblasts (proerythroblasts (PE) and basophilic erythroblasts (BE)) to mature erythroblasts (polychromatic erythroblasts (PCE) and orthochromatic erythroblasts (OCE)). Thus, most in vitro investigations on the effect on erythropoiesis have been conducted using the cells at an early stage of erythropoiesis.

It is known that some chemicals show stage specificity of the effect on erythroid cells, and that the toxicity of dihydroartemisinin is limited to the PE and BE, immature erythroblasts (Finaurini et al., 2010). On the other hand, it has been reported that PCE was more susceptible to the effects of other chemicals in vivo animal tests (Kawamura et al., 1996; Yasuda et al., 1989). PCE is the major cell population in the bone marrow (Doing, 2007; Edamoto et al., 2007; Tokalov et al., 2007), and exhibits the highest activity of heme synthesis (Wickrema et al., 1992). Additionally, this is the last stage in which the cell
is capable of undergoing mitosis (Doing, 2007). Therefore, in some cases, in vitro investigations using mature erythroblasts such as PCE as well as progenitor cells and immature erythroblasts may also be desirable to better understand the effects of various stimuli on erythropoiesis. However, to date little has been reported regarding in vitro investigations, in particular using laboratory animal cells, on the effects of various stimuli on mature erythroblasts such as PCE.

Furthermore, to collect mature erythroblasts from bone marrow, marrow cells need to be separated, because bone marrow consists of many types of cells. Density gradient centrifugation, velocity sedimentation, and their combination are well-known methods for the separation of bone marrow cells (Harrison et al., 1981; Olofsson et al., 1980; Shortman and Seligman, 1969; Sitar et al., 1997). Density gradient centrifugation using Percoll is a convenient technique and commonly used for cell separation, and it has been reported that bone marrow cells could be separated into different types or stages of cells using discontinuous gradients consisting of various densities (Olofsson et al., 1980; Sitar et al., 1997). However, little has been reported on a method for enrichment of PCE from bone marrow of laboratory animals.

This study was designed to establish a convenient method for enrichment of PCE from bone marrow of rats, a common laboratory animal species, and to investigate the development of enriched PCE in culture in order to confirm its usefulness in in vitro experiments on the effects of various stimuli on late-stage erythropoiesis.

**MATERIALS AND METHODS**

**Animals and preparation of bone marrow cells**

All experiments were performed in accordance with The Guide for Animal Care and Use of Sumitomo Chemical Co. Ltd. Sprague-Dawley female rats aged 3 and 10 weeks were purchased from Charles River Japan Inc. (Shiga, Japan) and used for the preparation of bone marrow cells. The animals were euthanized by exsanguination under anesthesia with diethyl ether. Femurs were excised at 8, 24, and 48 hr of culture and used for further examinations. After removal of the supernatant, the cells were resuspended in PBS. Whole bone marrow cells suspended in PBS with 2% FBS were centrifuged at 800 x g for 5 min at 4°C. After removal of supernatant, the cells were resuspended in PBS with 2% FBS, carefully layered over the Percoll gradient, and centrifuged at 1,800 x g for 15 min at 4°C. The cells distributed into fractions at the interfaces or midpoint of the Percoll solutions and a fraction under 1.093 g/ml were collected separately. Collected fractions were washed once with PBS and subjected to cell morphological analysis to establish the PCE-enrichment method.

**Cell cultures**

The fraction at the 1.040/1.058 interface from 3-week-old rats was collected as described above and used for in vitro culture. The collected cells were cultured following a cell culture method for human and mouse erythroblasts (Wada et al., 1999; Zhang et al., 2003) with some modifications. The culture medium consisted of Iscove’s modified Dulbecco’s medium (Invitrogen, Tokyo, Japan) supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin (Wako Pure Chemical industries, Osaka, Japan), and 0.1 mg/ml streptomycin (Wako Pure Chemical industries). Additionally, to inhibit lymphocyte-monocyte growth, 1 μg/ml cyclosporine A (Wako Pure Chemical industries) was added to the culture medium (Ronzoni et al., 2008). The collected cells were suspended in the culture medium and washed once. After removal of the supernatant, the cells were resuspended in the culture medium at a concentration of approximately 10^6 cells/ml. Human recombinant erythropoietin (EPO, Calbiochem, La Jolla, CA, USA) was added to the cell suspension at a final concentration of 2 U/ml. The cell suspension was placed in a well of a 24-well flat-bottom culture plate and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 48 hr. Culture media were not exchanged during incubation. The cells were harvested at 8, 24, and 48 hr of culture and used for further examinations.

