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Fetal Hemopoietic Tissue Differentiation and Response to Erythropoietin Stimulation in an *in Vivo* Culture System[1] (40171)

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The problem of regulation of erythropoiesis by erythropoietin during fetal life, although the subject of several studies, remains unsolved. Induced polycythemia or bilateral nephrectomy in the rat or mouse mother does not alter erythropoiesis in the 16 day old fetus, while maternal erythropoiesis is profoundly depressed (1, 2). This indirect type of observation is in contrast with observations performed directly in hypertransfused polycythemic lamb fetuses, where the rate of red cell production appears to be reduced, suggesting the existence of a mechanism of humoral regulation of erythropoiesis similar to that of adult (3; 4).

Several studies on short term cultures have demonstrated that erythropoietin affects fetal liver cell suspensions from 13 to 16 day old fetuses, supporting the hypothesis of erythropoietin-dependent fetal erythropoiesis in mouse (1, 5, 6). However, analogous studies made in a culture system which reproduces the environmental characteristics of the *in vivo* situation (Diffusion Chambers (7)) did not confirm such hypothesis in the mouse (8).

Our studies with the Diffusion Chamber technique on the effect of erythropoietin on fetal liver cultured as whole in comparison to fetal liver cell suspensions were performed to elucidate this problem. Differentiation along all the hemopoietic lines was determined during a 10-day culture period.

Materials and methods. Female CF1 mice, 14–15 weeks old, were mated 16½ days before surgery and used as fetus donors. Virgin female CF1 mice, 10–12 weeks old were used as Diffusion Chamber (DC) hosts. They were randomized in two treatment groups. Host mice of the 1st group received no pretreatment at all. Host mice of the 2nd group were bled 0.5 ml by cardiac puncture on the 6th, 4th and 1st days before DC implantation. Reticulocytes and hematocrits of each mouse in this group were tested the day before surgery, and mice with hematocrit higher than 40% or reticulocytes lower than 6% were discarded. Fetal livers were inserted in 4 mm deep DCs either as whole organ or as cell suspension of two different concentrations: 50×10^6 cells/DC (same cell concentration of the whole organ, as direct control for an environmental action), and 1×10^6 cells/DC (better cell concentration for proliferation in DCs, as result of a previous pilot experiment); cells were cultured by the DC method described by Tyler (11) and cells were harvested at intervals up to 10 days after implantation from groups of 2–8 mice per experiment. Five experiments were performed. Imprints of tissue and cytocentrifuge smears were made, stained with Benzidine stain and counterstained with Wright–Giemsa stain. Two-three hundred cells were recognized on two different smears by a single observator. One-two DCs per group were used as histological controls.

Results. Hematocrits and reticulocyte percentages of both groups are shown in Table I.

In the nontreated group no over all difference in total cellularity per DC was found between fetal liver cultured as the whole organ or as a cell suspension (Fig. 1). The suspension showed a decrease in cellularity during the 1st day of culture, as described by Marmor *et al.* (12) in bone marrow cell DC cultures and Symann et al. (8) in fetal liver cell cultures. The initial drop in cell number was followed by a plateau. Although the total cellularity was in the suspension at every point slightly lower than in the liver, this is more likely to be an effect of the initial manipulation rather than a true difference. We assumed that cell viability was preserved mainly on the ground of previous works (9, 10) demonstrating in the same experimental conditions that stem cells were preserved and

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TABLE I. HEMATOLOGICAL PARAMETERS OF CF1 HOST MICE OF DCs (B = BLED, C = CONTROL UNBLED) VALUES ARE MEAN ± SE.

Days of culture in DCs	Number of samples	Treatment group	Hematocrit %	Reticulocytes %	Number of samples
−1	—	C	—	—	—
	206	B	34.5 ± 0.1	20.0 ± 0.9	78
1	9	C	47.2 ± 0.7	2.7 ± 0.3	8
	14	B	39.7 ± 0.7	21.2 ± 2.2	11
2	12	C	44.7 ± 0.6	1.8 ± 0.2	10
	15	B	40.0 ± 1.1	19.3 ± 1.9	10
3	20	C	46.6 ± 0.6	1.6 ± 0.4	13
	38	B	41.0 ± 0.6	16.6 ± 1.2	34
4	10	C	46.4 ± 0.5	1.8 ± 0.8	8
	10	B	41.8 ± 0.8	10.1 ± 0.9	10
6	6	C	45.2 ± 2.0	1.5 ± 0.9	6
	12	B	44.8 ± 0.2	5.5 ± 0.9	12
7	18	C	45.8 ± 1.0	2.6 ± 0.2	11
	30	B	46.1 ± 0.5	6.0 ± 0.5	25
10	10	C	44.9 ± 0.4	1.9 ± 0.2	9
	9	B	44.7 ± 1.2	3.5 ± 0.3	9

