ENHANCEMENT OF HEME AND GLOBIN SYNTHESIS IN CULTURED HUMAN MARROW BY CERTAIN 5β-H STEROID METABOLITES*

By HIDEAKI MIZOGUCHI, M.D., AND RICHARD D. LEVERE,‡ M.D.

(*From the Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 and The Rockefeller University, New York 10021*)

In erythroid precursors cells, it has been demonstrated (1, 2) that both heme and globin synthesis are limited by δ-aminolevulinic acid synthetase, the initial enzyme in the heme biosynthetic pathway. It appears that one of the primary steps in erythroid cell maturation is the initiation of synthesis of this limiting enzyme. The studies of Granick and Kappas (3, 4), which showed that certain steroids could cause the *de novo* synthesis of δ-aminolevulinic acid synthetase in liver cells, suggested that these compounds might also play a physiologic role in the regulation of erythropoiesis. Previous investigations have shown that these 5β-H steroids, derived metabolically from several hormonal precursors and intermediates in man, were capable of stimulating hemoglobin synthesis in the cultured chick blastoderm (5) and erythropoiesis in rodents (6) by a mechanism which is independent of the action of erythropoietin.

The present study was undertaken to extend these observations on the ability of certain 5β-H steroids to induce δ-aminolevulinic acid synthetase and thereby stimulate porphyrin and heme synthesis to human marrow erythroid cells growing in primary culture. The data obtained indicated that these steroids are capable of stimulating both heme and globin synthesis in cultured human erythroid cells.

Materials and Methods

Human bone marrow was obtained by aspiration in patients undergoing this procedure as part of an evaluation of the clinical status of their pulmonary tumors. Only morphologically normal marrow aspirates were used for these studies. Bone marrow cells aspirated by sternal puncture were immediately placed in cold NCTC 109 (Difco Laboratories, Detroit, Mich.) solution and cultured by the method of Krantz (7). In brief, the marrow cells were washed twice with cold NCTC 109 solution and then suspended in an incubation medium consisting of 60% NCTC 109 solution, 40% heat-inactivated human plasma of AB type, and 50 units/ml of penicillin so as to obtain a cell concentration of 2000 nucleated cells/mm³ of medium.

0.8 ml samples of this marrow cell suspension were placed into 35 × 10 mm plastic tissue culture dishes (Falcon Plastics Div. of B-D Laboratories, Inc., Los Angeles, Calif.) and

appropriate amounts of test steroids in propylene glycol or erythropoietin[1] in saline were added in a solvent volume of 2 μl. At this point, in some experiments, inhibitors of nucleic acid and protein synthesis were added to the cultures in addition to steroids or erythropoietin. For each experiment two sets of controls were established; one treated with propylene glycol alone, the other with saline alone. After these additions the cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air and terminated at 72 hr.

To determine the rate of heme synthesis in these cultures, 3 μCi of radioactive iron (^{59}FeCl$_3$, specific activity 7.2 mCi/mg) preincubated with 0.1 ml of type AB plasma for 1 hr was added to each dish. At the termination of incubation, the cells were washed and centrifuged three times with cold phosphate-buffered saline. Heme was extracted from the cultured cells by the method of Teale (8) into methyl ethyl ketone. The methyl ethyl ketone extracts were evaporated to dryness on steel planchets, and their radioactivity was determined in a gas-flow counter (background 22 cpm). Each test culture was done in triplicate. The rate of heme synthesis as determined by ^{59}Fe incorporation into isolated heme is expressed as a percentage of that observed in the control cultures.