**Cell morphology analysis**

Cell morphology was analyzed by light microscopy on cytocentrifuged smears stained with May-Grünwald-Giemsa. Five hundred cells were counted on each slide for differential cell counts and then compositions of cell populations were determined.
Cell number and viability determination

Cell viability was determined by trypan blue (Nacalai Tesque Inc., Kyoto, Japan) staining and microscopic evaluation. To measure cell proliferation, viable cells were enumerated with an improved Neubauer counting chamber. The number of each type of cell was calculated from the number of viable cells and differential cell counts.

Measurement of heme

Intracellular heme content was measured using colorimetric determination at 400 nm with a QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA, USA). In brief, 1.5 ml of cell suspension was centrifuged at 10,000 x g for 1 min at room temperature. After removal of supernatant, cells were washed twice with PBS, and cell pellets were stored at -80°C until the measurement. The cell pellets were diluted in 50 μl of distilled water and assayed to determine their heme contents based on the manufacturer’s instructions.

Statistical analysis

In comparisons of the number of viable cells and intracellular heme contents in the presence and absence of EPO, Student’s t-test was used for statistical analysis.

RESULTS

Enrichment of polychromatic erythroblasts from rat bone marrow cells by discontinuous density gradient using Percoll

The whole bone marrow cells of 3- and 10-week-old rats were separated into five fractions by the density gradient centrifugation (Fig. 1). Fraction 1 (F1) was collected from the region above the 1.040 g/ml layer. Fractions 2 and 4 (F2 and F4) were collected at the 1.040/1.058 and 1.076/1.093 interfaces, respectively. Fraction 3 (F3) was collected at the 1.058/1.076 interface and midpoint of the 1.076 g/ml layer. Fraction 5 (F5) was collected from the region under the 1.093 g/ml layer. The differential cell counts were performed on the whole bone marrow and fractionated cells (Tables 1 and 2). In the whole bone marrow cells, the percentages of total nucleated erythroid cells and PCE were higher in the 3-week-old rats. The percentages of total nucleated erythroid cells in the 3- and 10-week-old rats were 42.6% and 35.6%, respectively. The percentages of PCE in the 3- and 10-week-old rats were 37.1% and 31.0%, respectively, and showed the highest percentages among the erythroid cells. The ratios of myeloid cells to nucleated erythroid cells (M/E) were 0.49 in the 3-week-old rats and 0.95 in the 10-week-old rats. Lower M/E in the 3-week-old rats reflected both higher percentage of enucleated erythroid cells and lower percentage of myeloid cells.

After separation, although PCE was found in every fraction, the percentages in F2 collected from the 3- and 10-week-old rats were 62.1% and 45.9%, respectively, and showed the highest values among all fractions. On the other hand, the percentages of myeloid cells in F2 were lower in the rats at both ages and showed the lowest

Enrichment and in vitro culture of rat polychromatic erythroblasts

Fig. 1. Schematic drawing of the density gradient used for whole bone marrow separation. The arrows indicate the regions of fraction collection. Fraction 1 (F1) was collected from the region above the 1.040 g/ml layer. Fractions 2 and 4 (F2 and F4) were collected at density interfaces. Fraction 3 (F3) was collected at the density interface and midpoint of the 1.076 g/ml layer. Fraction 5 (F5) was collected from the region under the 1.093 g/ml layer.

Fig. 2. Changes in the number of viable cells in culture. The F2 cells collected from 3-week-old rats were cultured in the presence and absence of EPO for 48 hr. Values are presented as mean ± S.D. (n = 3-8). **p < 0.01.
value in the 3-week-old rats. Regarding other erythroid cells of the rats at both ages, the percentages of immature cells (i.e. PE and BE) were higher in lower density fractions, while the percentages of mature cells (i.e. OCE and enucleated cells) were increased in higher density fractions. Particularly, in the 3-week-old rats, the percentages of PE and BE were the highest in F1 while those of OCE and enucleated cells were the highest in F5.

