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Mouse yolk sac and intraembryonic tissues produce factors able to elicit differentiation of erythroid burst-forming units and colony-forming units, respectively

(mouse embryo/hematopoietic stem cells/burst-promoting activity/hemoglobin switch/erythropoietin)

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ABSTRACT **This work was aimed at elucidating the environmental conditions that account for the production of embryonic erythrocytes in the mouse yolk sac (YS), while the adult-type hemoglobin and erythrocytes are generated in the fetal liver. Differentiation of YS hemopoietic stem cells (YS-HSC) of 9.5-day mouse embryos (prior to the colonization of the liver rudiment by HSC) was investigated *in vitro*. The influence of well-characterized erythroid growth factors, burst-promoting activity (BPA) and erythropoietin (EPO), which trigger the differentiation of early erythroid burst-forming units (BFU-E) and late erythroid colony-forming units (CFU-E), respectively, was tested on the YS-HSC in two different systems of culture: (*i*) organ culture and (*ii*) clonal culture in methylcellulose. When studied in organ culture, where the YS microenvironment was maintained, YS-HSC required only additional EPO to attain complete maturation into adult erythrocytes within 7 days. In contrast, YS hemopoietic single cells grown in methylcellulose, and thus released from the influence of the YS, required the presence of both BPA and EPO to generate BFU-E-derived colonies synthesizing high concentrations of hemoglobin. It is concluded that 9.5-day YS from mouse embryos is by itself able to promote the first differentiation steps of the adult lineage YS-HSC due to an intrinsic production of a BPA-like activity. In contrast, these experiments demonstrate that EPO or an EPO-like activity is not produced by YS tissues. As demonstrated earlier, if embryonic tissue is added to YS organ culture, although separated from it by a filter preventing cell contact, YS-HSC differentiate into adult erythrocytes producing adult-type hemoglobins. This shows that, in contrast to YS tissues, the early embryo produces EPO or a factor that can substitute for EPO.**

Differentiation of erythrocytes from multipotential hemopoietic stem cells (HSC) involves a series of events, certain of which have been well documented. It is known that a relatively mature member of the erythrocyte pathway—i.e., the erythroid colony-forming unit (CFU-E)—requires erythropoietin (EPO) to divide and differentiate into erythrocytes of the adult type (1) and is itself derived from an ancestor cell, the erythroid burst-forming unit (BFU-E) (2), whose further development depends upon another growth factor referred to as burst-promoting activity (BPA) (3, 4).

During normal development of the mouse, erythropoiesis first appears in the yolk sac (YS) at embryonic day 7.5–8 (E 7.5–8) and yields only primitive erythrocytes synthesizing the embryonic forms of Hbs (5–8). Definitive erythropoiesis producing anucleated erythrocytes with adult Hbs occurs only when the fetal liver has been colonized by HSC around E 9.5 (from the 28- to 30-somite stage) (9, 10).

The problem was thus raised as to whether the YS contains HSC able to differentiate along the definitive erythropoietic pathway and, if so, why does it produce only embryonic erythrocytes before the onset of fetal liver erythropoiesis. A number of experimental data have shown that the YS actually contains HSC capable of reconstituting irradiated recipients with adult erythrocytes (11) and of producing *in vitro* the definitive erythrocyte lineage as the clonal progeny of single cells in agar or in methylcellulose. In the latter situation, stimulation was provided by the mixture of factors contained in mitogen-stimulated spleen cell-conditioned medium (SC medium) with or without the addition of EPO (12, 13). It was also demonstrated by our group (14) that YS cultured (organ culture) only in the presence of medium supplemented with 15% fetal calf serum produced exclusively primitive erythrocytes. However, if stimulated by a diffusible factor originating from various embryonic tissues (including the fetal liver rudiment), a second wave of erythropoiesis of adult type was elicited in the YS explant (14). Similar results were also obtained by addition of SC medium to the medium (14, 15).