The rate of globin synthesis was determined by measuring the incorporation of valine-^{14}C into hemoglobin isolated by column chromatography utilizing a modification of the method of Chernoff et al. (9). For these experiments 2 μCi of L-valine-^{14}C (International Chemical & Nuclear Corporation [ICN], Burbank, Calif., SA 175 mCi/mM) were added to the culture dishes 6 hr before the conclusion of the 72 hr incubation period. The marrow cells were then washed three times in phosphate-buffered saline and hemolyzed by freeze-thawing twice followed by the addition of 1 ml of water. Lipids were removed by the addition of 1 ml of toluene. The resultant hemolysate was dialyzed against potassium-phosphate buffer, 0.01 N, pH 6.2, for 24 hr in the cold. This dialyzed hemolysate was then applied to a carboxymethyl-cellulose column measuring 1 $\times$ 5 cm equilibrated with dialysate buffer. The hemoglobin was eluted from the column with a two-step potassium-phosphate buffer gradient (0.01 N, pH 6.2–8.2) and quantitated in the eluate by difference spectroscopy of ferrous hemoglobin (E_m-432 nm) as compared to CO ferrous hemoglobin (E_m-418 nm) in the Soret region (2). If hemoglobin was present in the eluate at a concentration greater than 20 mg% it was quantitated by its absolute absorbance at 540 nm ($E_m = 14.6 \times 10^4$). Nonhemoglobin protein was eluted from the column at pH 6.2, hemoglobin at pH 8.2 (Fig. 1). A portion of the eluted hemoglobin was next pipetted onto a steel planchet, dried, and counted in a gas-flow counter at infinite thinness. The specific radioactivity of the hemoglobin in the test cultures was then expressed as a percentage of the specific radioactivity of the hemoglobin isolated from the control cultures.

RESULTS

The effect of various steroids on heme synthesis in cultured human marrow cells was investigated by measuring the incorporation of ^{59}Fe into heme extracted in methyl ethyl ketone. The results of these studies are summarized in Table I. The radioactivity of heme found (at 72 hr) in the test cultures is expressed as a per cent of that observed in control dishes incubated similarly. At concentrations of 3 $\times$ 10^{-8} M, significant stimulation of heme synthesis was observed with the following 5β-H steroids: 11-ketopregnanolone, 163% of control (SE $\pm$ 19, $P < 0.01$); etiocholanolone, 142% of control (SE $\pm$ 3, $P <$

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[1] Sheep erythropoietin, Step III, specific activity, 200 units/58 mg dry weight. Purchased from Connaught Medical Research Laboratories, Toronto, Ontario, Canada.

0.05); and pregnanediol, 130% of control ($\text{SE} \pm 3$, $P < 0.05$). The 5α-H epimer of pregnanediol was without effect on heme synthesis, as was (5α) 11-keto-pregnanolone. The glucuronide of etiocholanolone and lithocholic acid were also ineffective in stimulating heme synthesis. The primary sex steroid hormones, testosterone and progesterone, at this concentration of 3×10^{-8} M failed to stimulate the incorporation of ^{59}Fe into heme. Erythropoietin at a concentration of 0.2 units/ml medium caused a marked stimulation of heme synthesis ($210 \pm 31\%$ of control) in this culture system.
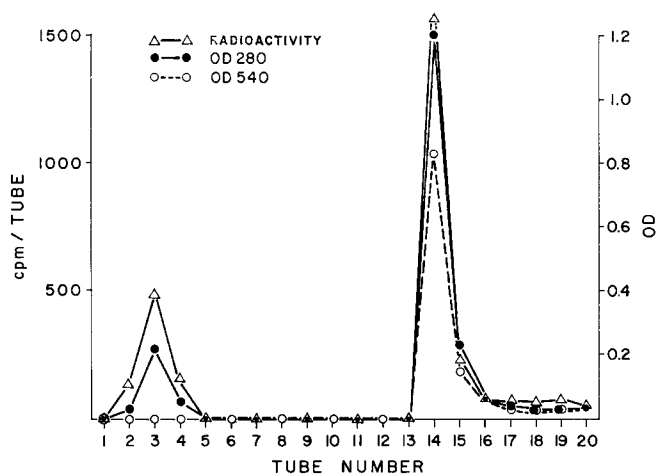

