Studies of the Hemopoietic Microenvironment.
II. Effect of Erythropoietin on the Splenic Microvasculature of Polycythemic CF<sub>1</sub> Mice

By Robert S. McCuskey, Howard A. Meineke, and Stephen M. Kaplan

The effect of erythropoietin on the splenic microvascular system of polycythemic CF<sub>1</sub> mice was studied using in vivo microscopic methods. Administration of a single dose (3 U) of erythropoietin resulted in an increase in the linear velocity of blood flow through the splenic sinusoids and a reduction in the number of sinusoids storing blood. This response was first seen 4–6 hr after injection; it persisted for 48 hr and was reduced markedly by 72 hr. By 120 hr the spleens were indistinguishable from controls. The response was specific for erythrogenic tissue, since no response was seen in the adjacent nonerythropoietic pancreatic tissue. The results suggest that the splenic microvascular response to erythropoietin may be indirect and may be mediated by the release of a vasoactive metabolite from the erythrogenic tissues surrounding the sinusoids. Erythropoietin-sensitive stem cells are suggested to be the source of such a metabolite.

The microenvironment of a hemopoietic organ may play a role in its activity. Using the spleen as an example, its hemopoietic microenvironment can be morphologically subdivided into: a microvascular compartment composed of arterioles, capillaries, sinusoids, and venules; a connective tissue compartment composed of fibers, ground substance, and cells; and neural elements associated with both the blood vessels and the stroma.

Several studies have suggested that erythropoietin, in addition to its action on erythroid stem cells, also may affect the microvascular compartment of the hemopoietic microenvironment. These reports suggest that erythropoietin is a vasoactive substance that causes vasodilation and/or vasoproliferation specifically in hemopoietic tissues. The mechanism of this action of erythropoietin, however, remains unknown. To elucidate this action further, a series of in vivo microscopic studies of hemopoietic tissues and organs have been in progress in this laboratory. In the most recent of these, the splenic microvascular system of the mouse was examined during erythropoietic regeneration and suppression. It was found that regenerating erythropoiesis in the red pulp of sublethally x-irradiated mice was accompanied by an elevation in blood flow through the microvascular system. Both the linear velocity of flow...
and the number of sinusoids with blood flow in them increased significantly. In contrast, in polycythemic and x-irradiated polycythemic mice with suppressed erythropoiesis, a decreased linear velocity of blood flow was observed, as well as a marked increase in the amount of blood being stored in the splenic sinusoids. These results lent further support to the reports that erythropoietin affected the microcirculation of blood in the sinusoids of erythropoietic tissue, since it was assumed that the circulating levels of endogenous erythropoietin were elevated in the sublethally, x-irradiated, anemic mice. In an attempt to establish whether erythropoietin was indeed vasoactive, the effect of exogenous erythropoietin on the microvascular system of the spleen of the polycythemic mouse was examined. The results form the basis of this report.

MATERIALS AND METHODS

Female, CF1 mice weighing approximately 20 g were used as experimental animals. The mice were made polycythemic by transfusion on 2 days successively with washed, packed red blood cells. On the third day following the second transfusion, 3 U of step III erythropoietin (Connaught Laboratories) suspended in 0.1 cc of saline were administered s.c. or i.v. Mice injected with 0.1 cc of saline served as controls. The spleens were subsequently observed using in vivo microscopic methods at 3, 4, 5, 6, 8, 24, 48, 72, 96, and 120 hr after injection. At 3, 6, 24, 48, and 96 hr, ten microscopic fields in each spleen were selected at random in the red pulp, and these were examined using a 75× water immersion objective and a 10× ocular containing a calibrated micrometer scale. All vessels with blood flow in each field were counted, measured, and classified as to type. In addition, the distance between sinusoids containing blood flow was measured, and the linear velocity of blood flow was estimated qualitatively using the following scale of 0-4+: 0 = zero flow, there was no movement of blood cells; 1+ = very slow flow, the blood oscillated with individual cells seen; 2+ = slow flow, there was steady forward progression of blood cells with individual cells seen; 3+ = moderate flow, the blood cells were blurred; and 4+ = rapid flow, the blood cells were not detected as individual entities. The remainder of the spleens (4, 5, 8, 72, and 120 hr after injection) were examined, and the microcirculation was compared qualitatively to the other experimental groups and controls. At least five spleens at each time were evaluated. The microvascular system of the pancreas in each mouse also was examined in vivo, in order to compare the response of the action