In the present work we have further analyzed the conditions required by YS-HSC to differentiate along the definitive erythrocyte lineage. This was done by using both organ and single cell cultures and by subjecting the YS-HSC selectively to sources of BPA and EPO. We found that organ cultures of YS required only EPO as an external stimulus to develop adult-type erythropoiesis. In contrast, E 9.5 YS cells cloned in methylcellulose require both BPA and EPO to undergo adult erythropoiesis, thus suggesting that the YS itself is responsible for BPA production.

MATERIAL AND METHODS

Mouse Embryos. The mice used were of the 129/SV strain. The morning on which the vaginal plug was found was designated as day 0 of gestation. On day 9.5, pregnant mice were killed by cervical dislocation and uteri were removed and dissected in phosphate-buffered saline at pH 7.4 (P_i/NaCl) under a stereomicroscope. Embryos and their YS were extensively washed in P_i/NaCl to prevent contamination by maternal erythrocytes before the YS was surgically removed. Only YS from 20- to 25-somite embryos were retained for further experimentation.

Culture Media. Dulbecco's modified Eagle's medium (DME medium) and Iscove's modified Dulbecco's medium (IMD medium) were purchased from GIBCO and supplemented with 15% fetal calf serum. Three batches of fetal calf serum were tested in these experiments and yielded identical

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Abbreviations: YS, yolk sac(s); HSC, hemopoietic stem cells; BPA, burst-promoting activity; EPO, erythropoietin; BFU-E, erythroid burst-forming unit; CFU-E, erythroid colony-forming unit; IU, international unit(s).

results. One of them was used for all of the experimental series reported here.

Growth Factors. As a source of BPA (16), conditioned medium obtained from murine myelomonocytic leukemic cells WEHI-3 (17) adapted to culture (WEHI-3 CM) was used at the final concentration of 20% in the culture medium. Choice of this concentration resulted from assays using a range of 10–50% WEHI-3 CM in DME medium; 20% was found to yield the best culture conditions for erythropoietic differentiation of YS disaggregated cells (data not shown). EPO purified from anemic porcine serum with a specific activity of 20 international units (IU)/mg (EPO_1) was purchased from Centre National de Transfusion Sanguine (Paris). In some experiments, homogeneously pure EPO (18) (EPO_2) with a specific activity of 70,400 IU/mg was used. Both types of EPO were used in a range of concentrations from 0.1 to 1 IU/ml of culture medium.

YS Organ Cultures. The YS were cultured as described (14). Briefly, they were placed on a Sartorius filter (cellulose acetate, 0.2-μm pore, S.M. 11107 Ac; Sartorius, Göttingen, Federal Republic of Germany), supported by a stainless steel grid put onto a 60 × 15 mm organ tissue culture dish (Falcon 3037) filled with 0.65 ml of culture medium. Thus, the explants were maintained at the atmosphere/medium interface and were cultured from 6 to 11 days at 37°C in 5% CO_2 in air without change of medium. The cultures were macroscopically observed daily and the blood islands were marked on a diagram of each culture dish to determine the appearance of new blood islands. The maturation of the blood foci was judged visually by an increase of both their size and Hb content. Moreover, preliminary studies had shown that this observation was correlated with an increased intensity of the hemoglobin benzidine staining after isoelectric focusing on polyacrylamide microgels.

Disaggregated YS Cell Cultures. YS from 20- to 25-somite embryos were incubated for 15 min at 37°C in a small volume of 0.1% trypsin (trypsin, 1:250; Difco) in P_i/NaCl without calcium and magnesium. They were then disaggregated by aspiration through successively smaller hypodermic needles. The cells were cultured at various concentrations (2×10^4–10^5 cells per ml) in 0.8% methylcellulose (Methocel MC 4000 mPa·s; Fluka) according to the method of Iscove and Schreier (19). Briefly, 0.5 ml of 2% methylcellulose in IMD medium was added in a tube containing 0.25 ml of stimulus (WEHI-3 CM or EPO or both) and 0.5 ml of the cell suspension in IMD medium containing 40% (vol/vol) fetal calf serum. After spinning in a vortex, the mixture was transferred to a 35 × 10 mm Petri dish (Falcon 1008). The cultures were incubated at 37°C with 5% CO_2 and 99% humidity in air. The colonies were observed daily under a stereomicroscope using semi-indirect lighting and were harvested after 8 days in culture.

