ERYTHROPOIETIN ACTION IN RAT MARROW CELL CULTURES IN COMPLETE ABSENCE OF DNA SYNTHESIS I. EARLY EFFECT ON RNA SYNTHESIS

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Received July 9,1975

SUMMARY: Incubation of rat marrow cells with hexachloroiridate ($4.5-5.5 \times 10^{-4}$M) at 36.5° for 45 hours totally abolishes their DNA synthesis. One hour exposure to erythropoietin considerably stimulates the rate of RNA synthesis of such Iridium treated marrow cells. Since hexachloroiridate is reported to be an agent which inhibits mammalian cell division by blocking cells in the G_1 phase, it seems that early erythropoietin action resulting in stimulated RNA synthesis is independent of DNA synthesis and can take place in cells arrested in G_1.

INTRODUCTION

The regulatory role of erythropoietin (EPO), a glycoprotein hormone, in the control of red cell production is well accepted. Krantz and Goldwasser (1) have reported that the first observable molecular event which can be brought about in rat marrow cell cultures by EPO is an increase in RNA synthesis, 15 minutes after the addition of the hormone. Increases in DNA (2) and hemogobin synthesis (3) appear 3-10 hours later. Employing fluorodeoxyuridine and hydroxyurea Gross and Goldwasser (4) prevented the occurence of any increase in DNA synthesis during the first ten hours of culture in the presence of EPO and were still able to demonstrate an increase in RNA synthesis. However their experimental design still permitted a measurable amount of DNA synthesis to take place in the cultures. Similar observations have been reported with fetal mouse liver erythroid cell cultures (5,6) utilizing the above mentioned inhibitors and cytosine-arabinoside. All these agents seemed to be useful in reducing DNA synthesis

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Abbreviations: EPO, erythropoietin; Ir, sodium hexachloroiridate; PBS, phosphate buffered saline; TCA trichloroacetic acid

of cultured cells to a great extent but failed to abolish it completely at dose levels which were not also unacceptably toxic. The question whether or not a low level of DNA synthesis in cultures is a requirement for EPO stimulated RNA synthesis had remained unresolved. Thus, it seemed to be of interest to examine this aspect of early EPO action in a rat marrow cell culture system in which DNA synthesis has been completely abolished by sodium hexachloroiridate (Ir), an agent reported to inhibit division of mammalian cells by arresting them in the G_1 phase (7).

MATERIALS AND METHODS

Bone marrow cells were obtained from femora and tibiae of male Sprague-Dawley rats weighing between 170 g and 200 g. Cell culture was performed as described previously (8). DNA and RNA synthesis were measured based on incorporation of labelled precursors into acid precipitable materials. At the end of the labelling period the cells were washed with ice cold phosphate buffered saline (PBS), suspended in 4 ml of 5% trichloroacetic acid (TCA) and kept in an icebath for 30 minutes. The TCA precipitates were collected on Whatman GF/C glass fiber filters (2.4 cm diameter), washed three times with 4 ml 5% TCA and once with 4 ml 95% ethanol. The filters were dried in an oven at 60°, transferred to glass vials and digested with 0.8 ml NCS tissue solubilizer (Amersham/Searle Corporation) at room temperature away from fluorescent light. The samples were dissolved in a toluene based scintillation fluid and their radioactivity was determined in a Searle Analytic Mark II liquid scintillation counting system. Counting efficiency for ^3H was 29%.

Cell separation was performed by unit gravity velocity sedimentation according to the method of Miller and Phillips (9) in which cells sediment in a glass chamber containing a linear protein solution gradient. In our adaptation of the method we used a linear gradient of 15-30% calf serum (GIBCO) in PBS and collected 20 ml fractions after allowing 3 hours for sedimentation at room temperature. Nucleated cell numbers were determined in triplicate utilizing a Coulter counter model ZBI.

Sodium hexachloroiridate ($Na_2IrCl_6,6H_2O$) was purchased from Ventron Corporation Alfa Products, [^3H] uridine (21 Ci/mmole) was obtained from ICN Chemical and Radioisotope Division and [^3H] methyl-thymidine (6.7 Ci/mmole) from New England Nuclear.

The human urinary erythropoietin used was generously supplied by the National Heart and Lung Institute. It had been collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina and was processed further by our laboratory. In all experiments with one indicated exception, preparation H-10-TaLSL (85.2 units/ mg protein) was used. The concentration of EPO employed was always 0.14 units/ml cell suspension.