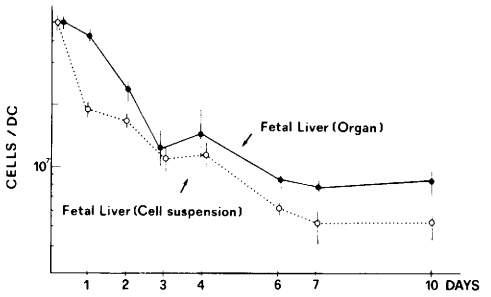

FIG. 1. Total numbers of nucleated cells after culture of fetal liver in DCs implanted in CF1 host mice. Values are mean ± SE of 6–11 DCs per group.

also able to self renew, before and after DC culture. Anyway, trypan blue viability test was done on the original suspension in the first two experiments showing respectively 85.5 and 89% viable cells.

Differential counts (Table II) showed that erythropoiesis was preserved better in the cultured organ than in the cell suspension. Immature erythroblasts were present until the 7th day of culture in the organ, while they disappeared from the suspension after the 4th day of culture. The total number of erythroblasts harvested from the cultured organ was at every point, except day 2, significantly higher than in the cultured suspension. Granulocyte number, which was higher during the first two days in the cultured organ, was then found to be below the conresponding suspension level, although never significantly. We also extrapolated the erythroid/granulocytic ratio finding at every point higher values for the cultured organ. Macrophages were always significantly higher in number in the organ than in the suspension. Lymphocytes, which were few in both groups, disappeared by the 6th day of culture. Megakariocytes were seen neither in the original suspension nor during the culture period.

Cellularity of DCs implanted in bled and unbled host mice during the 10 day culture period is shown in Fig. 2: in repeated experiments only on the 3rd and 4th days of culture did fetal liver show a better growth in bled hosts than in unbled. By the 6th day, the implanted whole organ in bled host mice reached the control level, and by the 10th day was slightly below it.

When a fetal liver cell concentration/DC of 1×10^6 cells was tested in contrast to the 50×10^6 cells/DC, no difference in cell numbers have been found in bled and control hosts throughout the duration of the culture.

When fetal liver cell differentiation was studied in bled and unbled host mice, the only difference which could be attributed to a response to the increased endogenous erythropoietin between cultured cells in bled and unbled hosts was found in the cultured organ (Table III). When observed in DCs implanted in bled mice at the 3rd and 4th days of culture, the erythroblasts were found to be significantly increased in number and a concomitant increase in cellularity and in erythroid/granulocytic ratio on the same days was

TABLE II. CELLULAR COMPOSITION OF FETAL LIVER ORGAN (O) AND FETAL LIVER CELL SUSPENSION (S) CULTURED IN DCs IN NONTREATED CF1 HOST MICE. VALUES × 10^6 ± SE OF THE MEAN.

Days of culture in DCs	Number of samples	Group	Erythroblasts × 10^6/DC	Granulocytes × 10^6/DC	Macrophages × 10^6/DC	Lymphocytes × 10^6/DC	Erythroid/Granulocytic Ratio
0	6	—	34.2 ± 3.4	5.4 ± 1.5	0.6 ± 0.2	0.7 ± 0.2	6.3
1	7	O	31.0 ± 1.8[c]	8.7 ± 0.9[d]	3.1 ± 0.5[d]	0.2 ± 0.07	3.6
	9	S	11.9 ± 1.0	4.3 ± 1.0	1.0 ± 0.3	0.1 ± 0.07	2.8
2	9	O	13.4 ± 1.4	6.4 ± 0.8[c]	3.3 ± 0.5[d]	0.2 ± 0.1	2.8
	10	S	11.0 ± 0.9	3.9 ± 0.5	1.6 ± 0.2	0.2 ± 0.04	2.1
3	8	O	4.9 ± 0.8[c]	5.2 ± 0.4	3.2 ± 0.5[b]	0.2 ± 0.05	0.9
	11	S	2.2 ± 0.5	6.1 ± 1.0	1.9 ± 0.4	0.1 ± 0.06	0.4
4	8	O	4.5 ± 1.0[b]	5.6 ± 0.7	3.3 ± 0.8[a]	0.1 ± 0.06	0.8
	9	S	2.0 ± 0.4	6.7 ± 0.6	2.2 ± 0.2	1.0 ± 0.03	0.3
6	9	O	2.0 ± 0.6[c]	3.9 ± 0.5	2.3 ± 0.4[d]	0	0.5
	7	S	0.5 ± 0.08	4.7 ± 0.4	1.0 ± 0.09	0	0.1
7	7	O	1.4 ± 0.2[c]	2.1 ± 0.6	2.8 ± 0.6[b]	0	0.7
	6	S	0.5 ± 0.1	4.0 ± 0.9	1.0 ± 0.09	0	0.1
10	11	O	1.9 ± 0.5[c]	2.8 ± 0.4	3.0 ± 0.5[d]	0	0.7
	7	S	0.5 ± 0.2	3.4 ± 0.7	1.2 ± 0.2	0	0.1

