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SOME MOLECULAR ASPECTS OF RED CELL DIFFERENTIATION

MASTER

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There is abundant evidence that the induction of erythrocyte formation in mammals is dependent on erythropoietin (1). The exact identity of the cells that are the target for erythropoietin action is not yet established, although it is clear that either some type of primitive cell, committed toward erythropoiesis, or some pluripotent cell is involved. There is also evidence that erythropoietin, once having initiated a wave of erythropoiesis, is not required for the full expression of that wave (2) and is therefore not necessary for the later events in the differentiation process. In this paper I will discuss the molecular mechanisms involved in the action of erythropoietin, with the intent of understanding the sequence of biochemical events which constitutes the process of erythroid cytodifferentiation.

Some years ago I proposed an operational division of the events in red cell differentiation into three distinct phases (1) (Fig. 1). The first phase, sensitization, is still not experimentally established and will not be discussed further. The second phase, induction, refers to the primary biochemical step in the specific action of erythropoietin on its target cells. The third phase, specialization, refers to the complex of biochemical changes which occurs when the induced cells acquire new functions that distinguish them as differentiated cells. The cells also lose some of their old functions

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during specialization, but we have no information yet regarding the mechanisms underlying these losses.

In the discussion to follow, I shall concentrate on induction and specialization in adult rodent bone marrow cells studied in primary cultures. A summary of the temporal sequences of effects induced by erythropoietin is presented in Figure 2. Details of methods used in our laboratory can be found in the pertinent references.

Induction

In attempting to study the nature of the inductive event in erythropoietin-induced red cell differentiation, we have sought to find the earliest function stimulated by erythropoietin (3,4). Within five minutes of exposure of adult rat marrow cells in vitro, to erythropoietin, they synthesize a small amount of a type of RNA not detected in control cells (4). This RNA, which appears to be unique to erythropoietin-affected cells, is very large, having a sedimentation coefficient of about 150S and a minimum molecular weight of about 30 million. The size of this RNA is not reduced by pronase, DNase, 8M urea, or 0.01M EDTA, but is rapidly broken down by RNase. These data indicate that it is a massive, single-chain RNA and not an RNA-protein complex, RNA-DNA complex, or RNA-RNA complex held together by magnesium ions or double stranded RNA. We have no evidence yet regarding the function of this RNA but do know that it is confined to the nuclei of the marrow cells, that it contains a small amount of methyl groups derived from methionine, has a relative base composition different from the other cellular RNA species, and has a very short half-life (approximately six minutes). Although we have not been able to detect any transfer of label to the cytoplasm in a chase experiment it is quite possible that it occurs to a small extent that is not detectable by

present methods. For instance, if the 150S RNA were a "unit of transcription" (5) and contained the nuclear precursor of the cytoplasmic 9S globin messenger RNA the 9S RNA would represent less than 1% of the 150S RNA and would be extremely difficult, if not impossible, to detect as a product. We consider that the rapidity of its synthesis and the fact that 150S RNA seems to be found only in erythropoietin-treated cells argues for its importance in the inductive action of the hormone. Clearly, much work remains to be done on the functional role of this massive nuclear RNA.

We have obtained data showing that the effect of erythropoietin on total marrow cell RNA synthesis is not due to increased uptake of labeled nucleoside or to alteration of nucleotide precursor pool size (4). We have not yet been able to determine whether the primary effect is on de novo synthesis of RNA or on inhibited breakdown of the labile species. Inhibition of induced RNA synthesis by actinomycin suggests, but does not prove, that it is the new synthesis of RNA which is stimulated.

The related questions of whether the new synthesis is due to new template being made available for existing RNA polymerase or whether the polymerase and possibly its associated factors are the limiting factors, are not yet answered. We have data showing that erythropoietin-stimulated RNA synthesis is not reduced by almost total inhibition of protein synthesis (6), which suggests that the rate-limiting factor may be DNA template or some other factor not requiring the formation of new protein.

Before erythropoietin can have an inductive effect on the target cell nuclei, some mechanism must operate for transfer of the extracellular signal (erythropoietin) to an intracellular one. The relatively simple question of

whether erythropoietin penetrates the cells has not been answered, because the ^{125}I -labeled pure hormone (7) is inactive. We must find a way to label minute amounts of purified erythropoietin without causing inactivation; such experiments are now being carried out. Some other important questions regarding the molecular mechanisms that may operate between cell recognition of extracellular erythropoietin and transcription in the nucleus are: (1) Are there specific receptor sites on the surface of erythropoietin target cells? (2) If erythropoietin is not taken up by the cell, what is the nature of the chemical signal that moves from the cell surface to the cytoplasm to the nucleus? (3) If erythropoietin is taken up by the cell, does it have a direct effect on the nucleus as such, or is a cytoplasmic carrier molecule needed for interaction with the nucleus? (4) What is the molecular nature of the effect on transcription? Is it a Jacob-Monod type of de-repression or might it be a repressive effect, i.e., causing the cessation of synthesis of a substance that acts as a repressor? (5) Could it be that neither of these mechanisms is operating and that the inductive effect is not on transcription per se but on "editing," i.e., erythropoietin may act to select which of the already transcribed nuclear RNA species will not be broken down and discarded or which are exported from the nucleus. For clear answers to these questions we would need a homogeneous population of erythropoietin-responsive cells and pure, active, labeled erythropoietin, neither of which is yet available.

