Effect of Testosterone on the Formation of Erythroid Spleen Colonies From Fetal Liver Precursor Cells

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Polycythemic x-irradiated female mice, injected with 12-day fetal liver cells, showed 81% suppression of erythroid spleen colonies as compared with nonpolycythemic recipients. On the other hand, in male recipients only 16% suppression was observed. Hence, androgenic hormones seem to play a role in regulating erythropoiesis of explanted fetal stem cells. To test this, we examined the effect of testosterone injected into polycythemic female recipients on the production of erythroid colonies from fetal liver cells. Testosterone was found to alleviate the suppressive effect of polycythemia. Antierythropoietin prevented the appearance of erythroid colonies in testosterone-treated animals. Thus, testosterone seems to act by increasing the levels of, or susceptibility to, erythropoietin. Under similar conditions, testosterone did not trigger the formation of erythroid colonies from bone marrow cells in polycythemic recipients. Hence, fetal cells can be induced to form erythroid colonies by doses of erythropoietin that are too low to induce erythroid colonies in bone marrow cells.

The control mechanisms of hemopoiesis in the developing mammalian embryo are not well understood. Whether there is a direct relationship between the control of erythropoiesis in the pregnant mother and the kinetics of erythroid cell differentiation of the fetus is still an open question. Thus, polycythemia induced in the pregnant mouse, which suppressed erythropoiesis in the mother, did not suppress the developing erythropoietic system of the fetus. This could be interpreted as indicating either that the control mechanism of the mother is independent of that of the fetus, or that polycythemia reduces erythropoietin to a level that is sufficient to suppress erythropoiesis of the adult organism but not that of the fetus.

Applying the in vivo cloning method for hemopoietic cells, we have previously found that in polycythemic recipients erythroid colonies of bone marrow origin are completely suppressed, whereas erythroid colonies produced by fetal liver are only partially inhibited. This could be explained on the basis of either of the following assumptions: (1) Erythroid cell differentiation from bone marrow is controlled by erythropoietin, whereas erythropoiesis from fetal cells is controlled by a different inducer, the production of which is not suppressed by polycythemia. (2) Erythropoiesis of bone...
marrow and fetal cells is induced by erythropoietin. However, the level of the inducer required to switch on erythroid differentiation from fetal cells is lower than that required for bone marrow cells. Hence, polycythemia might have suppressed erythropoietin production to a level below that able to signal bone marrow cells but still sufficient to induce fetal cells to differentiate to red blood cells.

Androgen hormones were shown to affect erythropoietin production and thus to stimulate erythropoiesis. It seemed possible that they might be involved in raising erythropoietin production to levels stimulating for fetal cells, although not sufficient to stimulate bone marrow stem cells. The present study was carried out to test these assumptions.

MATERIALS AND METHODS

Animals used in the present study were 10–14 wk old (C3H X C57BL)F1 male or female mice. They were exposed to 850 R total-body irradiation as previously described and were then inoculated intravenously with hemopoietic cells from either adult or fetal donors. Donors of adult hemopoietic cells were syngeneic mice, aged 6 wk, from which bone marrow cells were pooled in Tyrode’s solution and then injected intravenously at doses of $3 \times 10^4$ cells in 0.5 ml solution per recipient animal (donors and recipients being of the same sex). Fetal hemopoietic cells were taken from livers of syngeneic fetuses at 12 days of gestation. Cell suspensions were prepared by passing the liver tissue through a 25- or 27-syringe needle into Tyrode’s solution. Liver cells were injected at doses of $3 \times 10^4$ cells in 0.5 ml solution per recipient.

To produce polycythemia, animals were injected twice intraperitoneally with packed red blood cells from male and female C57BL mice on day 0 (1.0 ml) and day 1 (0.5 ml) postirradiation. Prior to transfusion, the blood was exposed to 1500 R to inhibit the colony-forming capacity of potentially contaminating nucleated cells. The hematocrit level of polycythemic animals was checked before sacrifice and was found to be equal to or greater than 60%. All experiments were terminated on day 6 postirradiation and hemopoietic cell injection.

The spleens of the x-irradiated animals were treated as previously described.3 Colonies were typed by testing from each spleen five sections, 100 µ apart. The registered erythroid colonies comprise both erythroid and mixed colonies, i.e., colonies containing erythroid and granuloid cells. Number of erythroid colonies is given ± SD.

