In Vitro Effect of Erythropoietin on the Spleen of The Polycythemic Mouse

II. Radiosensitivity of Stem Cells

By Yasusada Miura, Fumimaro Takaku and Kiku Nakao

In view of the function of hemopoietic stem cells maintaining a hematologically steady state, radiosensitivity of the stem cells has been regarded as responsible for postirradiation hemopoietic death. As a method for studying the effect of irradiation upon hemopoietic stem cells, the colony-forming cells first described by Till and McCulloch and the erythropoietin responsive cells in polycythemic animals have been used.

The present study was performed for the purpose of developing an in vitro method to observe the radiosensitivity of stem cells. The previously reported tissue culture method of estimating the response of polycythemic mouse spleen to erythropoietin was used to observe an in vitro and in vivo radiosensitivity of hemopoietic cells and the recovery from irradiation.

Materials and Methods

Young adult dd mice weighing approximately 18 Gm. were bred in the laboratory of the University of Tokyo and were made polycythemic according to the method of Jacobson and co-workers. Five to eight days after the second intraperitoneal transfusion, the spleens were removed and incubated. Only mice with hematocrit values above 60 percent were used.

The methods of in vivo and in vitro irradiation and the tissue culture of the spleen are described schematically in Figure 1. The source of cobalt irradiation was a teletherapy unit containing 1400 curies of cobalt-60. The distance from the source to the sample was 75 cm., and the exposure rate was 40 r per minute.

With in vivo irradiation, each group of mice was placed in a lucite box and received a dose of irradiation, as indicated in Table 1. Spleens were extirpated and incubated immediately at 1, 3, 6, 9, and 12 days after irradiation. For in vitro irradiation, spleen fragments from one mouse were divided into culture tubes in groups of three, each group receiving a different irradiation dose. These fragments were attached to the upper side of culture tubes and were irradiated on a wax sample holder with a dose of cobalt irradiation, as indicated in Table 2. The fragments of the spleen which were not irradiated served as the nonirradiated control. Immediately after irradiation, culture media were added to each tube and incubation was begun.
RADIosenSITIVITY OF STEM CELLS

Fig. 1.—Diagram of the methods of in vivo and in vitro irradiation and tissue culture of spleen fragments from polycythemic mice.

Table 1.—Effect of ^{60}Co Irradiation on Radioiron Incorporation in the Heme of Polycythemic Mouse Spleen Explants Incubated with Erythropoietin (Whole Body Irradiation)

<table>
<thead>
<tr>
<th>Dose of Irradiation (r)</th>
<th>Number of Mice</th>
<th>Heme Synthesis* (M ± Se)</th>
<th>% Rate of Heme Synthesis Compared to the Nonirradiated Control (M ± Se)</th>
<th>Erythroblasts† (1000 Nucleated Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
<td>104 ± 12.1</td>
<td>100 ± 0.0</td>
<td>56.2 ± 8.7</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>39 ± 8.6</td>
<td>37 ± 8.3</td>
<td>15.0 ± 2.5</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>27 ± 10.9</td>
<td>26 ± 10.5</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>3 ± 2.6</td>
<td>3 ± 2.6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>1 ± 1.7</td>
<td>1 ± 0.6</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Each specimen consists of spleen explants collected from two different tubes.

*The heme synthesis is calculated as follows: Per cent Fe^{59} uptake into heme × 10^{-5}/mg. wet weight of spleen explants.

†The erythroblasts were counted in the explants after 24 hours incubation.

Heme synthesis was measured from the explants after 48 hours incubation.

Table 2.—Effect of ^{60}Co Irradiation on Radioiron Incorporation in the Heme of Polycythemic Mouse Spleen Explants Incubated with Erythropoietin (in Vitro Irradiation)

<table>
<thead>
<tr>
<th>Dose of Irradiation (r)</th>
<th>Number of Experiments</th>
<th>Relative Heme Synthesis* (Mg. Spleen) (M ± Se)</th>
<th>Erythroblasts† (1000 Nucleated Cells) (M ± Se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
<td>100.0 ± 0.0</td>
<td>56.2 ± 8.7</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>52.8 ± 10.3</td>
<td>23.0 ± 5.2</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>47.2 ± 12.2</td>
<td>13.5 ± 8.7</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>19.7 ± 8.4</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>8.4 ± 5.6</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

One specimen consists of spleen explants collected from two different tubes.

