On the Mechanism of Erythropoietin-induced Differentiation

VII. THE RELATIONSHIP BETWEEN STIMULATED DEOXYRIBONUCLEIC ACID SYNTHESIS AND RIBONUCLEIC ACID SYNTHESIS*

(Received for publication, August 18, 1969)

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SUMMARY

Erythropoietin causes the conversion of primitive cells to erythrocytes. Some biochemical alterations seen in vitro during this induced process are increased RNA synthesis, DNA synthesis, iron uptake, and hemoglobin synthesis. In experiments of relatively long duration, inhibition of DNA synthesis prevents erythropoietin-stimulated iron uptake and hemoglobin synthesis. In shorter experiments, both these functions and stimulated RNA synthesis are completely expressed in the absence of stimulated DNA synthesis. Inhibition of either RNA or protein synthesis, however, causes complete suppression of the erythropoietin effect on iron uptake, hemoglobin synthesis, and DNA synthesis. These data, and earlier data on the time course of erythropoietin-stimulated RNA synthesis, indicate that the primary action of the inducer is not on cell division or DNA replication but on RNA or protein synthesis.

When primitive cells of the hematopoietic system are exposed to erythropoietin, a series of molecular events is initiated which culminates in the formation of mature erythrocytes. In order to understand the biochemical mechanisms underlying this process of cytodifferentiation we should know the temporal order of these events and their causal relationships to each other. Our studies in vitro with adult rat marrow cells have shown that erythropoietin causes increased RNA synthesis within a few minutes of its addition to the medium (1, 2). Other effects of erythropoietin, such as stimulated iron uptake (3) and increased formation of hemoglobin (4) and of stroma (5), occur later and are, most likely, not primary effects.

The relationship between erythropoietin action and DNA synthesis still needs to be clarified. In contrast to our earlier findings that erythropoietin had no effect on DNA synthesis in rat marrow cells (6), Dukes showed that there was an appreciable stimulation of thymidine incorporation in the same system after about 3 hours of incubation (7). Since one component of RNA synthesis is increased by 15 min it would appear that, in this system, the effect on DNA synthesis is secondary to an earlier effect, perhaps on a transcriptive step. In addition, we found that inhibition of cell division by colchicine had no effect on erythropoietin-induced stroma synthesis (8) and only partially inhibited increased hemoglobin formation (9). Paul and Hunter (10, 11), however, studying the effect of erythropoietin on fetal liver cells, found that DNA synthesis was markedly increased within the 1st hour of incubation, and that inhibition of DNA synthesis by FUDR caused the complete abolition of increased hemoglobin synthesis. From their studies with actinomycin and puromycin these authors suggested that RNA and protein may be formed prior to, and independent of, DNA synthesis (11). The results in the present paper confirm these suggestions and, taken with those from our previous paper, show that the action on DNA synthesis is preceded by and dependent upon the synthesis of RNA.

MATERIALS AND METHODS

Rat bone marrow cells were incubated in a medium consisting of 55% NCTC 109, 40% newborn calf serum, and 5% rat serum containing carrier iron, as described previously (2). When FUDR was used as inhibitor, the 109 medium was replaced by a similar, thymidine-free, formulation.

Incorporation of labeled thymidine or deoxycytidine into the trichloroacetic acid-insoluble fraction of the cells was used to evaluate DNA synthesis, and incorporation of uridine into the same fraction to measure RNA synthesis. The use of these simplified techniques was justified by our findings that hydroxyurea caused complete inhibition of incorporation of deoxycytidine but not of uridine. Sample preparation and counting of 3H and 14C were described in an earlier paper (2).

The erythropoietin-induced uptake of iron by marrow cells was measured by counting the washed cell pellet after the cells were incubated in the presence of 59Fe-labeled rat serum, the

* The preceding paper in this series is Reference 3. It is taken in part from the thesis submitted by Martin Gross to the University of Chicago in June 1969 for the degree of Doctor of Philosophy.
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1 The abbreviation used is: FUDR, 5-fluorodeoxyuridine.
transferrin of which was 75 to 100% saturated with iron. Heme was prepared from cell lysates by the method of Fox and Thomson (12). Since we had previously shown that virtually all of the labeled heme found in cultured marrow cells was in the form of hemoglobin (13), the heme radioactivity was taken as a measure of hemoglobin synthesis.