Fig. 1. Chromatography of cultured marrow hemolysate by carboxymethyl cellulose. Fractions were collected in 1-ml samples, absorbance at 280 and 540 nm was determined, and ^{14}C radioactivity was measured. Fractions 1–11 (tube No.) were eluted with potassium-phosphate buffer, 0.01 N, at pH 6.2. All subsequent fractions (tube No. 12–20) were collected with phosphate buffer, 0.01 N, at pH 8.2. Two protein peaks are observed, one with each pH gradient. Only the protein peak obtained at pH 8.2 contains hemoglobin as determined by absorbance at 540 nm which corresponds well with a protein absorbance peak at 280 nm and with maximum valine-^{14}C radioactivity.

The relationship between the dose of 5β-H steroid and the rate of heme synthesis is shown in Fig. 2. For these experiments 5β-H 11-ketopregnanolone was utilized as a prototype steroid. Maximum stimulation of heme synthesis was noted with a steroid concentration of 3×10^{-8} M. At higher concentrations there was a progressive falloff in stimulation.

The effects of actinomycin D and puromycin on both 5β-H steroid and erythropoietin stimulation of heme synthesis were also investigated in this culture system. At a concentration of 0.01 μg/ml, actinomycin D abolished the stimulatory effect on heme synthesis of both 11-ketopregnanolone and erythropoietin (Table II). Similar results were noted when puromycin (0.1 μg/ml) was added

to the cultures with either 11-ketopregnanolone or erythropoietin. Both actinomycin D and puromycin alone at the same concentrations significantly suppressed heme synthesis, but did not abolish it.

The effect of 5β-H steroids on globin synthesis, as measured by the rate of valine-^{14}C incorporation into isolated hemoglobin, is depicted in Fig. 3. With both etiocholanolone and 11-ketopregnanolone maximum effect was noted with a steroid concentration of 3 $\times$ 10^{-8} M in the culture dishes. This concentration

TABLE I

Effect of Steroids and Erythropoietin on Heme Synthesis of Cultured Marrow Cells

Compound	^{59}Fe radioactivity % of control ± SE	Significance
11-Ketopregnanolone (3 $\times$ 10^{-8} M) (5β pregnane-3α-ol, 11, 20-dione)	163 ± 19	$P < 0.01$
Etiocholanolone (3 $\times$ 10^{-8} M) (5β androstane-3α-ol, 17-one)	142 ± 3	$P < 0.05$
5β-pregnanediol (3 $\times$ 10^{-8} M) (5β pregnane-3α, 20α-diol)	130 ± 3	$P < 0.05$
(5α) 11-Ketopregnanolone (3 $\times$ 10^{-8} M) (5α-pregnane-3α-ol, 11, 20-dione)	98 ± 6	N.S.*
(5α)-pregnanediol (3 $\times$ 10^{-8} M) (5α pregnane-3α, 20α-diol)	99 ± 12	N.S.
Etiocholanolone glucuronide (3 $\times$ 10^{-8} M) (5β-androstane, 17-one, 3α-yl-β-D-glucosiduronic acid)	102 ± 8	N.S.
Progesterone (3 $\times$ 10^{-8} M) (Δ^4 pregnane-3, 20-dione)	101 ± 9	N.S.
Testosterone (3 $\times$ 10^{-8} M) (Δ^4 androstene-17β-ol, 3-one)	104 ± 15	N.S.
Lithocholic acid (3 $\times$ 10^{-8} M)	92 ± 8	N.S.
Erythropoietin (0.2 unit/ml)	210 ± 31	$P < 0.01$

* N.S. = not significant.

of steroid is identical with that needed for maximal effect of heme synthesis as noted above. As noted in the experiments measuring the steroid effect on ^{59}Fe incorporation into heme (Fig. 1), concentrations of active steroids greater than 3 $\times$ 10^{-8} M caused a progressive falloff in stimulation. The reason for this is not obviously apparent but may be related to the cytolytic effect of these steroids at high concentrations.