*The relative heme synthesis is calculated as follows: ^{59}Fe uptake into the heme per mg. wet weight of spleen irradiated in vitro ÷ ^{59}Fe uptake into the heme per mg. wet weight of nonirradiated explants from the same spleen.

†The erythroblasts were counted in the 24th hour incubated explants.
Tissue culture of the spleen was performed by the method previously described with slight modifications: two incubation tubes, each containing eight spleen fragments, were used for the extraction of heme, and the third tube was used for morphologic observations. In each series of experiments, calf serum from the same lot was used as an incubation medium.

Erythropoietin, extracted from urine of an anemic patient, was added to the incubation medium in a concentration of 0.2 cobalt unit per milliliter.

Radioiron was added to the media 6 hours prior to the termination of incubation. Heme was extracted at the 48th hour of incubation from spleen explants by the method already described. As we reported previously, morphology of the incubated cells was well maintained until the 48th hour of incubation. The morphologic observations were made from the stamp specimens of the spleen fragments at the 24th hour of incubation. The number of erythroblasts per 1000 nucleated cells was counted.

RESULTS

1. Effects of Irradiation on Heme Synthesis. As shown in Table 1 (in vivo irradiation) and Table 2 (in vitro irradiation), erythropoietin induced a decreased heme synthesis in the spleen fragments in proportion to the increase in the irradiation dose. Figure 2 demonstrates the accumulated results of the relationship between the dose of in vivo (solid circle) and in vitro (white circle) irradiation and heme synthesis in the spleen. Each plot represents a mean value of from three to seven experiments shown in Table 1 and 2.

These curves, linear on a semilogarithmic plot, have been fitted to the experimental points by the method of least squares, yielding D37 values of approximately 70 r for in vivo irradiation and 120 r for in vitro irradiation, respectively. Extrapolation numbers are considered to be near 1.

2. To confirm that the reduction in heme synthesis is due to an impairment of differentiation of stem cells responding to erythropoietin, the number of erythroblasts which appeared within each spleen fragment at the 24th hour of incubation were counted (Tables 1 and 2). As shown in Figure 3, a significant correlation was observed between heme synthesis and the number of erythroblasts per 1000 nucleated cells in the spleen imprint specimens (p < 0.01).

3. Recovery from Irradiation. Figure 4 and Table 3 show the recovery of heme synthesis from 300 r irradiation in the spleen fragment. Until the 6th day after irradiation, almost no response to erythropoietin occurred. On the 9th day, however, marked heme synthesis in response to erythropoietin occurred in the spleen fragments. This "overshooting" response continued until the 12th day after irradiation.

DISCUSSION

In this report, the effect of in vivo and in vitro cobalt irradiation on the response of the polycythemic mouse spleen to the erythropoietin in a tissue culture system was observed.

The impairment of heme synthesis in the spleen fragments showed a significant correlation with the decrease in the number of erythroblasts appearing. Thus, it is strongly suggested that the impairment of heme synthesis represents a reduction of stem cell differentiation in response to erythropoietin and not due to the reduction of heme synthesis within each erythroblasts.
Fig. 2.—In vitro response to erythropoietin in irradiated polycythemic mouse spleen. Each plot represents a mean value of from three to seven experiments as shown in Table 1 and 2.

Fig. 3.—Relationship between the appearance of erythroblasts and heme synthesis in polycythemic mouse spleen fragments incubated with erythropoietin.

The $D_{50}$ values could be estimated roughly to be 70 r in the spleen irradiated in vivo and 120 r in the spleen irradiated in vitro, respectively. As reported previously, some corrections would be necessary of the irradiation dose for the in vitro irradiation of the spleen fragments used in this experi-
Fig. 4.—Recovery of erythropoietin-responsive cells after 300 r. Response according to time after irradiation at which incubation was begun.

Table 3.—Response to Erythropoietin in the Polycythemic Mouse Spleen Incubated in Vitro Following a Single Dose of 300 R Gamma Ray from $^{60}$Co

<table>
<thead>
<tr>
<th>Initiation of Incubation Following Irradiation (Days)</th>
<th>Number of Experiments</th>
<th>$^{59}$Fe Uptake into the Heme $\times 10^{-2}$</th>
<th>mg. wet weight of spleen specimen</th>
<th>Erythropoietin 0.2 U/ml</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>1.0 ± 0.6</td>
<td>1.0 ± 1.7</td>
<td>2.0 ± 1.1</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>1.8 ± 1.2</td>
<td>2.0 ± 1.1</td>
<td>3.0 ± 1.1</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>11.5 ± 2.8</td>
<td>3.0 ± 1.3</td>
<td>3.0 ± 1.3</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>5.7 ± 1.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>134.7 ± 45.9</td>
<td>1.8 ± 1.0</td>
<td>1.8 ± 1.0</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>164.0 ± 18.3</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
</tr>
</tbody>
</table>