NCTC 109 was bought from Microbiological Associates; thymidine-free NCTC 109 was from Grand Island Biologicals; disposable culture dishes were from Falcon Plastics; 5H-deoxycoctydine (0.5 Ci per mmole), 5-methylthymidine (15 Ci per mmole), and 5H-5-uridine (28.3 Ci per mmole) were from Nuclear-Chicago; 5C-2-uridine (51.5 mCi per mmole) and 55FeCl₃ (95 mCi per mmole) were from New England Nuclear. Cycloheximide was a gift from Upjohn; FUDR was a gift from the Cancer Chemotherapy National Service Center of the National Cancer Institute; puromycin and hydroxyurea were bought from Nutritional Biochemicals; colchicine was from Eli Lilly; and actinomycin D was from Merek, Sharp and Dohme. Erythropoietin (Step IV) was a gift from the National Blood Resource Program, National Heart Institute, National Institutes of Health. Its potency was 19 units per mg of protein and it is extremely impure. Similar results, however, are obtained when fractions with much higher potency are used.

RESULTS

In Fig. 1 are shown the effects of FUDR and colchicine on erythropoietin-stimulated DNA synthesis. Both sets of controls (in complete and in thymidine-free medium) show an erythropoietin-induced increase in rate of DNA synthesis up to 9 hours and a sharp decrease thereafter. FUDR caused a complete inhibition of increased DNA synthesis at all three pulse times; colchicine had no effect at the earliest time, but caused progressive inhibition at the later times. These inhibitors also caused appreciable decreases in the rate of thymidine incorporation in cells not treated with erythropoietin. Cells exposed to FUDR showed a 50 to 65% inhibition at all three pulse times. Colchicine caused a 25% decrease at the two earlier intervals and a 70% decrease at 15 hours. Similar experiments showed that hydroxyurea (10⁻⁴ M) also completely inhibited the synthesis of DNA in response to erythropoietin when measured at 3 and 11 hours.

We have already shown that increased RNA synthesis caused by erythropoietin is not due to an effect on the specific activity of the precursor pool (2); this is also true for the incorporation of deoxyerythidine into DNA. When the pool was previously labeled by incubation with 3H-deoxyerythidine for 1 hour and the external label was removed, subsequent incubation in the presence of erythropoietin caused a small but significant increase in the DNA radioactivity, when compared with cells having the same labeled pool but no erythropoietin.

The effects of FUDR, hydroxyurea, and colchicine on erythropoietin-stimulated hemoglobin synthesis and iron uptake were determined in cultures that had been incubated for either 27 or 51 hours, the last 6 hours with 55Fe (Table I). At the later time both FUDR and hydroxyurea caused complete inhibition of stimulated hemoglobin synthesis and almost complete inhibition of stimulated iron uptake, the effect of colchicine being slightly less. At the earlier time the inhibition caused by all three inhibitors was somewhat smaller. Stimulation of hemoglobin synthesis was more sensitive to all three inhibitors than was stimulation of iron uptake. In this experiment, as in the one described in Fig. 1, the inhibitors showed a smaller effect on the control than on the stimulated cells.

When the incubation time was shortened to 4 hours (Table II) the effects of the inhibitors on the same stimulated functions were quite different. Hydroxyurea caused an inhibition of about 50% with respect to both stimulated iron uptake and hemoglobin synthesis; FUDR and colchicine had only a slight inhibitory effect on either of them. It is clear from Fig. 1 that FUDR at this time caused complete inhibition of erythropoietin-stimulated DNA synthesis. While the effects of erythropoietin so early in the culture are small, they are significant. The salient point is that early in the incubation period cells do not require stimulated DNA synthesis or cell division in order to express short term erythropoietin-stimulated iron uptake or hemoglobin synthesis.