Very shortly after synthesis of 150S RNA is induced, another species of large, rapidly labeled, heterogeneous, nuclear RNA is formed as a result of erythropoietin action (4). This RNA, with a range of sedimentation coefficients around 60-70S, is also found in control cells of the marrow and has

been described in other eukaryotic cells. Its function too is not established. Whether there is any relationship between 150S RNA and marrow cell 60S RNA is unknown, but observations that the half-life of the latter in rat marrow cells is of the order of hours rather than minutes, that 60S RNA is not labeled with methyl groups from methionine, and that the relative base composition is different, suggest that these two are not related as precursor and product. Whether 60S RNA is involved in induction or in specialization is not at all clear.

Specialization

Since the ultimate function of the differentiated erythroid cell is to make large amounts of hemoglobin, it is not surprising that one of the earliest events seen is the synthesis of new ribosomal RNA starting about 15 minutes after erythropoietin is added to the cells (4). The formation of ribosomal RNA precursor is followed at about 45 minutes by the appearance of "processed" ribosomal RNA, tRNA, some substance that allows iron to affect the cells function ("Fe effector" [8]), and by 9S RNA which is, most likely, the globin messenger. We do not yet know when the messengers for the enzymes of heme synthesis are formed nor when the enzymes themselves are formed, but have suggested that heme synthesis may, in part, be regulated by the availability of globin.

Our studies of the effect of erythropoietin on synthesis of 9S RNA (9) have shown that some 9S RNA is synthesized even under conditions of totally suppressed hemoglobin synthesis. We think this RNA may represent the message for a protein, not globin, of molecular weight about 16,000, which is not involved in erythropoiesis. When marrow cells from polycythemic animals are

given erythropoietin they synthesize increased amounts of 9S RNA some 9-10 hours before hemoglobin synthesis starts. It takes about three hours for the transposition of RNA from marrow cell nuclei to cytoplasm; this leaves about 6-7 hours during which the presumed globin messenger is in the cytoplasm but hemoglobin synthesis has not yet begun. Clearly there is some sort of post-transcriptional, post-transpositional regulatory mechanism operating in the control of hemoglobin synthesis. Whether this regulatory step is concerned with heme synthesis is still unknown, but is worth further study.

About two hours after erythropoietin is added to cultures, there is an increase in DNA synthesis (10,11). Since this is later than the events already mentioned, it is clear that in normal, adult bone marrow cells erythropoietin can exert its action prior to any increased cell division. This is also borne out by our finding that erythropoietin-induced DNA synthesis is inhibited by actinomycin and puromycin, although induced RNA synthesis is not inhibited by fluorodeoxyuridine. Erythropoietin-induced DNA synthesis may represent either the amplification divisions that occur during specialization or possibly the replication of erythropoietin-responsive cells (stem cells?) as a response to the vacancies left after the inductive stimulus has acted.

About four hours after addition of erythropoietin, the cells synthesize new proteins involved in uptake of iron from the medium (12). The exact biochemical identity of all these proteins is not established but the phenomenon can be used to advantage in the assay for erythropoietin in vitro. We have recently developed a micro culture assay that can detect as little as 0.001 i.u. in vitro (13). Cell cultures, however, are sensitive to some impurities in various crude preparations, making the use of this method of assay somewhat restricted.

The effect of erythropoietin on cellular uptake of glucosamine, both the formation of a glycolipid (14) and of stroma (15), is not apparent until about five hours. An hour later (at about six hours) the induced cells (normal) show increased hemoglobin synthesis (4). If cells from polycythemic animals are studied, hemoglobin synthesis is not detectable until about 10-12 hours after the addition of erythropoietin. The main difference between normal marrow cells and "polycythemic" marrow cells lies in the complete absence of differentiated cells of the erythroid series in the "polycythemic" population. The observation that erythropoietin has an effect on hemoglobin synthesis by normal cells in a considerably shorter time, than is required for suppressed cells argues that the hormone acts also on some differentiated cells (9). Earlier studies with whole animals suggested the same conclusion (16,17).