Each specimen consists of spleen explants collected from two different tubes. Heme synthesis was measured in explants after 48 hours incubation.

ment. However, our data on the radiosensitivity of the erythropoietin-responsive cells is similar to that described in several kinds of mammalian cell lines in tissue culture. No one has reported the in vitro effect of irradiation on the differentiation of the erythroblasts evoked with erythropoietin.

Using the colony forming method, McCulloch and Till reported that there was no significant difference in D$_{37}$ values between the in vivo and in vitro radiosensitivity curves. In vitro irradiation of the colony-forming cells, however, showed a significant increase in extrapolation number as compared to in vivo irradiation.

In the present report, the extrapolation numbers could be calculated approximately 1.0 in both in vivo and in vitro irradiation, which are lower than those described by other authors. Further study is necessary to determine
by this method extrapolation numbers of the stem cell radiosensitivity curve with greater accuracy.

The possibility still remains that the increased resistance to radiation in vitro could be due to relative hypoxia\textsuperscript{15,16} during irradiation, since no attempt was made to maintain an oxygen concentration to the in vivo level within the tissue fragments. McCulloch and Till,\textsuperscript{3} however, could not prove that the effect of anoxia caused a greater survival of cells irradiated in vitro than that in vivo. As Gurney and co-workers,\textsuperscript{4,14} Porteous,\textsuperscript{17} and Alexanian\textsuperscript{18} had observed, an "overshooting" response to erythropoietin occurred 9 and 12 days after irradiation in this experiment.

In our present study, the reduction in heme synthesis in spleen fragments, observed until the 6th day after irradiation, was considered to demonstrate a reduction in the occurrence of erythropoietin-induced differentiation of stem cells. In turn, this was thought to be caused either by a reduction in the number of erythropoietin-responsive stem cells or by an inactivation of stem cell response to erythropoietin. Further study is necessary to analyze the detailed mechanism involved in the recovery from irradiation.

**SUMMARY**

1. An in vitro method to observe radiosensitivity of stem cells was developed in the present study. In vivo and in vitro effect of \textsuperscript{60}Co irradiation on the erythropoietin-induced stem cell differentiation into erythroblasts was observed, using a tissue culture method of polycythemic mouse spleen. Response to erythropoietin was demonstrated by an appearance of heme synthesis and erythroblasts in spleen fragments.

2. A significant correlation between the rate of appearance of erythroblasts and heme synthesis of the spleen fragments was observed.

3. After irradiation, marked impairment of both heme synthesis and production of erythroblasts was observed, yielding D\textsubscript{37} values in the vicinity of 70 r in vivo and 120 r in vitro irradiation, respectively.

4. Marked recovery of erythropoietin-induced heme synthesis in the polycythemic mouse spleen was observed 9 days after 300 r irradiation, with an "overshooting" phenomenon on the 12th day.

**SUMMARIO IN INTERLINGUA**

1. Esseva disveloppate un methodo pro observar in vitro le radiosensibilitate de generalisate cellulas matre. Esseva observate in vivo e in vitro le effecto de irradiation a \textsuperscript{60}Co super le differentiation del cellulas in erythroblastos induce per erythropoietina. In isto, un metodo histocultural de polycythemic splen murin esseva usate. Le responsa a erythropoietina esseva demonstrate per le apparition de un synthese de hemo e de erythroblastos in fragmentos splenic.

2. Un correlation significative inter le apparition de erythroblastos e del synthese de hemo in le fragmentos splenic esseva observate.

3. Post le irradiation, un marcate interferentia in le synthese de hemo e etiam in le production de erythroblastos esseva notate. Le valores obtenite pro D\textsubscript{37} esseva in le vicinitate de 70 r in vivo e de 120 r in vitro.

4. Un marcate restablimento del synthese de hemo induce per erythropoietina in le splen de muses polycythemic esseva observate 9 dies post le irradiation con 300 r, con un phenomeno rebound le dece-secunde die.
REFERENCES


In Vitro Effect of Erythropoietin on the Spleen of The Polycythemic Mouse: II. Radiosensitivity of Stem Cells

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