We also determined the effects of these inhibitors on erythropoietin-induced RNA synthesis (Table III). Hydroxyurea and FUDR had essentially no effect on stimulated uridine incorporation either at 2.5 or 9 hours while colchicine had an inhibitory effect only at the later time. Hydroxyurea caused about 40% inhibition of RNA synthesis in the control (nonstimulated) cells at 2.5 hours and almost 70% inhibition at 9 hours. If FUDR had any significant effect on the control cells it was not inhibitory. Colchicine had no appreciable effect on controls at 2.5 hours but did cause 36% inhibition of RNA synthesis at 9 hours. Despite the effects of the inhibitors on the unstimulated cells, it is clear from these data that the expression of erythropoietin action with respect to RNA formation even as...
late as 9 hours does not require DNA synthesis. Essentially of the erythropoietin effect by hydroxyurea. There was no effect of FUDR or colchicine and 26% inhibition of *H-uridine and pulsing for 15 min after 4 hours of incubation. The same results were obtained in a similar experiment with the control cells and that the 60% increase in deoxycytidine incorporation due to erythropoietin was completely abolished. We found essentially similar results (Table V) when the incubation time was 100 min, but the erythropoietin effect was, as expected, quite small. The rather large effects of the inhibitors on the control cells make interpretation of these results somewhat equivocal, but we know that cycloheximide at the same concentration diminishes RNA synthesis only slightly, making

| Addition | Iron uptake | Inhibition of erythropoietin effect | Hemoglobin synthesis | Inhibition of erythropoietin effect | Hemoglobin synthesis |
|----------|-------------|-----------------------------------|----------------------|-----------------------------------|----------------------|
| A. None | 6,760 (±560) | 700 (±10) | 7,570 (±90) | 60 (±3) |
| Erythropoietin | 18,130 (±570) | 4,000 (±70) | 15,280 (±150) | 1,730 (±30) |
| FUDR | 5,880 (±70) | 570 (±30) | 1,970 (±30) | 22 (±1) |
| Erythropoietin + FUDR | 9,160 (±120) | 71 | 1,140 (±20) | 83 | 2,710 (±90) | 90 | 40 (±5) |

| B. None | 7,440 (±100) | 1,240 (±30) | 13,810 (±60) | 160 (±7) |
| Erythropoietin | 18,000 (±580) | 7,130 (±210) | 16,820 (±230) | 1,190 (±100) |
| Hydroxyurea | 4,600 (±80) | 620 (±30) | 8,270 (±330) | 46 (±3) |
| Erythropoietin + hydroxyurea | 5,880 (±100) | 89 | 940 (±80) | 95 | 8,210 (±340) | 100 | 41 (±1) |
| Colchicine | 4,420 (±150) | 710 (±40) | 650 (±20) | 19 (±1) |
| Erythropoietin + colchicine | 8,680 (±200) | 60 | 2,310 (±120) | 73 | 1,080 (±20) | 86 | 97 (±5) |

* Inhibitions expressed are those of the erythropoietin effect. Figures in parentheses are standard deviation of the mean.

**Table II**

Effects of inhibitors on early erythropoietin stimulated iron uptake and hemoglobin synthesis

Conditions were as in Table I except that labeling period was from 0 to 4 hours after which cultures were stopped. Cultures in Group B had 0.07 unit of erythropoietin per ml.

| Addition | 21 to 27 hrs | Inhibition | 45 to 51 hrs | Inhibition |
|----------|-------------|------------|-------------|------------|
| Iron uptake | cpm | % | cpm | % | cpm | % | cpm | % |
| A. None | 7470 (±130) | 1790 (±130) | 1590 (±30) | 1330 (±100) |
| Erythropoietin | 8040 (±200) | 2310 (±130) | 1590 (±30) | 1330 (±100) |
| FUDR | 7500 (±100) | 2100 (±20) | 1410 (±30) | 1120 (±30) |
| Erythropoietin + FUDR | 9150 (±150) | 11 | 2390 (±30) | 6 | 1580 (±30) | 6 | 1390 (±100) | 0 |
| B. None | 2150 (±60) | 840 (±10) | 700 (±30) | 680 (±50) |
| Erythropoietin | 3600 (±70) | 1040 (±40) | 700 (±30) | 680 (±50) |
| Hydroxyurea | 1910 (±30) | 670 (±20) | 330 (±15) | 210 (±10) |
| Erythropoietin + hydroxyurea | 2110 (±20) | 52 | 760 (±20) | 55 | 450 (±15) | 0 | 370 (±10) | 6 |
| None | 2140 (±50) | 560 (±20) | 1600 (±40) | 1440 (±60) |
| Erythropoietin | 2380 (±80) | 650 (±20) | 1900 (±100) | 1670 (±40) |
| Colchicine | 2180 (±40) | 510 (±20) | 1570 (±80) | 820 (±40) |
| Colchicine + erythropoietin | 2400 (±200) | 8 | 660 (±30) | 0 | 1850 (±50) | 4 | 1070 (±40) | 35 |
Effects of inhibitors on early stimulated DNA synthesis

