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Some Studies of Erythroid Differentiation *in vitro*

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Introduction

In recent years considerable evidence has accumulated in support of the hypothesis that erythropoietin (epo) is an inducer of erythrocyte differentiation in adult hematopoietic tissue (1). The system "epo-primitive hematopoietic cells" is, in principle, a very good one for the in vitro biochemical study of cytodifferentiation, since the process can be initiated at will by use of the inducer and since the end product cell has a protein, hemoglobin, that is unique and easily determined. In practice the system is somewhat less than ideal because the inducer, epo, is not yet available in a pure form and because the cell population obtainable from adult hematopoietic tissue is heterogeneous. Both of these limitations are the subjects of study in our laboratory but will be discussed only briefly in this paper.

Erythropoietin from anemic sheep plasma has been fractionated to a potency of about 6,500 units per mg of protein (representing a purification factor of 930,000) but the yield was too small for detailed chemical study. It appears to be a glycoprotein of molecular weight about 50,000 daltons (2) and even at this high degree of purity may be heterogeneous.

The problem of fractionating marrow cells is being studied in several laboratories at present without any spectacular success (3, 4, 5). It is fair to say that neither stem cells (capable of colony formation and/or repopulating an irradiated animal) nor epo responsive cells, if the two are different, have been isolated or identified morphologically.

For some years we have been studying the effects of epo on rat marrow cells in vitro in spite of having neither pure epo nor a homogeneous cell population. In part, these drawbacks can be circumvented by studying the effects induced by epo over the base line activities of the cells. We have demonstrated that epo causes increases in the rates of RNA synthesis (6, 7), hemoglobin synthesis (8, 9), stroma synthesis (10), and iron uptake (11). We have also been studying the relationships between these biochemical events and their possible roles in the sequential steps leading to the final erythrocyte. Our observations and hypotheses can be put into a coherent, if tentative and oversimplified, framework as illustrated in Fig. 1. The earliest functional change we have seen in marrow cells, so far, is the synthesis of an RNA species of very large molecular size (150S) which occurs in the nuclei within fifteen minutes of addition of epo to the culture medium (7). As yet we have no clear idea what role this "giant" RNA plays in the subsequent steps in cytodifferentiation, although we feel that it must have an important part in the process. A short time after formation of the 150S RNA, the nuclei of the induced cells synthesize another large sized RNA (55-65S) of unknown function and ribosomal RNA precursor (45S), followed in turn by formation of ribosomal RNA, transfer RNA, by increased protein synthesis, and the formation of the system that facilitates entry of iron into the cells. The molecular details of this last system are still being worked out, but we

know that part of it involves the new synthesis of ferritin and possibly of another intracellular iron-containing protein.

At about the same time, the induced cells begin to synthesize a component of their outer surface which we measure by the incorporation of labeled glucosamine into cell stroma. Some of the labeled material formed as a result of epo action appears in what seems to be a glycopeptide analogous to the M-N antigen of human erythrocytes (12). Induced hemoglobin synthesis also occurs during this time. Epo also induces an increase in DNA synthesis, but the time relationship suggests that this may be a secondary phenomenon, perhaps reflecting division of stem cells previously held in some sort of mitotic block that was released when cells were induced along the path toward erythroid differentiation. On the other hand, this increase in DNA synthesis may be due to an accelerated rate of division of differentiating erythroid cells. In the scheme shown in Fig. 1, epo may act on the original stem cell, on a cell that is differentiated (possibly in a reversible manner) to form a specific epo-responsive cell, or on both. There is also some evidence suggesting that epo, in larger than physiological amounts, may also act on cells that are irreversibly differentiated in the erythroid direction (13).

Most of our studies of marrow cells in vitro have involved short periods and have shown what appeared to be the rapid loss of epo-responsiveness during these short-term

incubations. In the remainder of this paper we will discuss problems concerning the maintenance of potential for cyto-differentiation (epo-responsiveness) in long-term cultures of rat marrow cells. As measures of epo-responsiveness, we have used RNA synthesis, protein synthesis, iron-uptake and hemo-globin synthesis, as measured by formation of both heme and globin.

Materials and Methods

Rat marrow cells were removed from tibias and femurs of male Sprague-Dawley rats by aspirating from the ends of the bones, and incubated in either plastic petri dishes (35 X 10 mm) or plastic bottles (25 cm²) bought from Falcon Plastics. The medium was 65% NCTC 109 (Microbiological Associates), 30% new-born calf serum (Colorado Serum Co.) and 5% rat serum containing 4 µg/ml of iron as ferric nitrate. All cultures were incubated at 37° in an atmosphere of 5% CO₂ 95% air.