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Table 1. Myelogram of bone marrow cells of 3-week-old rats separated by Percoll density gradient centrifugation

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Cells</th>
<th>WMCs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Erythroid</td>
<td>Nucleated cells</td>
<td>42.6 ± 1.6</td>
<td>54.8 ± 1.8</td>
<td>69.1 ± 4.1</td>
<td>54.1 ± 7.3</td>
<td>31.9 ± 4.8</td>
<td>23.3 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>Mitotic cells</td>
<td>0.5 ± 0.3</td>
<td>2.1 ± 0.1</td>
<td>1.0 ± 1.0</td>
<td>1.6 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>BE</td>
<td>2.9 ± 0.5</td>
<td>5.4 ± 1.4</td>
<td>3.5 ± 1.1</td>
<td>1.9 ± 0.5</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>37.1 ± 1.1</td>
<td>45.8 ± 2.7</td>
<td>62.1 ± 5.3</td>
<td>48.9 ± 7.0</td>
<td>29.0 ± 4.9</td>
<td>19.9 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>OCE</td>
<td>1.7 ± 1.3</td>
<td>0.6 ± 1.0</td>
<td>2.0 ± 1.2</td>
<td>1.7 ± 1.3</td>
<td>2.7 ± 0.5</td>
<td>3.2 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Enucleated cells</td>
<td>6.9 ± 1.8</td>
<td>3.7 ± 0.4</td>
<td>4.2 ± 1.1</td>
<td>2.3 ± 0.8</td>
<td>12.4 ± 2.4</td>
<td>30.5 ± 6.8</td>
</tr>
<tr>
<td>Myeloid cells</td>
<td></td>
<td>21.0 ± 1.8</td>
<td>17.1 ± 2.2</td>
<td>10.8 ± 3.5</td>
<td>27.9 ± 3.8</td>
<td>16.4 ± 1.6</td>
<td>29.1 ± 4.2</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td></td>
<td>28.9 ± 3.8</td>
<td>13.1 ± 3.3</td>
<td>15.0 ± 3.7</td>
<td>15.4 ± 4.6</td>
<td>39.3 ± 3.7</td>
<td>7.1 ± 2.6</td>
</tr>
<tr>
<td>Megakaryocyte</td>
<td></td>
<td>0.1 ± 0.1</td>
<td>10.2 ± 1.1</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Other cells</td>
<td></td>
<td>0.3 ± 0.2</td>
<td>1.1 ± 0.6</td>
<td>0.7 ± 0.4</td>
<td>0.2 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>M/E</td>
<td></td>
<td>0.49 ± 0.03</td>
<td>0.31 ± 0.04</td>
<td>0.16 ± 0.05</td>
<td>0.53 ± 0.14</td>
<td>0.53 ± 0.14</td>
<td>1.71 ± 0.27</td>
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</table>


Table 2. Myelogram of bone marrow cells of 10-week-old rats separated by Percoll density gradient centrifugation

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Cells</th>
<th>WMCs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Erythroid</td>
<td>Nucleated cells</td>
<td>35.6 ± 3.4</td>
<td>36.1 ± 4.6</td>
<td>50.1 ± 2.7</td>
<td>31.0 ± 5.9</td>
<td>18.7 ± 2.3</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Mitotic cells</td>
<td>0.4 ± 0.0</td>
<td>0.9 ± 0.6</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>BE</td>
<td>2.7 ± 1.0</td>
<td>3.1 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>0.4 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>PCE</td>
<td>31.0 ± 2.9</td>
<td>31.8 ± 5.2</td>
<td>45.9 ± 2.6</td>
<td>26.5 ± 4.9</td>
<td>15.7 ± 3.0</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>OCE</td>
<td>1.2 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>1.3 ± 0.3</td>
<td>1.8 ± 0.5</td>
<td>2.5 ± 0.6</td>
<td>1.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Enucleated cells</td>
<td>10.7 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>9.2 ± 2.5</td>
<td>4.3 ± 1.6</td>
<td>6.3 ± 2.7</td>
<td>66.7 ± 2.5</td>
</tr>
<tr>
<td>Myeloid cells</td>
<td></td>
<td>33.2 ± 3.5</td>
<td>21.5 ± 8.8</td>
<td>23.1 ± 1.1</td>
<td>48.9 ± 2.0</td>
<td>30.4 ± 1.6</td>
<td>27.1 ± 2.4</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td></td>
<td>19.8 ± 0.7</td>
<td>12.7 ± 5.1</td>
<td>16.9 ± 0.6</td>
<td>15.6 ± 6.6</td>
<td>44.4 ± 1.4</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>Megakaryocyte</td>
<td></td>
<td>0.1 ± 0.1</td>
<td>28.5 ± 11.3</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Other cells</td>
<td></td>
<td>0.6 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>M/E</td>
<td></td>
<td>0.95 ± 0.19</td>
<td>0.59 ± 0.21</td>
<td>0.45 ± 0.05</td>
<td>1.61 ± 0.27</td>
<td>1.63 ± 0.13</td>
<td>5.41 ± 0.49</td>
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</tbody>
</table>

