

In Vitro Production of Erythropoietin by Mouse Fetal Liver

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Mouse fetal liver tissue has been cultured and shown to produce and release into the culture medium an erythropoietically active substance for up to 30 days of culture. Since this substance can be completely neutralized by an antiserum to erythropoietin and shows a dose-response relationship in the plethoric mouse assay, it is

suggested that the culture medium contains erythropoietin, a hormone important in the regulation of erythropoiesis. Using this procedure, we have obtained the equivalent of about 20.7 units of erythropoietin from five T-flasks (75 sq cm) over the 30-day culture period.

ALTHOUGH THE KIDNEY has been established as the primary source of erythropoietin (Epo) production or activation in the adult, extrarenal sources have been shown to exist by a number of investigators,¹⁻¹⁰ and Fried¹¹ has implicated the liver as a source of this extrarenal erythropoietin. It has been suggested by the studies of Carmena et al.,¹² Lucarelli et al.,¹³ and more recently by the studies of Kaplan et al.,¹⁴ and Zanjani et al.,^{15,16} that unlike in the adult, where the kidney represents the major source of erythropoietin, in the fetus another site or sites exist which appear to serve as the primary organ in fetal erythropoietin production.

Recently, studies have been performed utilizing the culture of kidney tissue in an attempt to produce an erythropoietically active material in vitro. Using bovine kidney cell monolayers under a low O₂ environment, McDonald et al.,¹⁷ reported a significant elevation in erythropoietin levels in their culture media. Burlington et al.,¹⁸ culturing goat renal glomeruli also demonstrated elevated erythropoietin production over a period of 7 mo. Thus, these studies on the culturing of kidney tissue proved the feasibility of using tissue culture for the production of erythropoietin.

The present study describes a method of obtaining erythropoietically active material from the culture of mouse fetal liver. Such cultures have demonstrated the production of a significant quantity of erythropoietin for up to 30 days of culture.

MATERIALS AND METHODS

Fetal Liver Culture

Mouse fetal liver was obtained from the fetuses of Ha/ICR Swiss mice at 18-20 days gestation. Age of fetus was determined by the size of fetus at the time of culture. This tissue was minced in a petri plate containing a small amount of culture medium (Waymouth's MB752/1 medium,

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GIBCO, supplemented with 20% native horse serum, GIBCO, 0.5% lactalbumin hydrolysate, GIBCO, and sufficient penicillin-streptomycin to give 100 units of penicillin and 100 μ g streptomycin/ml of medium, GIBCO) with the use of two sharp scalpel blades. Tissue of less than 5 mm on a side was explanted into 75 sq cm Falcon T-flasks with 10 ml of culture medium. These cultures were then incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. At various times over a 30-day culture period, medium was collected for assay of erythropoietic activity. All media to be assayed were dialyzed against deionized water (pH 7.0) for 24 hr and concentrated in carbowax (Union Carbide). All assays were carried out with plain culture media treated in identical fashion as a control.

Erythropoietin Bioassay

Hypertransfused, plethoric Swiss mice were used to determine erythropoietic activity.¹⁹ Virgin female mice (6-wk-old) were injected intraperitoneally with 0.8 ml of packed red cells from isologous donors on 2 successive days. Six and 7 days after the last injection of red cells when the mice were polycythemic, 1 ml of saline, three different standard levels of sheep erythropoietin (step III) (Connaught Lab) or test materials were injected intraperitoneally. On day 8, ⁵⁹Fe citrate (0.5 μ Ci/0.2 ml) was injected intravenously through a lateral tail vein, and 48 hr later the animals were bled from the dorsal aorta. Erythropoietic activity was related to the uptake of ⁵⁹Fe into circulating erythrocytes. Each material was tested on groups of five to six polycythemic assay mice. Mice were demonstrated to be polycythemic by hematocrit determinations on the day of assay. Any mouse with a hematocrit less than 58 was eliminated.

Neutralization Studies

Neutralization of erythropoietic activity was conducted using antisera prepared in rabbits to sheep erythropoietin (0.4 ml of such antisera was shown to neutralize completely 1.2 U of the erythropoietic activity contained in 2 ml of hypoxic rat serum). Groups of material for neutralization were incubated with 0.4 ml of antierythropoietin for 2 hr at 37°C followed by 24 hr at 5°C. At the end of this incubation period, 1.5 ml of goat antirabbit gamma globulin (GARGG) (Antibodies, Inc.) was added and the incubation was continued at 37°C for 2 hr. The GARGG removed all excess antierythropoietin from the reaction mixtures and the precipitates formed were removed by centrifugation. The supernatant fluids were assayed for erythropoietic activity.