Radioactive precursors were bought from the following suppliers: ⁵⁹Fe, ¹⁴C-valine and ³H uridine from New England Nuclear Corp., ¹⁴C-glycine and ³H cytidine from Nuclear Chicago. Radioiron was counted in a well-type scintillation counter or as thin samples in a gas flow, thin-window beta counter. Radiocarbon and tritium were counted in a Packard TriCarb Model 3375 liquid scintillation spectrometer.

Hemoglobin was isolated by the method of Hrinda and Goldwasser (14) which depends on the specific complex formed with added haptoglobin. Total iron incorporation was determined by counting the washed cell pellet before other operations were performed. Total protein and RNA radioactivity were determined by precipitation with trichloroacetic acid, dissolving the washed pellet in formic acid, and counting in a 2:1 mixture of toluene: 2-ethoxyethanol containing 5 g/L of 2,5-diphenyloxazole.

Erythropoietin was a Step III preparation with a potency of about 3 units/mg of protein (15) and was generously supplied by the National Blood Resource Program of USPHS.

Results and Discussion

In earlier experiments (8), we found that the rate of heme synthesis in response to epo added at the start of incubation, showed an initial stimulus followed by a steady decline for three days. We therefore attempted to determine whether the ability of the marrow cells to respond to epo was impaired after longer periods or whether the decline was due to the requirement for epo throughout the incubation. The data presented in Fig. 2 show the results of an experiment in which epo was added after 0, 1, 2, 3, or 4 days of preincubation. Heme synthesis was not measured in this experiment because we had found that under some conditions the simplified method we use routinely may not distinguish between radioactivity in heme

iron and in ferritin iron. The effects of epo on rates of RNA synthesis, protein synthesis, and iron-uptake were studied and the results show clearly that rat marrow cells can respond to epo by changes in all of these functions even after 96 hours of pre-incubation.

Fig. 2A shows the changes in the control cells (no epo). Both iron uptake and protein synthesis fell steadily throughout the six days of incubation. After an initial rise during the first 14 hours, the rate of RNA synthesis by the control cells declined until about 120 hours when there seemed to be the start of a second rise. The data in Fig. 2B, C, and D are presented as percentages of the control values at each time the rates were measured. In the case of total RNA synthesis (B) the data show that epo caused a rapid increase whenever it was added to the cells, but that there were considerable differences in the mode of response dependent on the time of pre-incubation. When epo was added without pre-incubation the response was small and was followed by what seemed to be a small, secondary increase in rate of RNA synthesis at about 72 hours of incubation. Addition of epo at 24 or 48 hours was followed by a rapid rise in rate of RNA synthesis which declined quickly, and in both cases fell to levels considerably below the control values. These lower-than-control rates of RNA synthesis in the induced cells may represent a phase of activity not involving RNA synthesis, while the noninduced

cells still are making RNA. When epo is added at 72 or 96 hours there is again a rapid increase in the rate of RNA synthesis but this is not followed by a fall to values lower than control. In cultures that had epo added at 96 hours there appears to be a larger secondary response at about 120 hours, which is when the control rate of RNA synthesis showed some increase.

The pattern of epo effect on rates of protein synthesis (Fig. 2C) is similar to that seen for RNA synthesis but the response is somewhat slower, peaking at about 12 hours or more after addition of epo instead of at 3-4 hours as for RNA formation.

When iron-uptake was measured, the pattern of response was somewhat different (Fig. 2D). Addition of epo at the start of incubation caused a rapid, large and extended response; when it was added at later times there was generally a lag of about 8 hours before any increase in rate of iron-uptake was detected. The absence of a lag period when the cells were not pre-incubated might reflect the action of epo on already differentiated cells mentioned above. If this were the case it would imply that this type of responsive cell disappeared after 24 hours of incubation since epo added at that time did not cause a rapid response. Alternatively these observations might be interpreted as indicating that cells pre-incubated for 24 hours or longer have become temporarily refractory to epo and

become responsive again only after some intracellular process has had time to occur. Iron-uptake never decreased to values lower than the controls. All three parameters showed the largest effect of epo when it was added at 96 hours, suggesting that the number of responsive cells may have increased somewhat during the pre-incubation, or that the medium may have become conditioned during the pre-incubation and may then favor epo-responsiveness.

Clearly we have not been able to interpret the details of all of the biochemical changes in the responses of these pre-incubated cells. We can indicate, however, that for these relatively short periods the capacity of the cells to respond to epo is maintained and may even be somewhat increased.

Attempts to carry out experiments under similar conditions for even longer periods were not successful due to the loss of water from the culture dishes in spite of our efforts to keep the incubation chamber saturated with water vapor. To obviate this difficulty we used tightly stoppered plastic bottles for incubation with the results discussed below.