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>No. of Animals</th>
<th>Internal Diameters of Vessels (Mean±SE)</th>
<th>Distance (μ) Between Sinusoids With Flow (mean±SE)</th>
<th>Hematocrit (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hr after ESF*</td>
<td>5</td>
<td>4.67±0.10 8.05±0.31 6.25±0.31</td>
<td>42.99±1.90†</td>
<td>61.4±0.40</td>
</tr>
<tr>
<td>6 hr control</td>
<td>3</td>
<td>4.80±0.00 7.52±0.30 5.98±0.22</td>
<td>47.95±1.75</td>
<td>67.0±2.18</td>
</tr>
<tr>
<td>6 hr after ESF*</td>
<td>5</td>
<td>4.86±0.03 8.94±0.57 7.69±0.66</td>
<td>15.55±0.36</td>
<td>65.5±2.66</td>
</tr>
<tr>
<td>24 hr control</td>
<td>10</td>
<td>4.62±0.04 7.67±0.22 7.07±0.25</td>
<td>47.79±3.96</td>
<td>62.7±1.28</td>
</tr>
<tr>
<td>24 hr after ESF*</td>
<td>11</td>
<td>5.03±0.13 11.09±0.17 9.12±0.28</td>
<td>18.66±1.31</td>
<td>66.5±1.32</td>
</tr>
<tr>
<td>48 hr control</td>
<td>6</td>
<td>4.51±0.11 7.82±0.45 7.52±0.54</td>
<td>44.62±2.48</td>
<td>64.0±1.64</td>
</tr>
<tr>
<td>48 hr after ESF*</td>
<td>7</td>
<td>4.98±0.11 11.51±0.99 9.02±0.70</td>
<td>14.22±0.40</td>
<td>66.3±1.20</td>
</tr>
<tr>
<td>96 hr control</td>
<td>4</td>
<td>4.72±0.18 9.69±0.90 7.18±0.12</td>
<td>42.65±1.81</td>
<td>65.6±1.58</td>
</tr>
<tr>
<td>96 hr after ESF*</td>
<td>5</td>
<td>4.84±0.04 7.74±0.08 7.67±0.25</td>
<td>36.69±1.97</td>
<td>66.0±1.74</td>
</tr>
</tbody>
</table>

*Single (3 U) injection of step III erythropoietin (ESF).
†No control valve was obtained at this time interval because this valve was within the range of mean distances between sinusoids for all other groups of saline injected controls.
Fig. 1. Increase in numbers of arterioles, venules, and sinusoids with blood flow in ten microscopic fields (346, 185 sq μ) after single (3 U) injection of step III erythropoietin (ESF). Results are expressed by per cent increase in number of vessels compared to mean number of vessels of all controls injected with saline.

of erythropoietin on the microvasculature of a nonerythropoietic organ. Following in vivo study, a sample of blood was withdrawn from the heart for determination of hematocrit. Animals whose hematocrits were below 60% were excluded from the experiment.

To ensure that the single injection of erythropoietin was effective in stimulating erythropoiesis, the uptake of 59Fe was measured in the blood of the polycythemic mice 24 and 48 hr after simultaneous injection of the erythropoietin and of 0.5 μCi of 59Fe (ferric citrate) suspended in 0.5 cc of saline. The radioactivity of the blood was counted in a Nuclear-Chicago well-type scintillation counter. Total blood volume was estimated as 7.3% of body weight in the subsequent calculations.

RESULTS

Distinct differences in the splenic microvascular system were found between the untreated polycythemic mice and those administered erythropoietin. Dramatic increases in blood flow through the microvascular system, as well as a reduction in the number of sinusoids storing blood, were found. This was reflected by an increase in the number of arterioles, sinusoids, and venules containing blood flow and concomitantly by a reduction in the distance between sinusoids containing blood flow (Table 1, Fig. 1). The linear velocity of blood flow also increased from 3+ to 4+ in arterioles, from 0 or 1+ to 2+ or 3+ in sinusoids, and from 2+ to 3+ or 4+ in venules. This effect was time dependent in that no response was observed until 4–6 hr after administration of erythropoietin; the response persisted for 48 hr and was reduced markedly between 72 and 96 hr after the single injection of erythropoietin (Table 1, Fig. 1). By 120 hr, the spleens were indistinguishable from controls. The maximum response was observed between 6 and 48 hr. No difference in the time required for a response to occur was observed between mice administered erythropoietin s.c. or i.v. 59Fe uptake in the blood was increased at 48 hr (2.0 ± 0.39 cpm) as compared to 1.2 ± 0.35 cpm at 24 hr and 0.7 ± 0.063 cpm for the controls administered saline.