Dose Response Studies

Media were changed every 2-3 days and collected over a 30-day culture period from five mouse fetal liver cultures in 75 sq cm T-flasks. These media (600 ml) were pooled and concentrated ten times in carbowax (Union Carbide) and the concentrated medium was then diluted into three different concentrations (10 \times , 5 \times , and 2.5 \times) per ml for injection into assay animals. Each of these concentrations was assayed along with plain culture media concentrated ten times as a control in the hypertransfused, plethoric mouse assay. To determine the amount of erythropoietin produced by these five T-flasks, the amount found in 1.0 ml of 10 \times media was multiplied by the total amount of 10 \times media collected over the 30-day culture period.

RESULTS

The level of erythropoietic activity in mouse fetal liver culture media collected at various time periods is shown in Table 1. Significant activity was first detected in media collected between 9 and 15 days of culture. This activity was equivalent to 0.050 U Epo/ml of concentrated culture medium when compared to the known standards of erythropoietin. Media collected between 16 and 24 days of culture showed erythropoietic activity equivalent to 0.070 U Epo/ml of concentrated culture medium, while media collected between 25 and 30 days of culture demonstrated 0.075 U Epo/ml of concentrated culture medium. Addition of 0.4 ml of antierythropoietin to each of the different culture media neutralized the erythropoietic activity contained in those culture media (Table 1,

Table 1. Effect of Antierythropoietin (Epo) Serum on Erythropoietin (Epo) Production from Mouse Fetal Liver Cultures

Groups	Materials Injected	Mean Hematocrit	Per Cent ⁵⁹ Fe Incorporation	Equivalent Epo Units
1	Saline	63 ± 2.03*	0.15 ± 0.02	
2	0.05 U Epo	66 ± 0.59	0.90 ± 0.29	
3	0.20 U Epo	65 ± 1.27	6.48 ± 1.27	
4	0.80 U Epo	65 ± 1.86	15.54 ± 1.86	
5	2 ml Hypoxic Rat Sera + 0.4 ml NRS†	65 ± 0.93	6.16 ± 0.55	0.180
6	(2 ml Hypoxic rat sera + 0.4 ml anti-Epo) + 1.5 ml GARGG‡	61 ± 2.02	0.25 ± 0.06	
7	(0.4 ml anti-Epo + 1.5 ml GARGG) + 2 ml hypoxic rat sera	65 ± 2.36	6.56 ± 1.81	0.200
8	1.0 ml control media	63 ± 1.40	0.13 ± 0.02	
9	1.0 ml culture media D 1-9§	63 ± 1.76	0.14 ± 0.03	
10	1.0 ml culture media D 10-15	63 ± 1.35	0.87 ± 0.22	0.050
11	(1.0 ml culture media D 10-15 + 0.4 ml anti-Epo) + 1.5 ml GARGG	65 ± 2.05	0.10 ± 0.01	
12	1.0 ml culture media D 16-24	62 ± 1.52	2.01 ± 0.33	0.070
13	(1.0 ml culture media D 16-24 + 0.4 ml anti-Epo) + 1.5 ml GARGG	66 ± 1.84	0.11 ± 0.02	
14	1.0 ml culture media D 25-30	65 ± 1.80	2.32 ± 1.00	0.075
15	(1.0 ml culture media D 25-30 + 0.4 ml anti-Epo) + 1.5 ml GARGG	65 ± 1.70	0.15 ± 0.02	

* ± SE of the mean.

† Normal rabbit serum.

‡ Goat antirabbit gamma globulin.

§ Supernatant fluid from fetal mouse liver culture concentrated 2.5 times. (Each milliliter is equivalent to 2.5 ml of original culture media.)

Groups 11, 13, and 15). This result showed that the erythropoietic response observed in the assay mice was actually caused by erythropoietin present in the culture media. To prevent injection of antierythropoietin into the assay mice (which would neutralize the erythropoietin irrespective of the site of origin), goat antirabbit gamma globulin (GARGG) was added in sufficient quantity to neutralize completely the antierythropoietin effect observed. This effect can be seen in Group 7 of Table 1 in which the erythropoietin found in hypoxic rat serum was not neutralized when antierythropoietin was first removed by GARGG prior to addition to the hypoxic rat serum.