Some selected data from an experiment in which the pre-incubation time varied from zero to 20 days are shown in Figs. 3 and 4. These data are chosen to illustrate the observation that rat marrow cells kept in closed bottles without change in medium can still respond to epo added at 13 and 20 days. The data in Fig. 3 show the effects of epo addition at 13 or 20

days on the rates of RNA synthesis (A), protein synthesis (B), and iron-uptake (C) while Fig. 4 shows the effects on the rate of hemoglobin synthesis measured by glycine incorporation into globin (A) or iron-incorporation (B). In both of these figures the values for control and epo-treated cells are plotted as percentage of the control at 13 days of pre-incubation when the total nucleated cell number had fallen to about 10% of the original. This presentation of the data was chosen for simplicity and clarity of display and has no deeper significance.

When epo addition is made at 13 days there is a rapid increase in rates of RNA synthesis (Fig. 3A), protein synthesis (Fig. 3B), and hemoglobin synthesis (Fig. 4A and B). The effect on iron-uptake (Fig. 3C) is slower and smaller. This apparent discrepancy might be explained by the fact that the cells have been exposed to unlabeled iron during the pre-incubation period and may have accumulated a store of intracellular iron which is large enough to reduce their ability to respond to epo by increased iron-uptake. Inspection of Fig. 3C shows, however, that epo added at 20 days results in an appreciably greater iron-uptake than that caused at 13 days. If the argument about reduced iron-uptake at the earlier time is valid, then the increase at the later time must reflect an increase in the number of responsive cells. The data in Figs. 3 and 4 show that the response of all of the measured parameters after 20 days of pre-incubation is larger than after 13 days with the

exception of glycine incorporation into hemoglobin (Fig. 4B). We have no explanation for this difference at present, especially in view of the data in Fig. 4A which show a much greater stimulation of hemoglobin synthesis when determined by the incorporation of iron.

It is interesting to note that the three parameters that can be considered as functions specific for erythroid cells; iron-uptake, glycine incorporation into globin, and iron-incorporation into hemoglobin all show a secondary wave of response to epo added at 20 days. This second peak occurs at 27-28 days, i.e., 7-8 days after the epo was added and at a time when control rates were all falling. This is another phenomenon for which we have no explanation as yet.

Another general feature of this system may be seen by comparison of the times of maximal primary response to epo. In general the temporal sequence derived from earlier, indirect, experiments seems to hold for cells after various times of pre-incubation, i.e., RNA synthesis, then total protein synthesis, then iron-uptake followed by hemoglobin synthesis. For the purpose of studying this sequence it would seem to be advantageous to use cells that have been pre-incubated for at least 48 hours in order to eliminate any possible epo effects on already differentiated cells.

In all the experiments described in this paper the cells were incubated without change of medium. This probably accounts

for the decline after the fourth week in control cells' activities when measured by such nonspecific functions as RNA and protein synthesis, and we have found that use of fresh medium does prevent these changes.

Many of our observations on epo effects in marrow cell cultures are still only at the descriptive stage and we can produce no comprehensive concept linking them. Regardless of the details of the biochemical mechanisms underlying these observations it is clear, however, that epo-responsive cells can be maintained in vitro for considerable periods and that these cells are capable of expressing many of the functions characteristic of erythroid cells once they have been induced. The unraveling of the molecular mechanisms is the next order of business.

Summary

When rat marrow cells are pre-incubated for periods of up to 20 days they retain the capacity to respond to exogenous erythropoietin. This is true whether the response is measured in terms of increased rates of RNA synthesis, protein synthesis, iron-uptake, or hemoglobin synthesis. The patterns of response to erythropoietin by pre-incubated cells are complex and involve secondary waves of increased rates of synthesis that are not yet understood.

REFERENCES

1. Goldwasser, E. 1966. Biochemical control of erythroid cell development, p. 173-211. In A. Monroy and A. Moscona /ed/ Current Topics in Developmental Biology, Volume I. Academic Press, New York.
2. Goldwasser, E., and Kung, C. K. H. 1968. Progress in the purification of erythropoietin. Ann. N. Y. Acad. Sci., 149: 49-53.
3. Mel, H. C., and Schooley, J. C. 1964. Stable-flow free boundary fractionation of spleen colony-forming cells from mouse bone marrow. Exp. Hematol., 7: 26-29.
4. Turner, R. W. A., Siminovitch, L., McCulloch, E. A., and Till, J. E. 1967. Density gradient centrifugation of hemopoietic colony-forming cells. J. Cell Physiol., 69: 73-82.
5. Takaku, F., Dukes, P. P., and Goldwasser, E. 1964. In vitro studies of cell types responsive to erythropoietin. Endocrinology, 74: 968-972.
6. Krantz, S. B., and Goldwasser, E. 1965. On the mechanism of erythropoietin-induced differentiation. II. The effect on RNA synthesis. Biochim. Biophys. Acta, 103: 325-332.
7. Gross, M., and Goldwasser, E. 1968. Effect of erythropoietin on RNA synthesis. Fed. Proc., 27: 394.