Hb Analysis. *YS organ cultures.* Hb characterization was carried out by using the isoelectric focusing/polyacrylamide microgel technique previously described (20) with slight modification. The entire YS were removed from the culture dish and placed individually in a Microfuge plastic tube containing 1 μl of lysis buffer (0.5% Nonidet P-40/2% Ampholine, pH 7–9/20% sucrose/1% Trasylol). The YS were struck with a glass micropipette to burst the erythroid cells and were centrifuged for 1 min in a Beckman Microfuge. Electrophoresis of the supernatant was carried out at 4°C for 1 hr at 100 V. Uncultured E 10 YS and diluted adult mouse blood were used as references for embryonic and adult Hbs and were run on the same gel as the experimental sample. Gels were stained for 2 min in 1% 3,3'-dimethoxybenzidine in methanol, and peroxidase activity was revealed with 3% H_2O_2 in 70% ethanol. After washing, individual gels were dried on glass coverslips and photographed.

Disaggregated YS cell cultures. Erythrocyte colonies grown in methylcellulose cultures were removed individually with a glass micropipette and pooled in P_i/NaCl to dilute the methylcellulose. After centrifugation for 10 min at 80 × *g*, the supernatant was removed and the cell pellet of erythrocytes was resuspended in 1 μl of lysis buffer. Isoelectric focusing was then carried out as above.

RESULTS

YS Organ Cultures. *In DME medium with 15% fetal calf serum.* When the YS were cultured in DME medium containing 15% fetal calf serum without any additional stimulus, we observed further maturation of the erythroid cells of the primitive lineage until day 5 of incubation; this first wave of erythropoiesis then decreased from day 6 and disappeared around day 10. Biochemical analyses performed from day 6 to day 9–10 always revealed a migration pattern typical of embryonic Hbs (Fig. 1).

In DME medium with 15% fetal calf serum, WEHI-3 CM, and EPO. When WEHI-3 CM and EPO_1 were added together at the beginning of the culture, development of primitive erythropoiesis occurred as in the control cultures. Moreover, from about day 5, new blood foci appeared throughout the YS explant, reached maturation at day 7, and decreased rapidly after day 8. The migration patterns of the Hbs from the entire YS revealed both embryonic and adult patterns.

In DME medium with 15% fetal calf serum and WEHI-3 CM or EPO. In another series of cultures, only WEHI-3 CM or only EPO_1 was introduced into the culture medium. In the first case, embryonic erythrocytes developed, but the second phase of blood island differentiation did not occur and only embryonic Hbs were produced (Fig. 1). In contrast, in the cultures, where EPO_1 was the sole stimulus, the first wave of erythropoiesis was followed by new blood island formation commencing around day 6 of culture, and at 7–9 days Hbs were found to be a mixture of embryonic and adult types (Fig. 1). We obtained identical results with EPO_1 concentrations ranging from 1 to 0.1 IU/ml of culture medium (Table 1). This suggested that EPO was the specific stimulus allowing the production of definitive-type erythropoiesis within the YS. However, due to the relative impurity of the EPO extracted from porcine serum, we repeated the experiments, using the homogeneously pure EPO preparation (EPO_2) (0.1–1 IU/ml). As seen in Table 1, production of new blood islands composed of definitive erythrocytes took place