Proliferation of fraction 2 cells in culture

The F2 cells collected from 3-week-old rats were cultured in the presence or absence of EPO for 48 hr. At 8 hr of culture, the number of total viable cells was slightly increased in both the absence and presence of EPO. However, at 24 and 48 hr, the total number of viable cells in the presence of EPO was significantly increased while that in the absence of EPO had gradually decreased (Fig. 2).

In the differential cell count, the erythroid cells constituted 72.4% of the cell population at the initiation of the culture and showed prominent population throughout the 48-hr culture period. At 48 hr of culture, the erythroid cells in culture in the presence and absence of EPO still constituted over 70% and 60% of the total, respectively (Fig. 3). The changes in the absolute number of erythroid cells were reflected in those of total viable cells because of the prominent population of erythroid cells throughout the culture period (Figs. 2 and 4). The myeloid cells in cultures in both the presence and absence of EPO were gradually increased in absolute numbers during the culture period, and at 48 hr of culture constituted 15.0% and 28.0% of the total, respectively. Lymphocytes constituted 16.6% of the cell population at the initiation of the culture, but gradually decreased during the culture period.
and constituted less than 5.0% of the total at 48 hr of cultures in both presence and absence of EPO. Other cells, including megakaryocytes, macrophages, and monocytes, were slightly increased in the absolute number at 48 hr of culture in both the presence and absence of EPO, but constituted 4.0% or less of the total (Figs. 3 and 4).

Changes in the composition of the erythroid cell population in culture

The cultured F2 cells from 3-week-old rats were morphologically analyzed and the compositions of the erythroid cell population were determined (Fig. 5). PCE constituted 78.8% of the total erythroid cells at the initiation of culture. After 8 hr in cultures in both the presence and absence of EPO, these still constituted approximately 70% of the total. At 24 hr of culture in the absence of EPO, the composition of erythroid cell population was drastically changed, which means that the percentage of PCE had significantly dropped from 73.2% to 3.3% while that of OCE was increased from 6.7% to 66.2%. On the contrary, at 24 hr of culture in the presence of EPO, the erythroid cells did not demonstrate such drastic changes in the composition of cell population, although the percentage of PCE had slightly dropped, from 68.9% to 52.4%. However, at 48 hr of culture in the presence of EPO, the composition of erythroid cell population was significantly changed, as observed at 24 hr of culture in the absence EPO. At this time point, the percentage of PCE was significantly lower, from 52.4% to 3.7%, whereas that of OCE was increased from 21.3% to 69.8%. The composition of erythroid cell population at 48 hr of culture in the presence of EPO was similar to that at 24 hr of culture in the absence of EPO.

The absolute numbers of erythroid cells at each developmental stage were also determined (Table 3). The changes in the number of cells were similar to those in the composition of the cell population described above. Major differences between cultures in the presence and absence of EPO were observed at 24 hr in culture. At this time point, the accumulation of a large number of PCE was still observed in culture in the presence of EPO, whereas a significant decrease in the number of PCE was observed in culture in the absence of EPO. In addition, the numbers of OCE were increased in cultures in both the presence and absence of EPO, but the increment in cell number was greater in culture in the absence of EPO. Regarding immature erythroblasts, BE were observed until 48 hr in culture in the presence of EPO, but were not observed at 24 hr in culture in the absence of EPO.