8. Krantz, S. B., Gallien-Lartigue, O., and Goldwasser, E.
1963. The effect of erythropoietin upon heme synthesis
by marrow cells in vitro. J. Biol. Chem., 238:
4085-4090.
9. Gallien-Lartigue, O., and Goldwasser, E. 1964. Hemo-
globin synthesis in marrow cell culture: the effect of
rat plasma on rat cells. Science, 145: 277-279.
10. Dukes, P. P., Takaku, F., and Goldwasser, E. 1964. In
vitro studies on the effect of erythropoietin on
glucosamine-1-¹⁴C incorporation into rat bone marrow
cells. Endocrinology, 74: 960-967.
11. Hrinda, M. E., and Goldwasser, E. 1966. Facilitated iron
entry into marrow cells: an induced permease-like
activity. Fed. Proc., 25: 284.
12. Winzler, R. J., Harris, E. D., Pekas, D. J. Johnson, C. A.,
and Weber, P. 1967. Studies on glycopeptides released
by trypsin from intact human erythrocytes. Biochemistry,
6: 2195-2207.
13. Borsook, H. 1964. DNA, RNA and protein synthesis after
acute, severe blood loss. Ann. N. Y. Acad. Sci., 119:
523-539.
14. Hrinda, M., and Goldwasser, E., unpublished data.
15. Goldwasser, E., White, W. F., Taylor, K. B. 1962. Further
purification of sheep plasma erythropoietin. Biochim.
Biophys. Acta, 64: 487-496.

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FIG. 1. The pattern of erythropoietin action during erythrocyte differentiation: ER cell stands for erythropoietin responsive cell, rbc for erythrocyte, epo for erythropoietin, rRNA for ribosomal RNA, tRNA for transfer RNA.

FIG. 2. Effect of erythropoietin on marrow cells pre-incubated for 96 hours or less.

A: control values of rate of RNA synthesis ●, rate of iron-uptake ▲, and rate of protein synthesis ■.

B: effect of epo added at times indicated by arrows on rate of RNA synthesis measured by a 30-minute pulse of ^3H -uridine.

C: effect of epo added at times indicated by arrows on rate of protein synthesis measured by a 30-minute pulse of ^{14}C -valine.

D: effect of epo added at times indicated by arrows on rate of iron-uptake measured by a 4-hour pulse of ^{59}Fe .

In B, C, and D ▲ signifies epo addition at 0 hours, X at 24 hours, + at 48 hours, ■ at 72 hours and ● at 96 hours. All cultures had 10^7 nucleated cells/ml initially in a total volume of 1.0 ml in 30 X 10 mm petri dishes, epo was at 0.10 units/ml; all samples run in triplicate.

FIG. 3. Effect of erythropoietin on marrow cells pre-incubated for 13 and 20 days.

A: effect of epo added at 13 or 20 days on rate of RNA synthesis measured by a 30-minute pulse with ^3H -cytidine.

B: effect of epo added at 13 or 20 days on rate of protein synthesis measured by a 4-hour pulse with ^{14}C -valine.

C: effect of epo added at 13 or 20 days on rate of iron-uptake measured by a 4-hour pulse with ^{59}Fe .

All cultures had 6×10^6 nucleated cells/ml initially in a total volume of 3.0 ml in stoppered flasks: epo was at 0.25 units/ml: control, ; epo addition at 13 days $\bullet$; at 20 days X.

FIG. 4. Effect of erythropoietin on erythropoietin-induced hemoglobin synthesis by marrow cells pre-incubated for 13 and 20 days.

A: effect of epo added at 13 or 20 days on rate of incorporation of ^{59}Fe into hemoglobin measured by a 4-hour pulse.

B: effect of epo added at 13 or 20 days on rate of incorporation of ^{14}C -glycine into globin measured by a 4-hour pulse.

All cultures had 6×10^6 nucleated cells/ml initially in a total volume of 3.0 ml in stoppered flasks: epo was at 0.25 units/ml: control, ; epo addition at 13 days $\bullet$; at 20 days X.

DNA
Synthesis

150 sRNA

55-65 sRNA

rRNA precursor

tRNA

rRNA

Protein

Fe uptake system

Stroma

Hemoglobin

PERCENT OF CONTROL RATE AT 13 DAYS