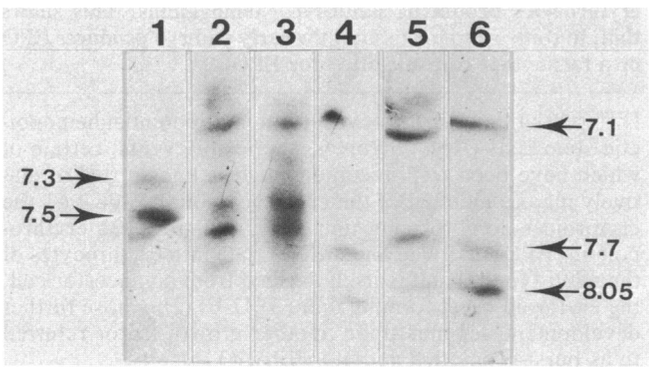

FIG. 1. Organ cultures of E 9.5 YS. Isoelectric focusing analysis of native Hbs on microgels with decreasing concentrations of EPO_1 from 1 to 0.1 IU/ml, as reported in Table 1. Lane 1, hemolysate of diluted adult blood (isoelectric points, 7.3 and 7.5). Lane 2, YS cultured for 6 days with 15% fetal calf serum and 0.75 IU of EPO per ml; embryonic and adult Hbs are present. Lane 3, culture for 8 days with 0.1 IU of EPO per ml; embryonic and adult Hbs are present. Lane 4, culture for 7 days with 15% fetal calf serum without other stimuli; embryonic Hbs alone. Lane 5, culture for 11 days with 15% fetal calf serum and 20% WEHI-3 CM, without EPO; embryonic Hbs alone. Lane 6, hemolysate of freshly harvested E 10.5 YS (isoelectric points, 7.1, 7.7, and 8.05).

Table 1. Effect of EPO concentration on YS blood island formation

EPO_1			EPO_2		
IU/ml	*n*	Positive result*	IU/ml	*n*	Positive result*
1	8	6	1	3	2
0.75	3	2	0.75	3	2
0.5	3	2	0.5	ND	ND
0.25	3	3	0.25	ND	ND
0.1	3	1	0.1	3	0
0	13	0	0	3	0

The new blood islands were always macroscopically visible after 6–7 days of culture. *n*, Number of experiments; ND, not done.
*We have considered as a positive result the macroscopically visible new erythrocyte foci. Moreover, most of the YS have been submitted to biochemical analysis.

in YS in the same way as with EPO_1 by using EPO_2 concentrations of 1 and 0.75 IU/ml.

Delayed addition of EPO. In further experiments, addition of EPO to the culture medium was delayed from day 0 to day 5. As seen in Table 2, the second wave of erythropoiesis arose in the YS after similar incubation periods independently of whether EPO_1 or EPO_2 was present from the beginning of the culture or after 1, 2, or 3 days. In contrast, when EPO was added 4 or 5 days after the onset of the culture, new erythrocyte foci developed in only a few YS, with an initial delay of 1 day. In addition, when EPO was added at 5 days, only 1 of 7 YS developed the second wave of erythropoiesis, and in this culture the dose of EPO used was twice that of the other experiments—i.e., 2 IU/ml. For all of these experiments, biochemical analysis of YS Hbs always displayed a mixture of embryonic and adult molecular species (Fig. 2).

YS Single Cell Cultures. Disaggregated YS cells from 20- to 25-somite embryos were cultured in 0.8% methylcellulose in IMD medium containing 16% fetal calf serum. The medium was supplemented either with 1 IU of EPO_1 per ml or 20% WEHI-3 CM or with both stimuli. No colonies were observed at day 2 (CFU-E) as well as at day 7 of culture (BFU-E) when only EPO_1 was added. When we compared the cultures in which WEHI-3 CM was added with or without EPO, in both cases we observed the growth of many large colonies that looked similar until day 5 independently of EPO. However, after 5 days, the Hb content of the colonies appeared conspicuous only in those cultures in which EPO (EPO_1 as well as EPO_2) was added; >90% of them synthesized high concentrations of Hb by day 6–7 of culture (Table 3). These large erythroid colonies were typical BFU-E-derived colonies (2, 21). The remaining 10% of the colonies did not synthesize Hb.

