Evidence for an Erythropoietin Receptor Protein on Rat Bone Marrow Cells

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SUMMARY

Rat bone marrow cells, in vitro, respond to erythropoietin by increased RNA synthesis even in the absence of protein synthesis. Trypsin treated cells lose their ability to respond to the hormone if protein synthesis is inhibited, but retain responsiveness if protein synthesis is permitted during the incubation. The data suggest that a protein receptor on the external surface of the responsive cells is required for the action of erythropoietin on marrow cells.

INTRODUCTION

A number of protein hormones and growth factors exert their effects on target cells by interacting with specific receptor molecules on the external surface of those cells [1-8]. We present, here, data suggesting that erythropoietin, a glycoprotein that is the primary inducer of erythrocyte development, operates by a similar mechanism.

Because of the unavailability of a sufficient quantity of pure, labelled, biologically active erythropoietin, and the unavailability of a homogeneous population of erythropoietin responsive cells (ERC), an indirect method for demonstrating the probable existence of erythropoietin receptor molecules was

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used. This method was adapted, in part, from earlier published work on insulin receptor sites [2,9,10,11].

The stimulation of nuclear RNA synthesis is among the earliest effects of erythropoietin on rat bone marrow cells [12,13] in vitro and on cultured fetal liver cells [14]. This action is mediated by a cytoplasmic protein (termed MCF) that is found in marrow ERC after their interaction with erythropoietin, and can be generated in the absence of protein synthesis [15]. In addition, inhibition of protein synthesis with cycloheximide does not affect the early stimulation of RNA synthesis due to erythropoietin by way of MCF action [16]. These observations formed the basis for our study of erythropoietin receptors on the outside surface of ERC.

MATERIALS AND METHODS

Marrow cell culture. Marrow cells from the femora and tibiae of male Sprague-Dawley rats (200g) were cultured in a medium at pH 6.7 consisting of 90% NCTC 109 containing 30mM morpholinopropane sulfonic acid (MOPS) and 10% newborn calf serum. In one experiment (Table 1, Experiment 1), the proportions were 65% 109:35% newborn calf serum. Trypsinization was done with the cells suspended in PBS (0.145 M NaCl, 0.013 M phosphate pH7.3); the concentration of trypsin was 0.25%. The cells were stirred in the trypsin solution at 25° for one minute then washed three times with PBS containing 0.1% bovine serum albumin. After the last wash, the cells were suspended in the culture medium at a concentration of 15×10^6 nucleated cells per ml and 0.9 ml aliquots were pipetted into culture dishes (35x10mm). In those experiments where RNA synthesis was measured 0.13 µmoles/ml of carrier uridine were added to the medium. Other conditions are indicated in the table legends. After the incubation the cells were transferred to 13x100 mm tubes using 1.0 ml of PBS, washed once with PBS and twice with 1.5 ml of 5% TCA. The acid insoluble pellet was dissolved in 0.2 ml of hot formic acid and counted, after addition of 5 ml of Aquasol.

Newborn calf serum was bought from Grand Island Biological Co., NCTC

109 from Microbiological Associates, trypsin from Worthington Biochemical Corp, cyclohexiмide from Nutritional Biochemicals Corp., culture dishes from Linbro Chemical Co., ^{3}H-uridine (25 Ci/mmole) and ^{14}C-valine (280 mCi/mmole) from

Table 1

Effect of Trypsin or Cycloheximide on Marrow Cell RNA and Protein Synthesis

	labelled precursor	
	^{14}C-valine cpm	^{3}H-uridine cpm
Experiment 1		
Control	5010 ± 180	4450 ± 10
Trypsin	3330* ± 170	2840* ±110
Cycloheximide	60 ± 8	-
Trypsin & Cycloheximide	50 ± 5	-
Experiment 2		
Control	-	850 ± 30
Erythropoietin	-	950* ± 30
Cycloheximide	-	720 ± 8
Cycloheximide + erythropoietin		810* ± 35

In Experiment 1 cells were trypsinized and washed as described in the text. They were cultured at a concentration of $15x10^{6}$ nucleated cells/ml in a medium of 65% NCTC 109:30% newborn calf serum and 5% rat serum, without carrier uridine, incubated at 37° in 10% CO_2:90% air for 30 minutes at which time cycloheximide (50 µg/ml) was added, at 75 minutes 1.0 µCi of ^{3}H-uridine and 0.5 µCi of ^{14}C-valine were added to each dish and the cultures stopped at 115 minutes.

In Experiment 2 cycloheximide (50 µg/ml) was added at the start of the incubation. At 20 minutes, erythropoietin (0.10 unit/ml) was added and at 50 minutes 1.0 µCi of ^{3}H-uridine. The cultures were stopped at 80 minutes. The cell concentration was $15x10^{6}$ nucleated cells per ml. in 90% NCTC 109:10% newborn calf serum.

The figures expressed are the means of 5 replicate cultures ± one standard deviation. The figures marked with an asterisk are significantly different from their respective controls at $P < 0.001$ by th "t" test.

Amersham-Searle, and Aquasol from New England Nuclear Corp.

The erythropoietin used was a sheep plasma preparation, with a potency of 240 units per mg. of protein, prepared in this laboratory.

RESULTS and DISCUSSION

In control experiments (Table 1) we found that trypsin treatment of rat bone marrow cells caused a decrease of about 35% in both uridine and valine incorporation.

Cycloheximide inhibited protein synthesis in untreated or trypsinized cells almost completely, while it had a small effect (15% inhibition) on control RNA synthesis and essentially no effect on erythropoietin-induced RNA synthesis by untrypsinized cells in agreement with our previous findings [16].