Changes in heme contents of the cultured erythroid cells

Heme contents of the cultured erythroid cells were determined at each time point (Fig. 6). At 8 and 24 hr in cultures both in the presence and absence of EPO, heme contents, which indicate amount of heme per 10⁶ erythroid cells, were comparable to those at the initiation of culture. At 48 hr of culture, heme contents were increased regardless of EPO stimulation, but the value was significantly lower in culture in the presence of EPO.
DISCUSSION

The main objective of this study was to establish a simple density-gradient method for the enrichment of PCE from rat bone marrow. In this study, Percoll was used as a density solution, and the bone marrow cells were separated using density gradients consisting of four layers, at which densities were 1.040, 1.058, 1.076, and 1.093 g/ml. As a result, the percentage of PCE was the highest in the fraction collected at the 1.040/1.058 interface. A study using murine bone marrow cells demonstrated that a large number of PCE distributed into densities of 1.070 g/ml and above, although the percentage in the density fraction was not determined (Shortman and Seligman, 1969). In the present study, it was possible that the actual number of PCE in the higher density fraction, such as an interface of 1.076/1.093, might have been larger than that in the fraction collected at the 1.040/1.058 interface, as demonstrated in the mouse study. However, even if the actual number was not so large, a fraction with the highest percentage of PCE population, like the fraction collected at the 1.040/1.058 interface in the present study, can be more appropriate for in vitro assays, since it is likely that the effects of various stimuli on PCE can be more easily detected as the purity of PCE in culture is higher. When compared between ages, the percentage of PCE in the fraction collected at the 1.040/1.058 interface was higher in 3-week-old rats. This result was considered to be attributable to the fact that in the present study the bone marrow cells of 3-week-old rats showed higher erythroid cell population than that of 10-week-old rats. Several studies have also demonstrated that the percentage of erythroid cells among whole bone marrow cells was higher in younger rats than in older rats (Dörmer, 1982; Endicott and Ott, 1945; Tokalov et al., 2007). In addition, the percentage of myeloid cells in the fraction collected at the 1.040/1.058 interface was significantly lower in 3-week-old rats, which could also have contributed to the higher percentage of PCE.

Regarding erythroid cells, the pattern of distribution into density fractions differed among the stages of cell maturation. As the erythroid cells matured from PE to enucleated cells, these cells showed a tendency to distribute into higher density fractions. Similar results had also been shown in studies where the mouse and human bone marrow cells were separated (Shortman and Seligman, 1969; Sitar et al., 1997). These differences in the distribution of erythroid cells into density fractions were considered to be caused by the differences in their hemoglobin contents. It is known that density of erythroid cell is increased depending on the accumulation of its hemoglobin content (Shortman and Seligman, 1969). During erythroid cell differentiation,
detectable hemoglobin synthesis occurs at a stage of BE and its content in a cell is increased with maturation (Doing, 2007). Although densities of erythroid cell were different depending on the stage of cell maturation, the distributions of cells at any stage were not limited to a single density fraction as shown in the mouse study (Shortman and Seligman, 1969). In particular, PCE was found in every fraction, although the percentages were highest in the fraction collected at the 1.040/1.058 interface. It is possible that intracellular hemoglobin content was also responsible for such a distribution pattern within cells at the same stage. It has been shown that hemoglobin contents in cells varied within different phases of the cell cycle, and there was a progressive increase in hemoglobin value from G1 through S to G2 phase of the cell cycle (Campbell et al., 1971; Steiner, 1973). Therefore, a possibility is that, in the present study, the cell cycle stages of PCE differed among the density fractions. The PCE in the fraction collected at the 1.040/1.058 interface might be abundant among cells at earlier stages of the cell cycle when compared with those in the higher density fractions. However, further investigations will be needed to analyze relation between density and cell cycle stage of fractionated PCE.

Another objective of this study was to investigate the proliferation and maturation of the enriched PCE in culture in order to confirm its usefulness in in vitro experiments. The cells collected at the 1.040/1.058 interface from 3-week-old rats were incubated in growth medium supplemented with 20% FBS for 48 hr. Also, the development of enriched PCE was compared between cultures in the presence or absence of EPO, the major stimulatory cytokine for erythroid cells, in order to investigate its effect in this culture system. As a result, in both the absence and presence of EPO, the proliferation and maturation of PCE were observed, and heme content was also increased during the culture period, suggesting the usefulness of the enriched rat PCE in in vitro investigations. However, those changes observed in cultures differed depending on the presence or absence of EPO stimulation. It is known that EPO exerts anti-apoptotic and mitotic effects via its receptor on cell surface, which results in the survival and proliferation of erythroid cells (Doing, 2007; Koury, 1992; Koury and Bondurant, 1992; Krantz, 1991). Also, it has been reported that the erythroid cells could respond to EPO stimulation until the early PCE stage (Wickrema et al., 1992). These effects of EPO could account for significant increase in the total number of erythroid cells, including mitotic cells observed in culture in the presence of EPO.

