Brief Report

In Vitro Effect of Erythropoietin on the Spleen of the Polycythemic Mouse. I.

By Kiku Nakao, Yasusada Miura and Fumimaro Takaku

The in vitro erythropoietic activity of “erythropoietin” has been reported by several authors. The in vivo effect of erythropoietin on the differentiation of stem cells into erythroblasts has been demonstrated by several workers. However, studies of the detailed biochemical changes in the differentiating stem cells has thus far been incomplete.

To demonstrate the in vitro effect of erythropoietin on the differentiation of stem cells into erythroblasts, the present study was undertaken. Serial morphologic changes as well as the incorporation of radioiron into the heme of the polycythemic mice spleen, occurring when erythropoietin was added, are described.

Materials and Methods

D-D strain female mice weighing 17 to 24 gm. bred in the Research Institute of Infectious Disease at Tokyo University were used throughout the experiments. The transfusion-induced polycythemia was produced by the method described by Jacobson et al. The spleen was extirpated 5 to 8 days after final transfusion. Only mice with hematocrits of more than 70 per cent were used for the present experiments.

The spleen was washed with saline and cut with a cataract knife into small cubes of approximately 1 cm. Five cubes were attached directly on the wall of the sterile culture tube with plasma clot. Two ml. of incubation medium consisting of 50 per cent NCTC109 and 50 per cent inactivated calf serum plus 50 U/ml. penicillin added to each tube.

Erythropoietin precipitated with alcohol from urine of anemic patients was dissolved in water and added to the medium in a concentration of 0.1 U/ml. The erythropoietin solution, passed through a millipore filter, showed an activity of approximately 5 U/ml. by the assay of starved rats.

Roller tube culture was performed at 37 C. in an atmosphere of 5 per cent CO2 and 95 per cent air. The tubes were rotated in 15 revolutions per hour. In each incubation, 4 tubes were incubated: 3 for heme extraction and one for morphologic studies. The control tubes without the erythropoietin were similarly incubated.

Three microcuries of 35FeCl3 in 0.2 ml. NCTC 109 solution were added to each tube 6 hours before the termination of the incubation. At the end of the indicated incubation intervals, the specimens were removed from the walls of the tubes. They were placed on a piece of aluminum foil, the wet weight determined, and then transferred into a test tube. Cells were lysed by the addition of 1.0 ml. of ice cold diluted hemolysate and 1.0 ml of

From the Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, Japan.
First submitted July 19, 1965; accepted for publication Nov. 24, 1965.
Kiku Nakao, M.D.: Professor of Medicine, 3rd Department of Internal Medicine, Faculty of Medicine, University of Tokyo. Yasusada Miura, M.D.: Research Fellow, 3rd Department of Internal Medicine, Faculty of Medicine, University of Tokyo. Fumimaro Takaku, M.D.: Assistant, 3rd Department of Internal Medicine, Faculty of Medicine, University of Tokyo.
IN VITRO EFFECT OF ERYTHROPOIETIN

Drabkin’s solution. The diluted hemolyzate was prepared by adding several drops of mouse blood to 100 ml. of water. The solution was allowed to stand in the cold for 24 hours, during which the container was shaken several times.

The heme was then extracted from the clear supernatant fluid with methylethylketone by the method of Teale and the radioactivity was counted with a well-type scintillation counter.

For morphologic studies, the specimens from the tube were cut off with a razor blade and their surfaces were gently pressed on a glass slide and stained with Wright-Giemsa stain.

RESULTS

Morphologic Observations

In the spleen imprints prepared before incubation, the reticulum cells were prominent, with a few lymphoid and myeloid cells. Erythroblasts were rarely observed (Fig. 1).

After 24 hours incubation with erythropoietin, immature large erythroblasts were observed, some of them were in clumps (Figs. 2 and 3). Figures of mitoses were also observed (Fig. 3). Forty-eight hours after incubation, small basophilic erythroblasts were frequently observed (Fig. 5). In the control experiment without erythropoietin, no erythroblasts were observed at the 24th (Fig. 4) and the 48th hours (Fig. 6). Table 1 shows the quantitative changes in erythroblasts in the spleen specimen.