RESULTS

Total abolition of DNA synthesis. The dose of Ir and the time of

exposure to Ir capable of eliminating DNA synthesis entirely were determined. Figure 1 shows the dose dependence of the Ir effect, marrow cells pre-incubated for 45 hours with 4.5×10^{-4}M Ir ended up with a complete inhibition of thymidine incorporation. In some experiments 4.5×10^{-4}M Ir did not supress DNA synthesis completely but the addition of 5 or 5.5×10^{-4}M Ir always resulted in total supression. Only experiments in which DNA synthesis was completely abolished in the treated cultures form the basis of this report. A number of preliminary experiments indicated that DNA synthesis in cultures preincubated with Ir for 24 hours was reduced to 1/3 of the control value whereas 45 hours treatment resulted in total cessation of DNA synthesis.

Cell survival. At the start and end of the incubation period nucleated cell numbers of control and Ir-treated cultures were determined. This was done in order to find out whether the elimination of the DNA synthesis of the treated cultures was due to an effect of Ir on surviving cells or simply due to a drastic reduction of the number of cells persisting in culture. As seen in Table 1, doses of Ir which abolished DNA synthesis actually reduced the cell loss which normally occurs due to maturation

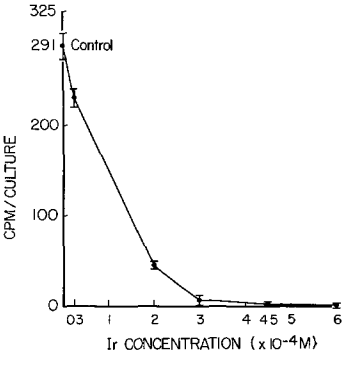

Figure 1. Inhibition of DNA synthesis in rat marrow cell cultures as a function of different concentrations of Ir. [^{3}H] -thymidine (1 μCi/ml) was added at the end of 45 hours and incubation continued for 1 hour. Values are the means ± S.E. from 3 determinations.

Table 1. Survival of Nucleated Cells.

Cell Counts at time zero ($\times 10^{-6}$/ml)	Survival after 45 Hrs.* (%)	
	Control	Iridium (4.5 to 5.5×10^{-4}M)
3.7 - 7.9	42.5 ± 3.6	67.0 ± 2.6

* Values are the means $\pm$ S.E. from six different experiments

and lysis during culture. A study was performed, utilizing the technique of cell separation at unit gravity, in an attempt to characterize the nature of the cells surviving after 45 hours incubation with and without Ir. It was found that the most pronounced apparent protective effect of Ir was exerted on large size cells sedimenting with the first peak fractions of the separated cells (figure 2). It is known from other studies (10,11) that cell fractions with such sedimentation characteristics are enriched in cells highly responsive to EPO in terms of increased hemoglobin synthesis and glucosamine incorporation. It seems therefore that Ir-treatment augments the survival of EPO-responsive cells.

Early EPO action on RNA synthesis. Cell cultures treated with Ir increased their RNA synthesis within one hour of the addition of EPO in the complete absence of DNA synthesis (table 2). The increases ranged from 13.0 to 28.1% of control values. RNA synthesis of untreated cultures similarly increased in response to EPO (range: 10.3 to 51.7%). The EPO specificity of the observed action on RNA synthesis was tested in an experiment in which the effects of the relatively crude EPO (85.2 units/mg protein) used in this study were compared with the effects obtained with a preparation highly purified by hydroxylapatite chromatography (11980 units/mg protein) (table 3). No difference was found between the effects of the two EPO preparations.

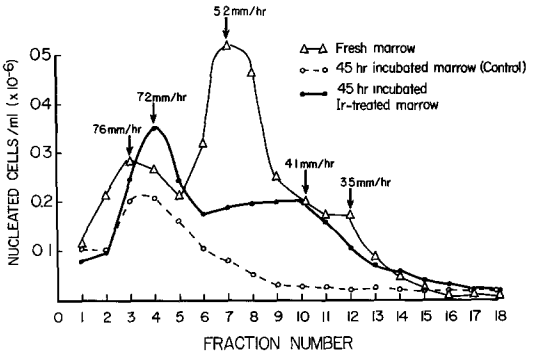

Figure 2. Velocity sedimentation profiles of rat marrow cells. Conditions as described in Materials and Methods.

Constancy of precursor pool size in the presence and absence of EPO.

Interpretation of the incorporation of more [^{3}H] - uridine radioactivity into acid insoluble material as an increase of RNA synthesis is only valid if the addition of EPO did not lead to a change in the intracellular pool of RNA precursors. Relative pool size was compared by incubating EPO-treated and control cultures with 3 different levels of [^{3}H] - uridine per ml (table 4). The results showed that, both in the presence and absence of Ir, EPO did not appear to influence the RNA precursor pool size.