Testosterone Injection

“Homosterone” (Teva, Jerusalem, Israel) was diluted in peanut oil to a concentration of 5 mg/ml; 0.2 ml, containing 1 mg testosterone, was injected subcutaneously on different days, as described.

Erythropoietin Injection

Erythropoietin was prepared by Dr. D. Hammond (University of Southern California, Los Angeles, Calif.) by collodion adsorption of human urine. Erythropoietin was injected subcutaneously in doses of 2.1 U in 0.6 ml saline and 1.5 U in 0.4 saline on days 4 and 5, respectively.

Antierythropoietin Injection

Antierythropoietin prepared in rabbits was supplied by courtesy of Dr. J. C. Schooley, Donnor Laboratory, University of California, Berkeley, Calif. The lyophilized material was suspended in 0.9% NaCl to a concentration that was found to have a neutralizing capacity of 4 erythropoietin U/ml. This antierythropoietin was injected subcutaneously, 2.5 U on day 4 and 1.5 U on day 5 postirradiation.
ERYTHROID SPLEEN COLONIES

RESULTS

Male and female animals are known to have different levels of steroid hormones. Hence, the first experiment aimed at testing the effect of polycythemia on the suppression of erythroid colonies produced by fetal liver cells in male and female mice. Polycythemic animals of both sexes were injected with fetal liver cells and tested for the incidence of various spleen colonies in relation to control, nonpolycythemic recipients. Female mice receiving cells from 12-day fetuses manifested 81% suppression of erythroid colonies, whereas in males only 16% suppression was observed. Cells from 19-day fetuses showed 100% suppression in females, as compared to 66% in males (Fig. 1).

These results raised the possibility that androgenic hormones play a role in the regulation of erythropoiesis of fetal stem cells. Therefore, we first tested whether testosterone applied to polycythemic females that had been inoculated with fetal liver cells will stimulate erythropoiesis. Irradiated polycy-
Fig. 3. (A) Effect of antierythropoietin (Anti-Epo) on erythropoietic function of testosterone (Test) in polycythemic (Pol) female mice inoculated with fetal liver (Fl) cells. (B) Effect of testosterone on formation of erythroid colonies in polycythemic (Pol) female mice inoculated with bone marrow (BM) cells.

Polycythemic female mice were injected subcutaneously with testosterone (1.0 mg), either on days 0, 1, 3, 4, 5 (group 1) or on days 4 and 5 (group 2), following inoculation of fetal liver cells. The result was that testosterone alleviated the suppressive effect of polycythemia (Fig. 2). In the first group 82% (6.4 erythroid colonies per spleen) of nonpolycythemic control were registered, whereas in the second group, consisting of animals that had been treated with testosterone just 2 days prior to the termination of the experiment, 66% (4.9 colonies per spleen) of the nonpolycythemic control were recorded.

To test whether testosterone, when stimulating erythropoiesis in polycythemic animals, acts directly on the erythropoietic cells, or whether it stimulates erythropoietin production, the following experiment was made. Polycythemic x-irradiated female mice, inoculated with fetal liver cells, were treated on days 4 and 5 with testosterone. Half of the animals were treated on days 4 and 5 with antienrythropoietin (2.5 U and 1.5 U, respectively), and the spleens were tested on day 6. Figure 3A summarizes the results; antierythropoietin antibodies suppressed the appearance of erythroid colonies of testosterone-treated polycythemic animals. (Granuloid and megakaryocytic colonies were not affected.) To ascertain the effect of antierythropoietin on the function of erythropoietin assumed to be induced by testosterone, the following subgroups were set up: (1) Polycythemic animals were injected with erythropoietin at doses almost equivalent with those of the antiserum used (2.1 and 1.5 U on days 4 and 5). The results (Fig. 3A) indicate that these doses of erythropoietin elicited erythroid colonies in the polycythemic recipients. The incidence of these colonies was similar to that of polycythemic animals treated with testosterone. (2) In parallel, we tested the effect of testosterone in polycythemic recipients on the production of erythroid colonies from bone marrow cells. An irradiated polycythemic subgroup of the experiment presented in Fig. 3 was inoculated with $3 \times 10^4$
bone marrow cells and treated with testosterone on days 4 and 5. The results (Fig. 3B) were that testosterone at doses that did trigger the formation of erythroid colonies from fetal cells did not trigger the production of erythroid colonies from bone marrow cells.