As shown in Table III the 5α-H epimer of 11-ketopregnanolone was ineffective in stimulating the incorporation of valine-^{14}C into hemoglobin. Glucuronidation of etiocholanolone as noted in the experiments on heme synthesis was associated with the loss of ability to stimulate globin synthesis. Both puromycin (0.1 μg/

ml) and actinomycin D (0.01 μg/ml) caused a marked inhibition in the stimulatory effect of 11-ketopregnanolone on globin synthesis.

Table IV shows the results of the effects of erythropoietin on globin synthesis. Stimulation of globin formation (152 ± 2% of control) was noted with a concentration of erythropoietin of 0.2 units of culture medium. As noted with
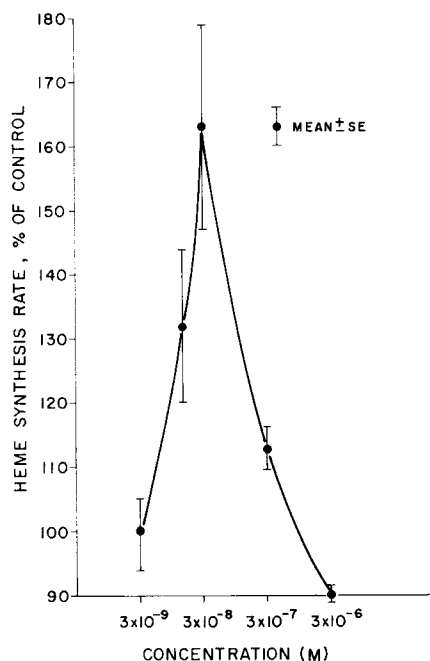

FIG. 2. Measurement of ^{59}Fe incorporation into heme of cultured human marrow cells as a function of increasing concentrations of 11-ketopregnanolone. The radioactivity of ^{59}Fe heme in the test cultures is expressed as a per cent of that observed in control cultures treated with solvent vehicle alone. Maximal steroid effect is noted at a concentration of 3×10^{-8} M. Higher concentrations are associated with progressive falloff in stimulation.

active 5β-H steroids, both puromycin and actinomycin D almost completely abolished the stimulatory effect of erythropoietin on globin synthesis.

DISCUSSION

In all cell types studied, to date, the heme biosynthetic pathway has been found to be limited by δ-aminolevulinic acid synthetase, the initial enzyme in this synthetic pathway. When δ-aminolevulinic acid, the product of this initial enzyme reaction, is added to a variety of cell types, including reticulocytes, embryonic and adult liver cells, chick blastoderm erythroid cells, chick embryo kidney cells, myeloid cells, and muscle, brain, and heart cells, it is rapidly con-

TABLE II

Effect of Antimetabolites on Stimulated Heme Synthesis

| Addition to culture	[59]Fe radioactivity % of control ± SE
(5β) 11-Ketopregnanolone (3 × 10⁻⁸ M)	172 ± 10
(5β) 11-Ketopregnanolone (3 × 10⁻⁸ M) + actinomycin D (0.01 μg/ml)	24 ± 2
(5β) 11-Ketopregnanolone (3 × 10⁻⁸ M) + puromycin (0.1 μg/ml)	95 ± 4
Erythropoietin (0.2 unit/ml)	210 ± 12
Erythropoietin (0.2 unit/ml) + actinomycin D (0.01 μg/ml)	80 ± 4
Erythropoietin (0.2 unit/ml) + puromycin (0.1 μg/ml)	100 ± 5
Actinomycin D (0.01 μg/ml)	22 ± 3
Puromycin (0.1 μg/ml)	75 ± 2
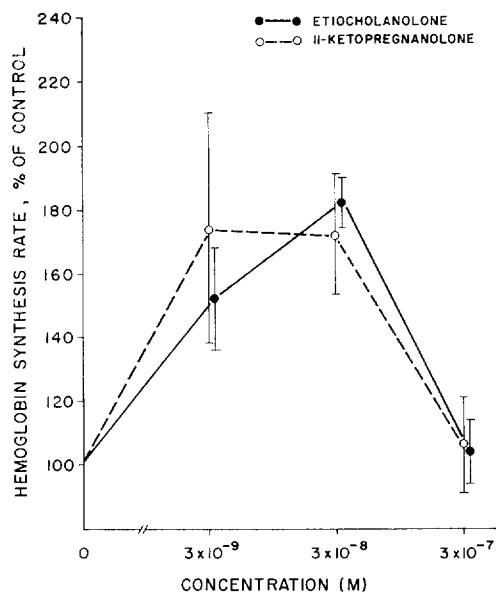
FIG. 3. Measurement of valine-^{14}C incorporation into hemoglobin of cultured human marrow cells as a function of increasing concentrations of 11-ketopregnanolone and etiocholanolone. The specific radioactivity of labeled hemoglobin-^{14}C in the test cultures is expressed as a per cent of that observed in control cultures treated with solvent vehicle alone. With both of these 5β-H sex steroid metabolites, significant stimulation of hemoglobin synthesis was noted at a concentration of 3 × 10⁻⁸ M.