The term specialization is applied to a complex of processes which is very far from being completely understood. One of the more important questions relating to the molecular events that eventually lead to a mature erythrocyte is that of their interrelationships. For instance, is the initiation of one function, e.g., stroma synthesis causally related to the induced synthesis of another function, e.g., the iron-uptake system? At present there are no hard facts on which to base such a speculation but it remains an attractive one.

Other Inducing Factors

There have been recent reports (18-23) that substances other than erythropoietin may induce erythropoiesis. We have tested several androstane and pregnane steroids, with a 9 β hydrogen, both in vitro and in polycythemic

mice (21). None of the steroids affected hemoglobin synthesis by "polycythemic" mouse marrow cells in vitro under conditions where erythropoietin had a marked effect. There was, however, a small effect on normal mouse marrow cells in culture. The steroids had no effect when they were injected into mice made plethoric by hypertransfusion, although similar mice responded well to erythropoietin. Etiocholanalone did, however, cause increased erythropoiesis in normal mice and in mice made polycythemic in a low pressure chamber if the latter were tested shortly after removal from the chamber. We conclude that the reported effect of these steroids is not on the induction of erythropoiesis but on cells that are already on the pathway toward the erythrocyte.

The almost ubiquitous role of 3,5-cyclic adenosine monophosphate (cAMP) in hormone action has raised the question of whether erythropoietin action is mediated by the same mechanism. Reports have been published (24,25) which show an effect of cAMP (and an even larger effect of dibutyrlcAMP) on activity of δ -amino levulinic acid synthetase activity and on incorporation of iron into red cells of polycythemic mice, but not on heme synthesis. We (23) have confirmed the observation that dibutyrlcAMP has no effect on hemoglobin synthesis by "polycythemic" rat cells in vitro. In our hands, however, neither cAMP nor the dibutyrl compound has any effect on iron uptake by the same cells. When RNA synthesis by marrow cells in vitro was measured we found that neither theophylline nor dibutyrlcAMP had any effect, although cAMP did stimulate. The effect of cAMP was maximal at a concentration of about 0.1mM: When erythropoietin was added to cells exposed to 0.1 or 0.2mM cAMP the response with respect to RNA synthesis was essentially the same as that seen

in the absence of cAMP. The fact that erythropoietin still affects marrow cell RNA-synthesis under conditions of saturation with cAMP suggests to us that the two substances act independently, perhaps on different cell populations.⁽²⁶⁾ This finding, plus the observation that cAMP has no effect on hemoglobin synthesis leads us to conclude that erythropoietin does not exert its effect on the cells via an adenylcyclase mechanism. The effect of cAMP on the activity of δ -amino levulinic acid synthetase activity is very interesting and may be more related to its observed action on the stability of the enzyme (25) than to any function in the induction of red cell differentiation.

Conclusions

The central problem in understanding the action of erythropoietin is that of regulation of gene expression. This is also the problem found in embryonic development or in cytodifferentiation in general. It is fairly clear now that somatic cells can retain all the genetic information contained in the zygote, most of it in an unexpressed form and that differentiation is the result of the selective expression of one set of characteristics as against some other sets. In the case of erythropoietin action, the hormone causes the cells to eventually express those genes, the products of which, such as hemoglobin, define the cell as erythroid. The detailed molecular mechanisms by which an external signal, carried to the hemopoietic cells by the circulation, instructs the "hit" cells to embark on the pathway toward becoming erythrocytes, are still obscure. Our data suggest that once a "sensitized" cell is hit, the signal, whether it is erythropoietin taken up by the cell or some secondary signal, acts on the nucleus to initiate a new transcription. The function of the first transcript, 150S RNA, has not been

made clear, nor has that of the second transcript, 60S RNA. An understanding of the biochemical roles of these RNA species may well be the key to the mechanism of selective gene expression in erythroid differentiation.

Erythropoietin not only initiates the erythropoietic sequence, starting with very primitive, non-erythroid, cells, but it can also affect cells that have progressed some way toward the erythrocyte. Studies of the cells of normal marrow take into account the contributions of both of these erythropoietin-responsive populations. We may assume, as a first approximation, that the same general biochemical mechanisms operate in both types of responsive cell, but eventually the observations concerning induction which have been made on normal marrow cells will have to be confirmed in cells from polycythemic animals.

The study of molecular mechanisms underlying erythropoietin action in the initiation of red blood cell formation is still in its infancy. The few fragments of information so far gained have not yet led to a generalized model of the process and, as indicated above, have left unanswered many more questions than have been answered. We can trust that with the potential availability of pure erythropoietin and with a widening interest in this field, progress in our understanding of how differentiation occurs at a molecular level will be made at an ever increasing rate.

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