The dose-response relationship of media collected from fetal mouse liver cultures over 30 days in culture is shown in Table 2. It can be seen that these culture media gave a dose-related response in our plethoric mouse bioassay and that this activity could be neutralized by addition of antierythropoietin serum. A total of 60 ml of 10 × concentrated medium was obtained from five T-flasks (75 sq cm) over 30 days in culture. Each milliliter of 10 × medium gave a response equivalent to about 0.345 U erythropoietin, thus the equivalent of about 20.7 U erythropoietin was obtained from these cultures.

DISCUSSION

The fetal liver culture system appears to have important potentials in the study of erythropoietin production. We have demonstrated that mouse fetal

Table 2. Dose-Response Effect of Mouse Fetal Liver Culture Media

Groups	Materials Injected	Mean Hematocrit	Per Cent ⁵⁹ Fe Incorporation	Equivalent Epo Units
1	Saline	71 ± 2.20*	0.19 ± 0.03	
2	0.05 U Epo	66 ± 2.70	0.70 ± 0.12	
3	0.20 U Epo	70 ± 1.88	3.53 ± 0.25	
4	0.80 U Epo	71 ± 0.58	13.54 ± 2.45	
5	1.0 ml control media (10.0 ×)	66 ± 0.88	0.12 ± 0.01	—
6	1.0 ml culture media† (2.5 ×)	69 ± 0.65	0.96 ± 0.30	0.060
7	1.0 ml culture media (5.0 ×)	69 ± 0.75	3.00 ± 0.38	0.165
8	1.0 ml culture media (10.0 ×)	66 ± 0.51	7.12 ± 2.40	0.345
9	(1.0 ml culture media (5.0 ×) + 0.4 ml anti-Epo)‡ + 1.5 ml GARGG§	65 ± 0.48	0.11 ± 0.01	—

* ± SE of the mean.

† Supernatant fluid from fetal mouse liver culture collected over 30-day culture period and concentrated.

‡ Antierythropoietin serum.

§ Goat antirabbit gamma globulin.

liver cultures are capable of producing an erythropoietically active material which can be neutralized by antisera to erythropoietin. This activity appears to increase with time in culture at least through 30 days and the activity we see has been shown to be due to the presence of detectable erythropoietin in the culture media. The results presented here also demonstrate the importance of the liver in erythropoietin production by the fetus. Such an organ might in fact be the major tissue responsible for fetal erythropoietin, especially when one notes the results of Zanjani et al.,¹⁶ dealing with fetal goats. They found that nephrectomized fetal goats were able to respond to bleeding with elevated erythropoietin levels not significantly different from those found in control fetuses (with intact kidneys). This observation suggests that at least in fetal goats the kidney is not required for the full expression of erythropoietin production. These results are in accord with those of Carmena et al.,¹² Lucarelli et al.,¹³ and Kaplan et al.¹⁴ in the newborn rat and Schooley and Mahlmann²⁰ in the weanling rat (21 day old), who reported that partial hepatectomy depressed the ability of these animals to produce erythropoietin in response to a hypoxic stress.

Cole, Paul, and others²¹⁻²⁴ have studied the effects of exogenous erythropoietin on hemoglobin synthesis by mouse fetal liver cells in culture. These authors have shown that hepatic erythropoiesis in the developing mouse fetus can be divided into two phases. An early stage with cells that are responsive to exogenous erythropoietin and a later stage in which cells are unresponsive to the actions of exogenous erythropoietin. The transition from one phase to the next is sudden, occurring on day 14 or 15 of gestation in the mouse. Cole et al.²² have suggested that this change in sensitivity may be due to an increase in the amount of endogenous erythropoietin available to the fetus. According to this hypothesis, erythropoietin production in the fetus would be minimal during the early phase and increase to excess shortly before the second phase. Large quantities of endogenous erythropoietin would account for the lack of responsiveness to exogenous erythropoietin since the cells are already fully stimulated.

Our fetal liver culture system offers us the opportunity to approach this problem directly. Studies are currently in progress to determine when in fetal development the liver obtains the capability of producing erythropoietin in culture and when in development it obtains the capacity to produce large quantities of this hormone.

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