When marrow cells, however, were briefly exposed to trypsin, washed, and incubated in the presence of cycloheximide, we found that the response to erythropoietin was almost completely lost (Table 2). When protein synthesis was permitted after trypsinization, there was a significant effect of erythropoietin on uridine incorporation. The magnitude of this effect (14-16% increase due to the hormone) is very similar to that found with untrypsinized cells incubated under the same conditions (Table 1).

The lack of response to erythropoietin by trypsinized cells, incubated in the presence of cycloheximide, cannot be due to the inactivation of the hormone by a small amount of trypsin still associated with the washed cells. This is seen from the fact that the trypsin treated cells incubated without cycloheximide do respond to added erythropoietin. In addition, comparison of rates of RNA synthesis by trypsinized and control cells after four hours of incubation shows that while the baseline RNA synthesis is depressed by about 40% by trypsin, the response to erythropoietin (Δ cpm) is not significantly affected (Table 3).

These data suggest that there is a protein on the external surface of the ERC which is accessible to trypsin and is required for the erythropoietin effect on RNA synthesis. After removal, by trypsin, this protein receptor

Table 2

The Effect on Cycloheximide on Response of Trypsinized Marrow Cells to Erythropoietin

	Uridine incorporation	
	no addition	Cycloheximide
	cpm	cpm
Experiment 1		
Control	450 ± 30	410 ± 15
Erythropoietin	520* ± 10	420 ± 10
Δ cpm	70	10
Experiment 2		
Control	660 ± 20	520 ± 10
Erythropoietin	760* ± 20	540 ± 10
Δ cpm	100	20

Cycloheximide, where present, was at a concentration of 50 µg/ml; erythropoietin at 0.10 unit/ml. Cycloheximide was added at the start of incubatic and the cells were incubated at 37° in 10% CO_2; 90% air. At 20 minutes, erythropoietin was added and at 50 minutes, 1.0 µCi of ^{3}H-uridine was added to each dish. The cultures were stopped at 80 minutes. The cell concentration in Experiment 1 was $15x10^6$/ml; in Experiment 2 $16x10^6$/ml. The figures expressed are the means of 5 replicate samples ± one standard deviation. The values labelled with an asterisk are significantly different from their respective controls at the $P < 0.001$ level by "t" test. The apparent effect of erythropoietin in the cycloheximide-treated cultures is not statistically significant.

may be rapidly resynthesized by the same cells in the absence of cycloheximide. Alternatively, another cohort of cells may become responsive to erythropoietin by forming receptor protein.

The evidence presented here is consistent with our earlier finding that erythropoietin exerts its effect on ERC nuclei by an indirect mechanism [15]. The hormone interacts with an external receptor protein causing the generation,

Table 3

Response of Trypsinized Marrow Cells to Erythropoietin

Cells	cpm in RNA		
	Control	Erythropoietin	Δ cpm
untreated	610 ± 30	680* ± 20	70
trypsinized	360 ± 15	420* ± 20	60

Conditions of this experiment were as in Table 2, except for total incubation time in the presence of erythropoietin which was four hours. Results are expressed as means of 5 replicate cultures (4 in the case of trypsinized cells treated with erythropoietin). Values with an asterisk are significantly different from their respective controls at $P < 0.01$ by "t" test. The difference between the two responses to erythropoietin is not significant.

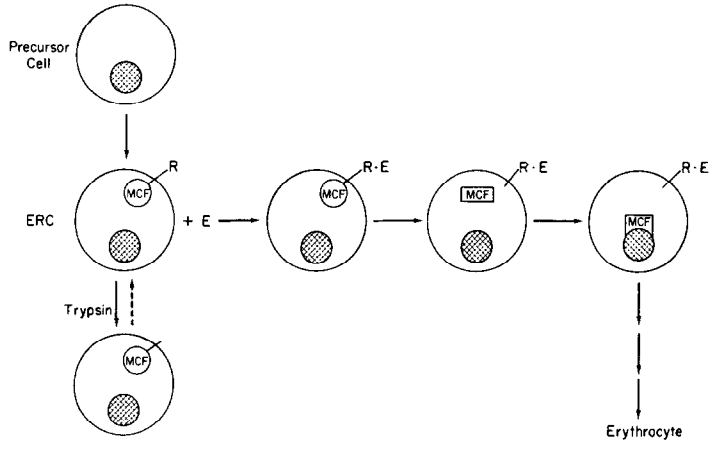

Figure 1

A Scheme for the Action of Erythropoietin on ERC

Legend: ERC = erythropoietin responsive cell, R = erythropoietin receptor, E = erythropoietin, MCF = marrow cytoplasmic factor. The shaded circle represents the cell nucleus; the change of MCF from circular to rectangular represents the formation of the active factor which can interact with the cell nucleus.

(perhaps by a conformational change) or the release, in the cytoplasm of MCF which is the direct effector of increased nuclear RNA synthesis (Fig. 1). While we indicate in the figure that erythropoietin (E) remains bound to the receptor (R) after the release of the active form of MCF (here portrayed by the conversion from a circle to a rectangle) it is quite possible that the hormone is dissociated from the receptor during, or after, MCF generation. If this is the case, it could explain our earlier finding, in which we could not detect loss of erythropoietin from the medium after the ERC had been triggered to synthesize hemoglobin [17].

These data also suggest that cells of the hemopoietic system become ERC and capable of being induced to differentiate, eventually to become erythrocytes, by virtue of possessing receptors for erythropoietin. ERC can then be defined as cells with functional receptors for erythropoietin.

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