Incorporation of the Radioiron into the Heme

As previously described, the radioiron was added to the incubation media 6 hours before the termination of the incubation. As shown in Table 2, when the spleen was incubated for 6 hours, no incorporation of the radioactive iron into the heme component was observed in the samples either with or without erythropoietin. In this experiment the radioactive iron was added to the media from the start of the incubation.

As shown in Table 3, after 24 hours incubation, a slight incorporation of the radioiron into the heme was observed in the samples to which erythropoietin was added. The incorporation of the radioiron into the heme was prominent after 48 hours incubation with erythropoietin (Table 4). No incorporation of the radioiron into the heme was observed in the control samples to which no erythropoietin was added. These observations occurred consistently as repeated studies demonstrated (see Tables 2–4).

DISCUSSION

Recently Nakao et al. confirmed the in vivo observations of Filmanowicz et al., regarding the appearance of erythroblasts in the spleens of transfusion-induced...
Figs. 2 and 3.—The spleen after 24 hours incubation with erythropoietin. Immature large basophilic erythroblasts are shown. Mitosis of the erythroblast is shown in Figure 3.

Fig. 4.—The spleen after 24 hours incubation without erythropoietin. No erythroblasts are found.
Fig. 5.—The spleen after 48 hours incubation with erythropoietin. Small basophilic erythroblasts are shown.

Fig. 6.—The spleen after 48 hours incubation without erythropoietin. No erythroblasts are found.

duced polycythemic mice following an erythropoietin injection. In their study they also demonstrated increased nucleic acid synthesis, radioiron incorporation and heme synthesis in the spleen.

Studies on the effect of erythropoietin upon erythroid cells in vitro have been extensive.1-9 Krantz et al. and Powsner and Berman demonstrated an increase in heme synthesis in vitro. However, the experimental erythroid tissue was prepared from the bone marrow of normal animals or normal and anemic humans, which contained “stem cells” as well as differentiated erythroid cells and other types of hematopoietic cells. In such an experimental model it would be difficult to determine the specific cells responding directly to the erythropoietin. Erslev reported the increase in radioiron incorporation into the bone marrow cells from normal and polycythemic animals by the erythropoietin in vitro. In his experiment, the radioiron might be incorporated into nonhemin iron components within erythroid cells as well as in other kinds of cells.
Table 1.—Changes in Erythroblasts Percentages in the Imprint Specimen from Polycythemic Mice Spleens after Incubation with and without Erythropoietin

<table>
<thead>
<tr>
<th>Incubation Time (hr.)</th>
<th>Number of Experiments</th>
<th>Large Erythroblasts (%)</th>
<th>Small Erythroblasts (%)</th>
<th>Large Erythroblasts (%)</th>
<th>Small Erythroblasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0.1 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>5.7 ± 4.2</td>
<td>0.4 ± 0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>4</td>
<td>0.4 ± 0.2</td>
<td>9.8 ± 1.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Figures represent the average and the standard deviation of each experiment. Percentage rate is calculated from the numbers of erythroblasts among 500 nucleated cells in the imprints.

Takaku et al.9 reported that the spleen from the polycythemic mouse responded to the erythropoietin in vitro with an increased incorporation of 14C-1-glucosamine. Moreover, they reported an independence of the degree of the response from the percentages of the erythroblasts in the incubated rat bone marrow cells. Although the incorporation of glucosamine-14C into cells is not specific to the erythroid cells, their findings suggested that erythropoietin effects differentiation of the stem cells.

In the present studies, the spleen explants from the polycythemic mice with hematocrit over 70 per cent contained almost no erythroblasts. After exposure to erythropoietin, the erythroblasts appeared at the 24th and the 48th hour and iron uptake into the heme was quite evident at the 48th hour as compared with control experiments. The increase in heme synthesis is seen to correspond well to the sequences of observed morphologic changes: Immature large basophilic erythroblasts appearing at the 24th hour are not yet undertaking active heme synthesis, whereas mature erythroblasts appearing after 48 hours incubation are actively synthesizing heme.