Morphologically, the maturation of PCE to OCE was observed at 48 hr of culture regardless of EPO stimulation. However, at 24 hr of culture, the accumulation of large numbers of PCE was still maintained in culture in the presence of EPO, whereas the PCE had almost disappeared from the culture in the absence of EPO. Similar results had been reported in the study using fetal mouse erythroid cells, and one possibility was that EPO stimulation prolonged the period during which immature erythroblasts were maintained in culture and were contributing to the yield of PCE (Chui et al., 1971). It is likely that this prolongation of the period during which immature erythroblasts were maintained in culture also occurred in the present study, as BE were maintained in culture in the presence of EPO throughout the culture period. Another possible explanation for the accumulation of large numbers of PCE is that the maturation of PCE to OCE was delayed by the effects of EPO. Actually, the numbers of OCE, more mature erythroid cells, at 24 hr of culture in the presence of EPO were smaller than those in the absence of EPO. Also, a study using Ba/F3-EPO-R cells showed that high concentration of EPO blocked their differentiation (Carroll et al., 1995). Further study will be needed to clarify the cause of the accumulation of large numbers of PCE in cultures with EPO. Thus, though there were differences between the presence and absence of EPO, the enriched rat PCE could proliferate and mature to OCE, suggesting the usefulness of this culture system for the evaluation of the effects of a variety of chemical, biological and physiological stimuli on proliferation and maturation of PCE. Furthermore, the culture of enriched PCE in the presence of EPO might be a more sensitive system to detect the effect of various stimuli than that in the absence of EPO, because of higher proliferation and longer period of existence of PCE in culture.

Intracellular heme contents were increased at 48 hr of culture regardless of EPO stimulation. However, it was lower in the presence of EPO than in the absence of EPO, although EPO is known to stimulate heme synthesis (Koury and Bondurant, 1992). Iron is essential for heme synthesis; and transferrin, a plasma protein, transports it into cells (Coleman, 2007). Therefore, it is possible that the amount of iron and transferrin in the culture medium was insufficient for adequate heme synthesis, which resulted in lower intracellular heme content in highly proliferating cells under EPO stimulation. Actually, iron-saturated transferrin is essential in erythroid cell cultures when serum-free media are used (Sandstrom et al., 1994). However, in the present study, the culture medium was supplemented with FBS mostly containing iron and transferrin. Also, a study using human BFU-E had demonstrated that cellular hemoglobin content in FBS-supplemented
culture was comparable to that in serum-free culture supplemented with iron-saturated transferrin (Migliaccio and Migliaccio, 1987). Based on these facts, it was considered unlikely that insufficient amount of iron and transferrin in the culture medium caused lower heme content in the cells cultured with EPO. Considering the relation between hemoglobin contents and cell cycle phases, one possible explanation for lower intracellular heme content in the presence of EPO might be the shorter elapsed time after cell division. At 48 of culture, the elapsed time after the completion of differentiation into OCE was shorter in the presence of EPO, and therefore the cells might have a shorter duration of intracellular heme accumulation. Additionally, at 8 and 24 hr of culture, intracellular heme contents were not increased despite the presence of EPO. The erythroid cells through 8 to 24 hr of culture in the presence of EPO showed high proliferation and therefore might have higher population of G1-phase cells with the lowest value of intracellular hemoglobin content (Steiner, 1973). Thus, at least after 48 hr in culture, increased heme contents in the erythroid cells were confirmed, and therefore it may be possible to evaluate the effect of a variety of chemical, biological and physiological stimuli on heme synthesis using this culture system.

In conclusion, a simple method using Percoll density gradient centrifugation for the enrichment of PCE from rat bone marrow was established. The PCE could be highly enriched in the fraction at the density interface between 1.040 and 1.058 g/ml from the bone marrow of 3-week-old rats. Also, it was confirmed that the enriched PCE could proliferate, mature to OCE, and synthesize heme in in vitro culture regardless of EPO stimulation. In particular, the culture in the presence of EPO revealed higher proliferation of mature erythroblasts, and therefore might be more appropriate for in vitro experiments on the effects of a variety of chemical, biological and physiological stimuli on the development of mature erythroblasts.

REFERENCES