Table 2. Effect of delayed EPO addition upon YS blood island formation

Day EPO added*	IU/ml	*n*	Positive result†	Day islands visible‡
		EPO_1		
0	1	8	6	7
1	0.75	4	3	6–7
2	1	3	2	7
3	1	6	5	7
4	1	6	3	8
5	1	4	0	—
		EPO_2		
0	1	3	2	7
1	0.75	2	2	6
2	0.75	2	1	7
3	ND	ND	ND	ND
4	1	2	1	8
5	2	3	1	8

n, Number of experiments; ND, not done.
*Day at which EPO was added in the culture medium.
†We have considered as a positive result the macroscopically visible new erythrocyte foci. Moreover, each YS has been submitted to biochemical analysis.
‡Day at which new blood islands were visible.

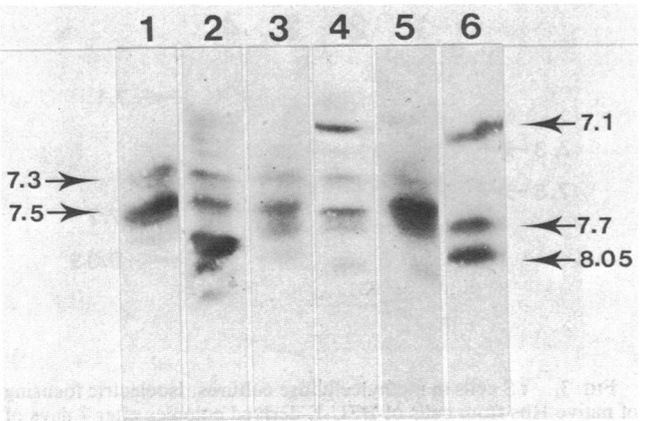

FIG. 2. Organ cultures of E 9.5 YS. Isoelectric focusing analysis of native Hbs after 8 days of culture with EPO_2 addition delayed from day 0 to day 5, as reported in Table 2. Lane 1, hemolysate of diluted adult blood. Lane 2, YS cultured for 8 days with 0.75 IU of EPO_2 per ml added after 1 day of culture; embryonic and adult Hbs are present. Lane 3, 0.75 IU of EPO_2 per ml added after 2 days; embryonic and essentially adult Hbs are present. Lane 4, 1 IU of EPO_2 per ml added after 4 days; embryonic and adult Hbs are present. Lane 5, 2 IU of EPO_2 per ml added after 5 days; embryonic and adult Hbs are present. Lane 6, hemolysate of freshly harvested E 10.5 YS. Isoelectric points are shown as in Fig. 1.

Hb Analysis. Biochemical studies were performed at day 7 of culture. As seen in Fig. 3, only adult-type Hbs were detected in erythroid colonies grown in methylcellulose cultures.

DISCUSSION AND CONCLUSIONS

As shown in previous work from our group (14), YS-HSC do not differentiate into adult erythrocytes in YS organ cultures when the medium contains DME medium and 15% fetal calf serum and no other stimulus is provided. Addition of a known source of BPA (WEHI-3 CM) alone does not induce development of CFU-E and their further maturation into adult erythrocytes in any detectable amount. In contrast, purified EPO_2 used at 0.75 IU/ml induces the differentiation of definitive erythrocyte foci, which appear about 6–7 days after the onset of the culture. Addition of EPO could be delayed for 3 days without changing the time when the erythroid foci develop. If EPO was added later (4–5 days after the beginning of culture) the number of explants undergoing the second wave of erythropoiesis decreased significantly and the erythrocyte foci were visible at only 8 days of culture. This and the fact that no erythrocyte colonies with definitive erythrocytes and Hbs appear within the first 2 or 3

Table 3. Erythroid colonies derived from 10^5 YS cells cultured in methylcellulose in relation to the culture conditions

WEHI-3 CM (20%, vol/vol)	EPO_1 1 IU/ml	EPO_2 1 IU/ml	Hb-synthesizing colonies
−	+	−	0
+	−	−	0
+	−	+	64
+	+	−	100

All of the Hb-synthesizing colonies identified were BFU-E-derived colonies.