In contrast to the microvascular system of the spleen, the microvasculature of the pancreas exhibited none of the changes described above. No significant
changes in the pancreatic microcirculation were seen during polycythemia or in polycythemic mice administered erythropoietin. In all mice, the pancreatic microvasculature was similar to that observed in normal mice.11

DISCUSSION

The results of this study strongly support previous observations that erythropoietin has an effect on the microvascular compartment of erythrogenic tissue.1-7 This was indicated by increased blood flow through the splenic sinusoids and a reduction in the storage of blood in the sinusoids following a single injection of erythropoietin. That the response was due to the erythropoietin and not to some contaminant in the hormone preparation was suggested by the duration of the response, which coincided closely with the time required for a single stem cell to differentiate into a mature erythrocyte. In addition, the increased uptake of 59Fe in the blood 48 hr after administration of erythropoietin indicated that the hormone was active and effective in stimulating erythropoiesis.

The microvascular response to erythropoietin appeared to be specific for erythrogenic tissue, since no response was seen in the microvasculature of the pancreas, an organ that is not erythropoietic. Thus, the results agreed with previous reports from this laboratory that erythropoietin influences the microvasculature in splenic transplants in the mouse back chamber,2 in the fetal liver in situ,3,4 and in the bone marrow,5 but not in transplants of non-erythropoietic tissue6 or in the adult liver, which is nonerythropoietic.9

The mechanisms by which this effect is accomplished is not clear at the moment. However, the 4-hr lag before the effect was seen would suggest that the effect was an indirect one, perhaps resulting from the release of a metabolite from the stimulated erythrogenic tissue. The erythropoietin-sensitive stem cells would be a likely source for such a metabolite, especially since they begin to incorporate 3H-thymidine and 3H-uridine during the first 6 hr after administration of erythropoietin,18 and nucleoli are first seen 4–12 hr after administration.12 The lag in the appearance of a response was not due to slow absorption of the hormone when injected subcutaneously, since no difference was seen in the time required before the onset of the response when the hormone was given intravenously.

Further support for this interpretation has been reported in previous studies.1 For example, no microvascular recovery was seen in mice that showed no erythropoietic recovery 10 days after x-irradiation. In contrast, in comparably anemic mice whose spleens contained extensive erythroid colony formation, the flow of blood through the sinusoids was increased greatly, and there was essentially no storage of blood in the sinusoids. While caution must be exercised in interpreting the results in x-irradiated animals due to the possible direct effects of the irradiation on the splenic microvascular system, the results in the x-irradiated animals showing no evidence of erythropoietic recovery were interpreted as indicating that this tissue also was unable to initiate a microvascular response. Since the stem cells in these animals probably were inactivated by the irradiation, these results again suggested that the stem cells might be the source of the postulated vasoactive metabolite.
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This close specific interrelationship between erythropoietin, erythropoiesis, and the microvascular system was supported further by the observation that, in spleens with suppressed erythropoiesis in polycythemic X-irradiated mice, granulocytic colony formation occurred in the poorly perfused microenvironment, a microenvironment in which erythropoiesis appeared to be inhibited. While the observation by Feleppa et al. that the effect of erythropoietin was seen in the absence of active erythropoiesis in transplants of splenic tissue, appears to pose a contradiction, their results might be explained on the basis that erythropoietin-sensitive stem cells were present but that the chemical composition of the microenvironment was not conducive for erythroid differentiation. For example, if the stroma contained considerable amounts of sulfated acid mucopolysaccharide, differentiation and maturation of the erythropoietin-sensitive stem cells could not occur. A vascular response to erythropoietin might be seen, however, since the stem cells would be present to liberate metabolite. Histochemical studies are in progress to clarify this point.

In conclusion, while this report strongly suggests that erythropoietin indirectly acts as a specific vasoactive agent in erythrogenic tissue, definitive proof of such an action for this hormone must await a demonstration that the microvascular response can be prevented when erythropoietin inactivated with antierythropoietin serum is administered.

REFERENCES

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