DISCUSSION

The precise target cell for erythropoietin action has not been critically determined, nor has the mechanism of action of erythropoietin been clarified. Yet, it is generally accepted that erythropoietin functions as an inducer of erythroid cell differentiation in the adult organism. Whether erythropoietin has a similar role in embryonic or fetal erythropoiesis is still an open question. The kidney appears to be the main organ in which erythropoietin is produced. Nephrectomy in the adult organism was found to suppress erythropoiesis. However, nephrectomy of newborn rats did not seem to affect erythropoiesis. Furthermore, unlike in adult organisms, induced hypoxia in the newborn was not associated with the stimulation of erythropoiesis. These observations raised the possibility that the control mechanism of erythropoiesis in adult animals differs from that of the fetus. This conclusion seemed to be supported by our observation that in male recipients polycythemia hardly suppressed the formation of erythroid colonies produced by fetal liver cells. However, polycythemia did suppress erythroid colonies in female recipients. Hence, certain factors existing in the male animal could have a stimulating effect on red blood cell formation. Fried et al. demonstrated that testosterone activates erythropoiesis in the adult organism, and Schooley provided evidence that the hormone functions in this case by increasing secretion of erythropoietin. Antierythropoietin prevented the stimulating effect of testosterone on erythropoiesis of the adult animal. The indirect effect of testosterone, i.e., the effect via erythropoietin production, could be deduced also from the kinetics of the phenomenon. Whereas erythropoietin activated incorporation of $^{59}$Fe 48 hr following its injection, testosterone did so only after 96 hr. Similar results were obtained by Naets and Wittek. On the other hand, the kinetics of formation of erythroid colonies in our experiments do not seem to reflect an indirect effect of testosterone. In our earlier studies, we have demonstrated that at 48 hr following the application of erythropoietin to polycythemic animals treated with bone marrow, erythroid colonies developed consisting each of about 3 x 10$^4$ cells.

On the assumption that testosterone acts by triggering the production and secretion of erythropoietin, one would expect the time required for the hormone to stimulate erythroid colonies in polycythemic animals to be longer than 48 hr. Yet, in the present study, testosterone elicited the production of erythroid colonies within 48 hr (Fig. 2). It is clear, however, that testosterone did function in our system via erythropoietin, since antierythropoietin prevented its effect. Hence, the relatively short lag of 48 hr following testosterone application in our experiments does not contradict the assumption of an indirect action of testosterone. It should be noted, however, that this applies only to erythroid colonies developed from fetal liver cells. The
same doses of testosterone had no effect on bone marrow cells injected into x-irradiated polycythemic animals and tested 48 hr after hormone administration. These differences between fetal liver cells might, in fact, be only temporal and had tests been made at later stages such differences would not have been registered. The differences we obtained might be attributed to either the possibility that more fetal cells are at cycle at the time of inoculation, or that colonies from fetal cells are manifested earlier because of their shorter generation time. It could be inferred, therefore, that fetal cells can be induced by doses of erythropoietin that are too low to induce erythropoiesis in bone marrow cells. Thus, there are two mechanisms that could account for the effect of testosterone on erythropoiesis of fetal cells in our experimental system. (1) Testosterone raises the secretion of erythropoietin to a level that is sufficient to trigger erythropoiesis of fetal cells but not of bone marrow cells. This implies that fetal hemopoietic cells are susceptible to doses of erythropoietin that do not affect adult cells. (2) Testosterone does not affect the level of erythropoietin but makes fetal cells more susceptible to the inducer. This would imply, however, that antierythropoietin antibodies reduce the level of active erythropoietin below its ordinary level in polycythemic animals, since treatment with antibodies to the inducer prevented formation of erythroid colonies in testosterone-treated recipients. Although this second interpretation seems less likely, further experiments are required to distinguish between the two possible mechanisms.

Another possibility should be considered, i.e., that the difference between males and females is due to the suppressing effect of estrogens on erythropoiesis. This possibility is now being tested in our laboratory.

Tarbutt and Cole claimed that they were unable to measure any $^{59}$Fe uptake in polycythemic recipients inoculated with fetal liver cells. Thus, their results are incompatible with our observations based on colony formation. There are two possibilities to explain these differences: (1) The small number of normoblasts appearing in an erythroid colony is not sufficient to reflect a measurable level of $^{59}$Fe uptake, and (2) in the absence of erythropoietin cells might differentiate with no measurable haemoglobin synthesis. Experiments are now in progress to differentiate between these two alternatives.

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REFERENCES

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