Cultures in quadruplicate contained 25 x 10^4 nucleated rat bone marrow cells in 1.0 ml of medium. Inhibitors were added at zero time and 0.05 unit of erythropoietin 10 min later. At 5.25 hours 1 µCi per ml of ^3H-deoxycytidine was added, and cultures were stopped at 6.75 hours.

| Addition          | Control     | Erythropoietin | Inhibition of erythropoietin effect |
|-------------------|-------------|---------------|-----------------------------------|
| None              | 270 (+30)   | 270 (+30)     | 99                                |
| Actinomycin D (8 µg/ml) | 1390 (+110) | 1390 (+110)   | 99                                |
| Puromycin (100 µg/ml) | 1900 (+30)  | 1900 (+30)    | 100                               |

It is plausible that its inhibition of DNA synthesis is not due to general cytotoxicity but to its effect on protein formation.

**DISCUSSION**

Erythropoietin initiates the process by which some unknown precursor cells develop into mature erythrocytes. The most striking changes occurring during this developmental process, such as synthesis of massive amounts of hemoglobin, loss of nuclear function, and, in mammals, loss of the nucleus, are not the immediate consequences of the induction of erythropoiesis. In order to determine the molecular locus of the primary inducive step and the sequence of biochemical events leading to the immediate consequences of the induction of erythropoiesis, we may study changes in primitive cells to red cells we may study changes in function with time or use inhibitors with known specificities.

In previous papers we reported on the time course of erythropoietin stimulation of RNA synthesis (1, 2). Since the earliest change that we have seen in adult marrow cells exposed to erythropoietin is an increase, at 10 to 15 min, in synthesis of a very large (150 S) nuclear RNA (2) and since both DNA synthesis and protein synthesis are affected only at a later time we have suggested that the primary action of erythropoietin may be on a transcripive step.

TABLE IV

Effects of inhibitors on stimulated DNA synthesis

Cultures in quadruplicate contained 15 x 10^4 nucleated rat marrow cells in 1.0 ml of medium. The inhibitors were added at zero time and 0.05 unit of erythropoietin 10 min later. At 5.25 hours 1 µCi per ml of ^3H-deoxycytidine was added, and cultures were stopped at 6.75 hours.

| Addition          | Control     | Erythropoietin | Inhibition of erythropoietin effect |
|-------------------|-------------|---------------|-----------------------------------|
| None              | 2580 (+200) | 2580 (+120)   |                                   |
| Actinomycin D (8 µg/ml) | 340 (+10)   | 4150 (+440)   | 100                               |
| Puromycin (100 µg/ml) | 270 (+20)   | 270 (+30)     | 99                                |
| Cycloheximide (100 µg/ml) | 270 (+30)   | 270 (+30)     | 99                                |

In this paper we have shown by use of FUDR and hydroxyurea that stimulated total RNA synthesis is independent of DNA synthesis for at least the first 10 hours of incubation. This is in marked contrast to the observations of Paul and Hunter (11) who found that stimulated RNA synthesis by fetal liver cells was completely inhibited by FUDR. We have no explanation for this difference at present, except to suggest that adult bone marrow cells may be less dependent than fetal liver cells on DNA replication for the expression of erythropoietin action.

The data of Paul and Hunter show an increase in DNA synthesis by fetal liver cells exposed to erythropoietin considerably earlier than we have found to be the case for adult marrow cells (6) (1 hour as opposed to 3 hours). They did not find as rapid an effect on RNA synthesis as we have reported, the earliest effect in fetal liver being seen at about 2 hours. These differences between the two systems are, in reality, not very great. Our studies of stimulation of total RNA synthesis by marrow cells gave results similar to those of Paul and Hunter. Only when the RNA was fractionated on a sucrose gradient did we see the very rapid effect which was confined to the small amount of large sized RNA.

The effect of erythropoietin on marrow cell DNA synthesis, like that in fetal liver cells, is abolished by actinomycin D, puromycin, and cycloheximide. In both cases it is clear that erythropoietin acts on RNA and protein synthesis prior to any increase in DNA synthesis.