verted to porphyrins and heme (10). This indicates that the enzymes beyond δ-aminolevulinic acid synthetase, which convert δ-aminolevulinic acid to heme, are, under normal conditions, present in these cells in nonlimiting amounts. In the erythroid precursor cells of the chick blastoderm (2), bypassing δ-amino-

levulinic acid synthetase by the addition of δ-aminolevulinic acid to the cells not only causes increased porphyrin and heme production but also stimulates globin synthesis. These observations suggest that the induction of synthesis of δ-aminolevulinic acid synthetase is a "key" step in the maturation of erythroid

TABLE III

Effect of Steroids and Erythropoietin on Globin Synthesis of Cultured Marrow Cells

Compound	^{14}C specific radioactivity % of control ± SE	Significance
11-Ketopregnanolone (3 × 10^{-8} M) (5β-pregnane-3-ol, 11, 20-dione)	172 ± 19	P < 0.05
Etiocholanolone (3 × 10^{-8} M) (5β-androstane-3α-ol, 17-one)	182 ± 6	P < 0.01
(5α) 11-Ketopregnanolone (3 × 10^{-8} M) (5α-pregnane-3α-ol, 11, 20-dione)	102 ± 8	N.S.*
Etiocholanolone-glucuronide (3 × 10^{-8} M) (5β-androstane, 17-one, 3α-yl-β-D-glucosiduronic acid)	102 ± 15	N.S.
Erythropoietin (0.2 unit/ml)	153 ± 2	P < 0.01

* N.S. = not significant.

TABLE IV

Effect of Antimetabolites on Stimulated Globin Synthesis

Addition to culture	^{14}C specific radioactivity % of control ± SE
(5β) 11-Ketopregnanolone (3 × 10^{-8} M)	141 ± 4
(5β) 11-Ketopregnanolone (3 × 10^{-8} M) + actinomycin D (0.01 μg/ml)	56 ± 1
(5β) 11-Ketopregnanolone (3 × 10^{-8} M) + puromycin (0.1 μg/ml)	110 ± 14
Erythropoietin (0.2 unit/ml)	154 ± 2
Erythropoietin (0.2 unit/ml) + actinomycin D (0.01 μg/ml)	52 ± 1
Erythropoietin (0.2 unit/ml) + puromycin (0.1 μg/ml)	96 ± 2
Actinomycin D (0.01 μg/ml)	60 ± 6
Puromycin (0.1 μg/ml)	99 ± 2

cells and may even play a role in the commitment of precursor cells into the erythroid cell line.

It has been proposed by Granick (11) that δ-aminolevulinic acid synthetase formation, in the liver, is under the control of a repressor-operator system which can be influenced by a diverse group of drugs and chemicals. These compounds cause an increase in the synthesis of δ-aminolevulinic acid synthetase and lead, thereby, to enhanced hepatic porphyrin and heme formation. The description by Granick and Kappas (3, 4) of a group of natural substances which can also

induce δ-aminolevulinic acid synthetase and cause enhanced heme formation suggests that such compounds may be concerned with the physiologic regulation of heme synthesis. These chemicals are all endocrine in origin and specifically steroidal in type.