DISCUSSION

It has been reported previously (4,5,6,) that EPO stimulated DNA synthesis is not required for the expression of the initial stimulatory effect of this hormone on RNA synthesis. The salient finding of this communication is that the early EPO mediated increase in RNA synthesis of marrow cell cultures can take place in the total absence of DNA synthesis therefore, it seems to be possible to conclude that EPO, in the course of its action leading to erythroid cellular differentiation, can bring about the transcription of certain DNA sequences without the need for a loosening up of chromosomal structures which occurs during

Table 2. Early Effect of EPO on Stimulated RNA Synthesis In Complete Absence of DNA Synthesis.

Experiment	Condition	[^{3}H]-uridine incorporation (CPM/10^6 nucleated cells)		Stimulation due to EPO (%)	[^{3}H]-thymidine incorporation (CPM/10^6 nucleated cells)	
		-EPO	+EPO		-EPO	+EPO
1	Control	942 ± 31	1100 ± 18	16.8	69 ± 1	68 ± 1
	Ir	624 ± 13	752 ± 21	20.4	2 ± 1	2 ± 3
2	Control	323 ± 3	490 ± 5	51.7	161 ± 15	N.D.
	Ir	281 ± 22	360 ± 20	28.1	0	N.D.
3	Control	1626 ± 26	1794 ± 59	10.3	162 ± 3	N.D.
	Ir	1059 ± 55	1314 ± 84	24.0	0	N.D.
4	Control	573 ± 21	644 ± 35	12.3	206 ± 6	N.D.
	Ir	314 ± 11	355 ± 13	13.0	1 ± 1	N.D.

EPO and radioactive labels (1 μCi/ml) were added at the end of 45 hours and incubation continued for 1 hour. Incorporation values are the means ± S.E. from 3 determinations. N.D., not done.

Table 3. Early effect of EPO of different purity on stimulated RNA synthesis in complete absence of DNA synthesis.

Condition	[^{3}H]-uridine incorporation (CPM/10^6 nucleated cells)			Stimulation due to EPO (%)		[^{3}H]-thymidine incorporation (CPM/10^6 nucleated cells)
	-EPO	+EPO* (crude)	+EPO** (purified)	Crude	Purified	-EPO
Control	412 ± 14	493 ± 23	468 ± 17	19.7	13.5	210 ± 9
Ir	128 ± 1	148 ± 3	150 ± 2	15.6	17.3	3 ± 1

* Crude EPO: pool H-10-TaLSL (85.2 units/mg protein). ** Purified EPO: fraction HMI-55 (11980 units/mg protein). Other information as in table 2.

Table. 4. Constancy of Pool Size During EPO Action.

Level of [3H]-uridine (μCi/ml)	CPM/Culture				Stimulation due to EPO (%)	
	Control		Iridium			
	-EPO	+EPO	-EPO	+EPO	Control	Iridium
1	7035 ± 32	7783 ± 123	6933 ± 44	8310 ± 139	10.5	18.8
3	19842 ± 1059	21493 ± 1341	18341 ± 110	22253 ± 2165	7.9	20.2
5	30875 ± 1942	33843 ± 3173	25537 ± 554	30271 ± 783	9.5	18.4

Conditions as in table 2.

DNA synthesis. This should be contrasted with the requirement for DNA synthesis prior to the transcription and translation of hemoglobin genes both in the case when hemoglobin synthesis was caused by EPO action on normal hemopoietic cells in culture (5) or when it was caused by dimethylsulfoxide action on Friend virus infected cells (12). It may be assumed that the derepression of hemoglobin genes requires conformational rearrangements of regulatory proteins or their actual removal from the DNA and that these events may only take place in chromosomal structures during or after DNA synthesis. Certain other early transcriptive events involved in erythroid differentiation, on the other hand, may take place on regions of the DNA strands of chromosomes accessible to regulatory signals at all times.

We employed 45 hours treatment with $4.5\text{-}5.5 \times 10^{-4}$M Ir to abolish DNA synthesis in rat marrow cell cultures. Kolodny (7) has reported that such exposure to Ir blocked a number of different mammalian cells including 3T3, HeLa and primary human embryo cells in the G_1 phase of the cell cycle. It seems reasonable to assume that the rat marrow cells in our cultures were similarly blocked in G_1 by Ir. The present study therefore suggests that an early phase of EPO action may take place during G_1, although it does not exclude the possibility that cells may also initiate their response to EPO during other stages of the cell cycle. At any rate our findings are compatible with the suggestion made by several authors (13,14,15) that in order for EPO to exert its effect it must enter a target cell during G_1.

ACKNOWLEDGEMENTS

We thank Mr. Curtiss Polk for technical assistance and Mrs. Terri Jones for secretarial services. This research was supported in part by grants AM 16999, HL 10880 and HD 00048 from the National Institutes of Health.

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