Ortega and Dukes (14) have shown that inhibition of marrow cell DNA synthesis by cytosine arabinoside has no effect on erythropoietin-stimulated incorporation of glucosamine into the acid-insoluble fraction. The data in Table II show that inhibition of DNA synthesis did not affect erythropoietin-stimulated iron uptake or hemoglobin synthesis when measured at 4 hours, although when measurements were made later (Table I) there was almost complete inhibition. At present there is no completely satisfactory explanation for these differences. We can suggest that the early erythropoietin effect on hemoglobin synthesis may represent action on cells that are already differentiated to some extent, and may be independent of DNA synthesis. The effect on hemoglobin synthesis at later times may be the result of erythropoietin action on more primitive cells and their subsequent development may be dependent on DNA synthesis. Experiments testing the effect of erythropoietin on recognizably differentiated cells are in progress.

There are several possible relationships between induced differentiation and stimulated DNA synthesis. Cells that have been committed to the red cell pathway must still undergo three or four divisions before reaching the mature, nondividing differentiated stage. If these amplification divisions are inhibited, the induced cells, if they go on to differentiate, may form only 10 to 15% of the normal amount of hemoglobin. Alternatively, these cells may not manifest any of the characteristic erythroid functions, but they still synthesize a full complement of RNA after erythropoietin has acted. We have found that much of the RNA made by marrow cells after exposure to erythropoietin is retained in the nucleus for at least 3 hours. It may be that DNA replication or mitosis (or both) is required for the transfer of some fraction of the newly formed RNA to the cytoplasm where the specific phenotypic functions can be differentiated.

1 M. Gross and E. Goldwasser, unpublished observations.
expressed. This latter possibility is essentially the superposition of control of nucleus to cytoplasm flow on translational control.

In addition to these possibilities a second type of stimulated DNA synthesis may be considered to be secondary to the inductive step. If erythropoietin acts on a cell that is in equilibrium with a pool of stem cells, induction of differentiation would result in a deficit in that pool which would be made good by increased division of the remaining stem cells. Inhibition of this type of DNA synthesis would be expected to have no direct effect on erythropoietin action until the supply of inducible cells became exhausted. If only a small fraction of the cells was inducible, and if generation of such cells depended on division of stem cells, then inhibition of DNA synthesis would apparently exert a direct effect on erythropoietin action. At present, study of these problems is greatly impeded by our being able to work only with heterogeneous cell populations. When homogeneous populations of stem cells (or erythropoietin-responsive cells if they are different) become available for study we shall be able to resolve some of these difficulties.

REFERENCES
1. Krantz, S. B., and Goldwasser, E., Biochim. Biophys. Acta, 103, 325 (1965).
2. Gross, M., and Goldwasser, E., Biochemistry, 8, 1795 (1969).
3. Hrinda, M. E., and Goldwasser, E., Biochim. Biophys. Acta, 126, 166 (1966).
4. Krantz, S. B., Gallien-Lartigue, O., and Goldwasser, E., J. Biol. Chem., 238, 4085 (1965).
5. Dukes, P. P., Takaku, F., and Goldwasser, E., Endocrinology, 74, 960 (1964).
6. Goldwasser, E., in A. Monroy and A. A. Moscona (Editors), Current topics in development biology, Vol. 1, Academic Press, New York, 1969, p. 173.
7. Dukes, P. P., Ann. N. Y. Acad. Sci., 149, 437 (1968).
8. Dukes, P. P., and Goldwasser, E., Biochim. Biophys. Acta, 108, 447 (1965).
9. Gallien-Lartigue, O., and Goldwasser, E., Biochim. Biophys. Acta, 103, 319 (1965).
10. Paul, J., and Hunter, J. A., Nature, 219, 1362 (1968).
11. Paul, J., and Hunter, J. A., J. Mol. Biol., 42, 31 (1969).
12. Fox, J. B., and Thomson, J. S., Biochemistry, 3, 1323 (1964).
13. Gallien-Lartigue, O., and Goldwasser, E., Science, 145, 277 (1964).
14. Ortega, J. A., and Dukes, P. P., Fed. Proc., 28, 600 (1969).
On the Mechanism of Erythropoietin-induced Differentiation: VII. THE RELATIONSHIP BETWEEN STIMULATED DEOXYRIBONUCLEIC ACID SYNTHESIS AND RIBONUCLEIC ACID SYNTHESIS
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J. Biol. Chem. 1970, 245:1632-1636.

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