The present observations on in vitro heme synthesis are in accordance with
IN VITRO EFFECT OF ERYTHROPOIETIN

Table 3.—Radioiron Incorporation into the Heme of the Spleen Explants from Polycythemic Mice with and without Erythropoietin (24 Hours Incubation)

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Background Count (c.p.m.)</th>
<th><em>Fe Added</em> (c.p.m.)</th>
<th>Erythropoietin 0.1 U/ml in the Medium</th>
<th>c.p.m. into the Heme</th>
<th>Mg. Wet Weight of Specimen</th>
<th>Mg. Wet Weight of Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>69</td>
<td>411</td>
<td>+</td>
<td>26</td>
<td>9.4</td>
<td>6</td>
</tr>
<tr>
<td>66</td>
<td>69</td>
<td>411</td>
<td>−</td>
<td>9</td>
<td>12.5</td>
<td>1</td>
</tr>
<tr>
<td>67</td>
<td>69</td>
<td>411</td>
<td>+</td>
<td>40</td>
<td>10.5</td>
<td>10</td>
</tr>
<tr>
<td>73</td>
<td>67</td>
<td>306</td>
<td>−</td>
<td>11</td>
<td>11.0</td>
<td>2</td>
</tr>
<tr>
<td>76</td>
<td>67</td>
<td>306</td>
<td>+</td>
<td>11</td>
<td>11.8</td>
<td>3</td>
</tr>
</tbody>
</table>

*One specimen consists of spleen explants from 3 tubes.

Table 4.—Radioiron Incorporation into the Heme of the Spleen Explants with Polycythemic Mice with and without Erythropoietin (48 Hours Incubation)

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Background Count (c.p.m.)</th>
<th><em>Fe Added</em> (c.p.m.)</th>
<th>Erythropoietin 0.1 U/ml in the Medium</th>
<th>c.p.m. into the Heme</th>
<th>Mg. Wet Weight of Specimen</th>
<th>Mg. Wet Weight of Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>70</td>
<td>328</td>
<td>+</td>
<td>346</td>
<td>8.7</td>
<td>121</td>
</tr>
<tr>
<td>59</td>
<td>70</td>
<td>309</td>
<td>−</td>
<td>2</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>70</td>
<td>311</td>
<td>+</td>
<td>252</td>
<td>10.4</td>
<td>78</td>
</tr>
<tr>
<td>61</td>
<td>70</td>
<td>311</td>
<td>−</td>
<td>13</td>
<td>4.7</td>
<td>1</td>
</tr>
</tbody>
</table>

*One specimen consists of spleen explants from 3 tubes.

From our previous in vivo observations with the polycythemic mouse, in which evidence of heme synthesis was first observed in the spleen 24 hours after erythropoietin injection and markedly on the 48th hour.

From the results of the present study, it appears that erythropoietin has an in vitro effect on differentiation of erythroid cells from stem cells.

The possibility still remains that erythropoietin has other effects such as maturation and division of erythroblasts as shown by Matot.⁶

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
SUMMARY

1. Spleen explants of transfusion-induced polycythemic mice were incubated in vitro with and without erythropoietin.
2. After 24 hours incubation with the erythropoietin, immature large erythroblasts appeared, whereas the mature small erythroblasts were first observed after 48 hours incubation.
3. Marked radioiron incorporation into the heme was observed after 48 hours incubation with erythropoietin.
4. The control incubations without erythropoietin did not show these findings.
5. These data strongly suggest that erythropoietin induced the differentiation of erythroblasts from stem cells in vitro.

SUMMARIO IN INTERLINGUA

1. Explantationes splenic de muses con polycythemia inducite per transfusion esseva incubate in vitro con e sin erythropoietina.
2. Post un incubation de 24 horas con le erythropoietina, immatur grande erythroblastos appareva, durante que matur micre erythroblastos esseva primo observate post 48 horas de incubation.
3. Marcate grados de incorporation de radio-ferro ad in le hem esseva observate post 48 horas de incubation con erythropoietina.
4. In le incubationes de controlo, in que nulle erythropoietina esseva presente, iste constatationes non esseva facite.
5. Iste datos suggestiona fortemente que erythropoietina induceva le differentiation de erythroblastos ab cellulas primordial in vitro.

REFERENCES

11. Erslev, A. J.: The effect of anemic an-
Brief Report: In Vitro Effect of Erythropoietin on the Spleen of the Polycythemic Mouse. I

KIKU NAKAO, YASUSADA MIURA and FUMIMARO TAKAKU

Updated information and services can be found at:
http://www.bloodjournal.org/content/27/5/646.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml