Stimulation of haem synthesis by erythropoietin in mouse yolk-sac-stage embryonic cells

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SUMMARY

Cultures of disaggregated cells from mouse embryos at the stage of yolk-sac erythropoiesis have been used to test sensitivity of primary erythroid cells to erythropoietin, the hormone which controls adult red cell production. Synthesis of haem was stimulated by 89% in cells of 8-day embryos, 23% in cells of 9-day embryos and by 19% in peripheral nucleated blood cells of 12-day embryos. Differentiation of primary embryonic erythroid cells may therefore be controlled by erythropoietin in situ. The haem precursor 5-amino-4-ethylvaleric acid (ALA) did not stimulate haem synthesis under similar conditions.

INTRODUCTION

The production of red blood cells (erythropoiesis) in adult mammals is controlled by the hormone erythropoietin (Ep) which is known to stimulate the differentiation of recognizable erythroid cells from unrecognizable precursors (Jacobson, Goldwasser, Plzak & Fried, 1957; Hanna, 1967), to effect the release of increased numbers of new red cells into the circulation after 24 h in vivo (reviewed by Hodgson, 1970) and also to reduce the cell cycle time of maturing erythroid cells (Hanna, Tarbutt & Lamerton, 1969) and to increase haem synthesis in marrow cells in vitro (Krantz, Gallien-Lartigue & Goldwasser, 1963).

Ep may also control erythropoiesis in the embryo. Mammalian erythropoiesis occurs in four successive organs throughout development. The first erythroid cells (the primary series producing large nucleated erythrocytes) occur in the yolk sac; then foetal liver, spleen and bone marrow become successive sites of erythropoiesis. Foetal liver and foetal spleen cells synthesize haem in vitro in response to Ep (Cole, Hunter & Paul, 1968) but haem synthesis in cultured whole yolk-sac-stage embryos is independent of added Ep (Cole & Paul, 1966; Hunter & Paul, 1969).

This study has used cultures of disaggregated cells from whole embryos and yolk-sac tissues, and also cultures of nucleated blood cells which result from yolk-sac erythropoiesis, to investigate the sensitivity of these cells to Ep.

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In a comparison with erythropoiesis in chick embryos, the haem precursor molecule δ-aminolaevulinic acid (ALA), which stimulates haemoglobin accumulation in cultured chick blastoderms (Levere & Granick, 1965; Wainwright & Wainwright, 1970), was added to cultures of mouse embryonic cells, then haem synthesis measured.

**MATERIALS AND METHODS**

Random-bred Swiss mice of the Porton strain were used. Females were primed with Folligon and Chorulon (Organon Ltd.) before mating: vaginal plugs were seen on the morning of day 0 gestation. Eight- and 9-day embryos were dissected as previously described (Cole & Paul, 1966). Embryos and yolk sacs were then disaggregated to give a suspension of single cells, after trypsin treatment similar to that used for foetal liver tissue (Cole & Paul, 1966). Foetal blood cells from 12- and 13-day embryos were collected in Hanks's saline (Paul, 1970). Twelve-day blood contains negligible numbers of enucleate liver-produced erythrocytes, 13-day blood contains nucleated cells with more condensed nuclei, and ca. 20% enucleate cells.

Waymouth's medium MB 752/1 (Waymouth, 1959), supplemented with 5% foetal calf serum and 2.5% Porton mouse serum was used for all cell cultures. Suspensions of \(1 \times 10^6\) embryonic cells or blood cells in this medium were cultured in 1 ml aliquots in 15 ml glass culture tubes at 37 °C in an atmosphere of 5% CO₂ in air.

Erythropoietin (Ep: National Institute of Health, step IV preparation) was added at the start of the culture period at concentrations of 0.1-0.3 units per ml. The haem precursor δ-aminolaevulinic acid (ALA; as the hydrochloride, Sigma) was added to a series of cultures at concentrations of 0.01-10 mM.

\(^{59}\text{FeCl}_3\) in HCl (Radiochemical Centre, Amersham, Bucks) was equilibrated with 50% mouse serum for several hours at 37 °C before addition to cultures at 1 μCi/ml. Labelled haem was extracted from lysed cells by the acid ketone technique (Teale, 1959; Krantz et al. 1963) and counted in a Nuclear Chicago gas-flow counter. Results are expressed as counts per min/h/10^6 cells.

**RESULTS**

No stimulation of incorporation of \(^{59}\text{Fe}\) into haem was observed in cells incubated with ALA. Concentrations greater than 1 mM inhibited haem synthesis. Lower concentrations had neither a short-term nor long-term effect on haem synthesis.

Erythropoietin did result in significantly raised levels of haem synthesis by the primary erythroid cells tested. \(^{59}\text{Fe}\) was added to cultures for periods of 4 h or longer after an initial pre-incubation period of at least 30 min. When haem synthesis at all times of incubation in all experiments is considered, pooled data
show that $^{59}$Fe incorporation into haem is stimulated 89% in cells from 8-day embryos, 23% in cells of 9-day embryos and 19% in 12-day blood cells. Table 1 presents the pooled data.

Table 1. *Stimulation of haem synthesis in blood cells of the primary series by erythropoietin at 0.15 to 0.3 units per ml.*

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>No. of 1 ml cultures</th>
<th>Mean c.p.m. $^{59}$Fe in haem/h</th>
<th>% increase above control ± S.E.</th>
<th>Probability that + Ep = control (Wilcoxon test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-day embryos (0–12 somites)</td>
<td>20</td>
<td>64.2</td>
<td>121.4</td>
<td>89 ± 24</td>
</tr>
<tr>
<td>9-day embryos (13–20 somites)</td>
<td>92</td>
<td>200</td>
<td>246</td>
<td>23 ± 12</td>
</tr>
<tr>
<td>12-day blood</td>
<td>65</td>
<td>684</td>
<td>778</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>13-day blood</td>
<td>12</td>
<td>278</td>
<td>297</td>
<td>6.3</td>
</tr>
</tbody>
</table>

There was no significant increase in haem labelling by peripheral blood cells from 13-day embryos, perhaps because the erythrocytes are more mature than those in 12-day blood. Significance is assessed by the matched-pair technique of Wilcoxon (Siegel, 1956). This non-parametric test was more sensitive than matched-pair *t*-tests applied to these data (because numbers are not from a Normal distribution).

The pattern of response to Ep at different times is shown in Fig. 1. An
experiment on 8-day-embryo cells is shown in Fig. 1A; Fig. 1B shows the time course of response of cells from 9-day embryos.

Experiments on cells of disaggregated 8- or 9-day embryos were limited because of shortage of material. Blood cells from 12-day embryos were more plentiful, and the time course of response was studied. Fig. 2 shows results of three experiments using 12-day blood cells. The percentage increase in haem synthesis in the presence of Ep, above control levels, varies with time, with a probable maximum increase after 24–36 h incubation with Ep in vitro. Thus the kinetics of response of primary series blood cells to Ep in vitro may be similar to those reported for foetal liver cells in vitro (Cole & Paul, 1966).

![Graph](image)

Fig. 2. The in vitro response to erythropoietin of nucleated erythrocytes from the blood of Porton embryos of 12 days' gestation. The percentage increase of haem synthesis in Ep-stimulated cells above control levels is calculated for each period of labelling with $^{59}$Fe, and is plotted at the midpoint of that period. Data of three experiments, A, B and C are shown.

**DISCUSSION**

$\delta$-aminolaevulinic acid (ALA) has been used in several studies of differentiation of erythroid cells in chick embryo blood islands developing in vitro. Levere & Granick (1965) and Wainwright & Wainwright (1966, 1967) showed that ALA increased the haemoglobin content of young blastoderms, and induced haemoglobin synthesis prior to the normal onset of synthesis at the 7-somite stage. But Wilt (1968) showed that ALA stimulation of the haemoglobin content was only detectable on non-rich media. A further analysis by Wainwright & Wainwright (1970) based on a new very sensitive assay for haem (Wainwright, 1970) showed that ALA causes an increase in haemoglobin, the pattern of which is specific to the stage of the embryo at which ALA is added.
It seems therefore that the production of ALA is the ultimate control factor of haem and haemoglobin synthesis in chick embryos. However, added ALA does not stimulate haem synthesis in cells of the mouse yolk-sac-stage embryo, nor in foetal liver cells of 12- to 14-day embryos (Bateman, 1971).

The hormone erythropoietin (Ep) does stimulate haem synthesis in cells of young embryos, and in blood cells of the primary generation. Previous experiments using whole embryo cultures found no stimulation of yolk-sac-stage haem synthesis in the mouse (Cole & Paul, 1966) and the rat (Hunter & Paul, 1969). These negative results could arise if the external endodermal layer of the yolk sac is impermeable to Ep, or if the cells within the intact embryo are maximally stimulated by a factor produced in situ.

Haem synthesized in response to Ep in this study has not been characterized as haemoglobin, and other haem proteins will be synthesized in embryonic cells but Cole et al. reported (1968) that synthesis of haem in hepatocytes was not stimulated by Ep and Ep-dependent haem synthesis probably occurs only in erythroid cells.

Table 1 shows a decrease in response to Ep with increasing embryonic age. Similar trends in response are found in cells of chick embryos at equivalent developmental stages (Malpoix, 1967) and in liver cells from mouse foetuses of increasing age (Cole & Paul, 1966; Bateman, Cole, Regan & Tarbutt, 1972). As both absolute and proportional amounts of Ep-induced haem synthesis in vitro are greater in cells of 8-day embryos than in cells at later stages of primary erythropoiesis the detected response is probably not due to small numbers of cells of the intermediate (hepatic) erythroid series which might exist prior to the development of the liver, as these cells would be more numerous at 9 days of gestation.

The ability of yolk-sac erythroid cells to divide in the circulation decreases progressively between the 11th and 13th days of development, and is paralleled by a decline in RNA content and RNA synthesis. During the early stages of the phase of yolk-sac erythropoiesis haemoglobin synthesis depends on newly synthesized RNA (Fantoni, de la Chapelle, Rifkind & Marks, 1968). The erythropoietin sensitivity of circulating yolk-sac erythroid cells is therefore limited to a period when there is active DNA synthesis and cell division, and this is consistent with the known effects of erythropoietin on both foetal liver and bone marrow erythroid cells.

The conclusion to this work is that Ep can stimulate haem synthesis in cells from 8- and 9-day embryos and nucleated erythrocytes of 12-day embryos in vitro. Electrophoretic studies of $^{59}$Fe labelled haemoglobins on polyacrylamide gels suggest that the pattern of synthesis of foetal haemoglobins by 12-day blood cells is not altered significantly by Ep. Ep therefore stimulates the synthesis of haemoglobins which the erythroid cell is already programmed to produce, as previously described in foetal liver cells (Cole et al. 1968).
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REFERENCES


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