[a] $P < 0.1$.
[b] $P < 0.05$.
[c] $P < 0.02$.
[d] $P < 0.01$.
[e] $P < 0.002$.

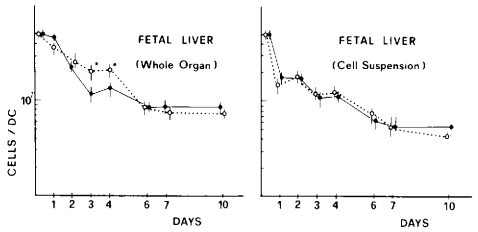

FIG. 2. Total numbers of nucleated cells after culture of fetal liver in DCs implanted in normal (●——●) and bled (○· · · · ·○) CF1 host mice. Values are mean ± SE of 6–14 DCs per group. * = $P < 0.02$.

observed. Erythroblasts continued to be slightly above the control values through day 7. No significant difference was found in the number of granulocytes and macrophages, in the two types of hosts. Megakaryocytes were not observed. Control histological sections showed a maintained lobular structure of the whole livers up to 10 days of culture.

When fetal liver cell suspensions were studied in the same conditions, no differences in number were found either in total or in differentiated cells (Table IV).

Discussion. The most important contributions given by the DC culture technique in studying developmental cell systems have been the evaluations of the growth fraction of the total number of cells inserted into the DC and the process of cell differentiation and maturation. The present experiment was done in the attempt to compare the cell growth pattern and the differentiation of fetal liver, cultured as whole organ or as cell suspension. A cellular growth over the initial number of cells in the DCs was not observed in culture of fetal liver either as whole organ or cell suspension. Both fetal liver cultures, after a 4-day period of decline in cellularity, reached a plateau phase at a lower and possibly optimal cell level. Growth failure may have been due to the high concentration of cells initially put into the chambers and to the absence of preirradiation of DC hosts. Niskanen *et al.* (13) in bone marrow cultures and Symann et al. (8, 10) in fetal liver cultures, have shown that total cellular growth and CFU-S recovery in DCs are dependent on cellular concentration.

The evaluation of the parameter of cell differentiation showed some differences between organ and cell suspension.

Differential counts showed that organ cultures of fetal liver maintained erythroid differentiation during all the culture period: erythroblasts were seen until the 7th day and represented ¼ of all the total cells at the 10th day, suggesting that the fetal liver constitued an ecological situation favorable for erythroid

TABLE III. CELLULAR COMPOSITION OF FETAL LIVER ORGAN CULTURED IN DCs IN BLED (B) AND CONTROL UNBLED (C) CF1 HOST MICE. VALUES × 10^6 ± SE OF THE MEAN.

Days of culture in DCs	Treatment group	Number of samples	Erythroblasts × 10^6/DC	Granulocytes × 10^6/DC	Macrophages × 10^6/DC	Erythroid/ Granulocytic Ratio
0	—	6	34.2 ± 4.0	5.4 ± 1.5	0.6 ± 0.2	6.3
1	B	7	25.1 ± 3.5	7.4 ± 1.0	1.6 ± 0.4	3.4
	C	7	31.0 ± 1.8	8.7 ± 0.9	3.1 ± 0.5	3.6
2	B	8	19.5 ± 3.7	6.0 ± 1.3	3.1 ± 0.7	3.2
	C	9	13.4 ± 1.4	6.4 ± 0.8	3.3 ± 0.5	2.8
3	B	8	11.8 ± 2.1[b]	4.8 ± 0.4	3.1 ± 0.6	2.5
	C	8	4.9 ± 0.8	5.2 ± 0.4	3.2 ± 0.5	0.9
4	B	8	8.9 ± 1.4[a]	6.4 ± 1.0	3.8 ± 0.4	1.4
	C	8	4.5 ± 1.0	5.6 ± 0.7	3.3 ± 0.8	0.8
6	B	8	2.5 ± 0.7	3.0 ± 0.4	2.3 ± 0.5	0.8
	C	9	2.0 ± 0.6	3.9 ± 0.5	2.3 ± 0.4	0.5
7	B	8	2.0 ± 0.5	2.7 ± 0.7	1.9 ± 0.3	0.7
	C	7	1.4 ± 0.2	2.1 ± 0.6	2.8 ± 0.6	0.7
10	B	8	1.8 ± 0.4	2.6 ± 0.3	2.6 ± 0.3	0.7
	C	11	1.9 ± 0.5	2.8 ± 0.4	3.0 ± 0.5	0.7

[a] $P < 0.02$.
[b] $P < 0.01$.