Previous studies (5) have shown that certain of these steroids, through their effect on heme production, are capable of increasing the rate of hemoglobin synthesis in the erythroid cells of the cultured, deembryonated chick blastoderm where heme synthesis is also limited by δ-aminolevulinic acid synthetase as it is in the liver (12). Steroids which are active in stimulating hemoglobin synthesis in this system all have a hydrocarbon nucleus of the 5β-androstane or 5β-pregnane type and have an alcohol or ketone substituent at the C_3, C_{17}, C_{20}, and possibly C_{11} positions. All of these compounds are either metabolites, or intermediates in the synthesis of the primary sex steroids testosterone and progesterone.

These 5β-H steroid metabolites have also been demonstrated to be capable of stimulating erythropoiesis in vivo in normal and posthypoxic polycythemic mice and normal rats (6, 13). The stimulation of erythropoiesis in the mouse and rat by these active steroids has been shown to be probably independent of the action of erythropoietin. This is unlike the action of the parent hormone testosterone which stimulates erythropoiesis in the polycythemic mouse only through a mechanism which involves erythropoietin (14). Since the 5β-H steroids are active in the posthypoxic polycythemic mouse where there is a virtual disappearance of nucleated erythroid cells, it would appear likely that part of this primary effect is exerted directly on erythroid precursor cells, inducing them to differentiate into the nucleated erythroid cell line.

The present study demonstrates that certain 5β-H steroids are also capable of stimulating heme synthesis in cultured human marrow cells. Significant stimulation of ^{59}Fe incorporation into heme was noted with 11-ketopregnanolone, etiocholanolone, and pregnanediol (Table I). The 5α-H epimers of 11-ketopregnanolone and pregnanediol were without effect on heme synthesis, indicating a degree of steric specificity in this steroid activity. The necessity for a 5β-H (A:B cis) or highly angulated A:B-ring junction in the molecule is also supported by the failure of the unsaturated A-ring steroids, testosterone and progesterone, to stimulate heme synthesis in this system and is identical to the structural requirements noted in other systems (3). Necheles and Rai (15) have noted similar effects on in vitro human marrow heme synthesis using 5β-H etiocholanolone and its 5α-H epimer.

Glucuronidation of etiocholanolone at the C_3 position was associated with complete loss of ability to stimulate heme synthesis. In addition, the C_{24} acidic steroid, lithocholic acid, was also devoid of activity. These structural requirements for stimulatory activity are the same as those noted by Granick and Kappas for steroid induction of hepatic porphyrin synthesis.

These studies also demonstrate that certain 5β-H steroids, as exemplified by 11-ketopregnanolone and etiocholanolone, can stimulate globin synthesis in cultured human marrow as measured by the incorporation of valine-^{14}C into chromatographically isolated hemoglobin (Fig. 3). The 5α-H epimers of these active steroids were also incapable of stimulating globin synthesis as was noted in studying their effect on heme synthesis. These observations on the effect of these 5β-H steroids on globin synthesis differ from those of Necheles and Rai (15), who were unable to demonstrate a definite effect of etiocholanolone on globin formation. This difference in observed effect on globin synthesis may be related to the fact that these investigators isolated glycine-^{14}C–tagged globin from their marrow cultures by acid precipitation, while in the present study hemoglobin labeled with valine-^{14}C was isolated by chromatography of hemolysates obtained from the cultured marrow cells.