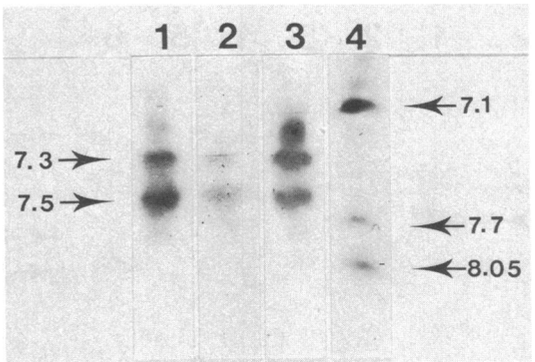

FIG. 3. YS cells in methylcellulose cultures. Isoelectric focusing of native Hbs from cells of BFU-E-derived colonies after 7 days of culture, as reported in Table 3. Lane 1, hemolysate of diluted adult blood. Lane 2, colonies very rich in hemoglobin, pooled from one culture dish: 5×10^4 cells, IMD medium, 15% fetal calf serum, 20% WEHI-3 CM, and 1 IU of EPO_1 per ml; adult Hbs alone. Lane 3, as in lane 2 with EPO_2; adult Hbs alone. Lane 4, hemolysate of freshly harvested E 10.5 YS. Isoelectric points are shown as in Fig. 1.

days of the methylcellulose culture suggest that CFU-E production has not started in E 9.5 YS before it is explanted *in vitro*. This process seems to be initiated during the culture period itself—i.e., when the YS is more mature.

Single cell cultures demonstrated further that, if removed from the YS microenvironment, the E 9.5 YS-HSC require both BPA and EPO to differentiate. This raises the possibility that in organ cultures the YS itself provides the BFU-E with BPA or a BPA-like activity. On the other hand, the diffusible factor of embryonic origin (not produced by the YS) demonstrated previously by Cudennec *et al.* (14), which promotes definitive erythrocyte differentiation in transfilter YS organ culture, can be considered as an EPO-like activity. In other words, the factor that is missing in the YS for it to produce, when isolated in culture, adult-type erythropoiesis is EPO or a factor that can substitute for EPO. Until now, EPO activity has been demonstrated in fetal liver of E 15–18 mouse fetuses (22, 23). Previous (14) and present experiments by our group support the assumption that the EPO-like substance is, in fact, produced by embryonic tissues much earlier and at least at the time of colonization of the fetal liver by HSC.

The fact that other authors (12, 13, 24) have been able to induce definitive erythropoiesis in YS-HSC with SC medium suggested that, in addition to BPA, this conditioned medium also contains an EPO-like activity. This is in agreement with the results obtained by Fagg with another experimental system (25).

It should be underlined that YS organ cultures grown in the presence of EPO yield a mixed electrophoretic pattern with embryonic and adult Hbs in the same explant. This is due to the superposition of some residual primitive erythropoietic foci along with the newly formed definitive ones. In our previous report (14) dealing with YS cultured in association with embryonic tissues and without any specific erythroid stimulus, only definitive Hbs were identified at day 8–10. Such conditions very likely did not permit the maintenance of the first wave of embryonic erythropoiesis.

An interesting point is that, in our organ culture system, definitive erythropoiesis never appeared without primitive erythropoiesis having first developed, even if 2 IU of EPO per ml was added from the very beginning of YS cultures (not shown). This result is consistent with the observations of Ripoche and Cudennec (24), who also reported the succession of primitive and definitive erythropoiesis in explanted mouse blastocysts cultured *in vitro* with SC medium or EPO. Thus, it appears that the very early addition (even at the blastocyst stage) of growth factors specific for adult erythroid lineage differentiation does not result in the bypass of the primitive erythropoiesis. This supports the contention that two distinct erythroid stem cells are responsible for the successive forms of erythropoiesis occurring in ontogeny (13).

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