TABLE IV. CELLULAR COMPOSITION OF FETAL LIVER CELL SUSPENSION (50 × 10^6 CELLS/DC) CULTURED IN DCs IN BLED (B) AND CONTROL UNBLED (C) CF1 HOST MICE. VALUES × 10^6 ± SE OF THE MEAN.

Days of culture in DCs	Treatment group	Number of samples	Erythroblasts × 10^6/DC	Granulocytes × 10^6/DC	Macrophages × 10^6/DC	Erythroid/ Granulocytic Ratio
0	—	6	34.2 ± 4.0	5.4 ± 1.5	0.6 ± 0.2	6.3
1	B	9	7.4 ± 0.7	5.6 ± 0.8	1.5 ± 0.4	1.3
	C	9	11.9 ± 1.0	4.7 ± 0.9	1.0 ± 0.3	2.8
2	B	7	10.8 ± 1.3	5.2 ± 0.6	1.9 ± 0.3	2.1
	C	10	11.0 ± 0.9	3.9 ± 0.5	1.6 ± 0.2	2.1
3	B	9	3.5 ± 0.7	5.4 ± 0.6	2.3 ± 0.6	0.6
	C	11	2.2 ± 0.5	6.1 ± 1.0	1.9 ± 0.4	0.4
4	B	10	3.1 ± 0.9	6.6 ± 0.5	2.4 ± 0.4	0.5
	C	9	2.0 ± 0.4	6.7 ± 0.6	2.2 ± 0.2	0.3
6	B	8	0.8 ± 0.2	5.4 ± 1.0	1.1 ± 0.1	0.1
	C	7	0.5 ± 0.08	4.7 ± 0.4	1.0 ± 0.09	0.1
7	B	6	0.8 ± 0.3	2.8 ± 0.4	0.7 ± 0.2	0.3
	C	6	0.5 ± 0.1	4.0 ± 0.9	1.0 ± 0.09	0.1
10	B	6	0.1 ± 0.04	3.1 ± 0.3	1.1 ± 0.2	0.03
	C	7	0.5 ± 0.2	3.4 ± 0.7	1.2 ± 0.2	0.1

cell differentiation. Granulopoiesis also was actively proceeding so that after the 3rd day of culture, granulocytes were as numerous as erythroblasts. Histology of cultured fetal liver tissue showed that most of the granulocytic cells were confined to the external part of the liver, so that they were exposed more directly to what is called 'the DC's milieu'.

McCuskey (14) has reported that erythropoietin influences the fetal liver hemopoietic microenvironment *in vivo* by dilating the sinusoid sphincters so that mature and maturing erythrocytes could pass from a storage stage to the circulation. Analogous studies done on the spleen *in situ* led to the suggestion that erythropoietin does not act directly on the vasculature but rather on the erythropoietin-sensitive stem cells which in turn release substances which are vasoactive (15) and also probably influences the stromal production of neutral mucopolysaccharides leading to the formation of a microenvironment conductive to erythropoiesis (16).

Our data suggest moreover that the fetal liver microenvironment may be maintained in an *in vivo* situation even in absence of blood flow and erythropoietin may have influence on such a model of environment.

The only response to erythropoietin in our studies was found on the 3rd and 4th days of

culture of whole fetal liver in DCs, when erythroblasts were significantly higher in DCs of bled than of unbled host mice. The fetal liver parenchyma, as judged from the histological sections and the number of macrophages, was not modified. Erythopoietin could act to enhance erythroid differentiation only in collaboration with the appropriate environment, in this case the fetal liver when present as the organ but not as a cell suspension. Therefore, the parenchymal and structural components of fetal liver appeared to play an important role in the erythropoietic effect of the hormone.

Summary. Murine fetal liver was cultured in diffusion chambers (DC) either as the whole organ or as a cell suspension of equivalent concentration. Only the cultured organ was able to maintain erythroid differentiation during the 10-day culture period, suggesting the influence of maintaining an environment favorable to erythropoiesis. When diffusion chambers were implanted in bled host mice, erythropoiesis was enhanced in the cultured whole organ, but not in the cultured cell suspension, suggesting a cooperative action of erythropoietin and the microenvironment in inducing erythroid differentiation.

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