Both heme and globin synthesis were maximally stimulated by active steroids at a concentration of 3×10^{-8}M (Figs. 2 and 3). However, stimulation of heme and globin production may even occur with steroid concentrations as low as 3×10^{-9} M. The ability of certain 5β-H steroids to enhance hemoglobin synthesis at such low concentrations is compatible with a possible physiologic role which these compounds might play in the normal regulation of hemoglobin synthesis. It is also of interest to note that at the optimal steroid concentration of 3×10^{-8} M both heme and globin synthesis were stimulated to an almost comparable degree, i.e., approximately 140–180% of control levels. This finding is consistent with previous observations of coordinate synthesis of these two moieties (16, 17), as well as a dependence on globin synthesis of heme availability as previously noted (1, 2, 18, 19).

Low concentration of either actinomycin D, which prevents the formation of messenger RNA, or of puromycin, which inhibits protein synthesis at the ribosome level, in the marrow cultures abolished the stimulatory effect of the active steroid 11-ketopregnanolone (Table II). This finding suggests that the action of 5β-H steroids on hemoglobin formation requires both new RNA and new protein synthesis. If the mechanism of steroid action was to activate an inactive form of δ-aminolevulinic acid synthetase, this action should not have been effected by the addition of actinomycin D to the cultures. The ability of puromycin to block the steroid action is to be expected because puromycin not only blocks the synthesis of globin but of the limiting enzyme δ-aminolevulinic acid synthetase as well.

As expected, erythropoietin caused a marked stimulation of ^{59}Fe incorporation into heme in these cultures, as has been previously noted (7). Erythropoietin also caused a striking stimulation of globin synthesis in the cultured human marrow, as has been observed in studies of erythropoietin action on globin formation in cultured marrow and spleen of other species (20, 21). The effect of erythropoietin on both heme and globin synthesis was abolished by either

puromycin or actinomycin D, indicating that the action of this hormone also requires *de novo* synthesis of RNA and protein.

The data presented here, in conjunction with the previous studies of 5β-H steroid action on erythropoiesis (5, 6), indicates that these steroids can exert a profound effect on hemoglobin synthesis in erythroid tissue obtained from birds, rodents, and man, suggesting that they may play a fundamental role in the process of erythropoiesis. The action of these 5β-H steroids is independent of erythropoietin and appears to be primarily mediated by their induction of δ-aminolevulinic acid synthetase in immature erythroid cells. The appearance of this enzyme in the erythroid cell allows heme synthesis to begin; the heme in turn stimulates the production of globin, the occurrence of these events being associated with progressive erythroid maturation. Still to be determined is at what developmental erythroid cell stage these steroids act and if their action is in some way related to the erythropoietin effect on erythroid tissue, either by potentiating or being potentiated by the action of that hormone. It will also be of further interest to learn if the action of these 5β-H steroids is specific for erythroid cells. The failure of these compounds to stimulate heme synthesis in phytohemagglutinin-transformed lymphocytes (22) suggests a degree of target cell specificity.

The ability of certain 5β-H steroids to independently stimulate hemoglobin synthesis in cultured human marrow suggests that these naturally occurring compounds may play a physiologic role in the regulation of normal human erythropoiesis. In addition, since these steroids are devoid of either classical androgenic or progestational activity, their use as therapeutic modalities in the management of certain chronic anemias of man seems worthy of extensive further investigation.

SUMMARY

These studies demonstrate that certain sex steroid metabolites are capable of significantly stimulating the synthesis of both heme and globin in cultured human bone marrow cells. These compounds, which are maximally effective at a concentration of 3×10^{-8} M, are steroids of the C_{19} and C_{21} neutral type; share a common 5β-H (A:B cis) configuration; and are derived from the in vivo biotransformation of testosterone or progesterone, or their intermediates, in man. Since these steroid metabolites have been shown to be capable in other systems of inducing the synthesis of δ-aminolevulinic acid synthetase, the limiting enzyme in the heme biosynthetic pathway, it is hypothesized that their action on human erythroid precursor cells is directed similarly at this enzymatic step leading thereby to increased heme production with consequent stimulation of globin synthesis. This steroid action is independent of erythropoietin, and since these compounds are effective at extremely low concentrations, it is suggested that they may play a physiologic role in the regulation of human erythropoiesis.

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