Oak Ridge

For presentation at the IV International Congress of Endocrinology, Wash. D.C., 18-24 June 1972. To be published in a separate volume approximately 9 months after the symposium

ACRH-1066-393

ERYTHROPOIETIN AND THE INDUCTION OF ERYTHROID CYTODIFFERENTIATION

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CONF-720633--2

Erythropoietin, first described more than sixty years ago (Carnot and Deflandre, 1906), fits the usual definition of a hormone. It is formed in one tissue (Jacobson, Goldwasser, Fried, and Plzak, 1957), is carried by the circulation, and acts on a different target tissue. Its primary function is to regulate the rate of formation of erythrocytes and, except in some pathological states (Friend, Patuleia, and de Harven, 1965), it is absolutely required for red blood cell differentiation to occur. The two major biochemical problems concerning erythropoietin are its chemical nature, including structure-function relationships, and its molecular mode of action.

The purification of erythropoietin from anemic sheep plasma to a condition of homogeneity has been reported from this laboratory (Goldwasser and Kung, 1971). The preparation of a fraction having about the same potency (approximately 9000 units per mg of protein) from human urine, has also been reported (Espada and Gutnisky, 1970); however, no data on the homogeneity or chemical properties of the urinary hormone have been published. The only significant contaminant of the plasma hormone, when isolated, is a small amount of desialated erythropoietin. When all the sialic acid is removed, the preparation is homogeneous by gel electrophoresis at pH 8.6 and 6.0.

Chemical Properties of Erythropoietin

Because of the extremely small amounts of pure plasma erythropoietin available, the chemical properties reported here must be considered preliminary.

DISCLAIMER

Sheep plasma erythropoietin is composed of 74% protein and 26% carbohydrate. The carbohydrate consists of 10% - 11% sialic acid (16-18 residues/mole), 6% galactose (18 residues/mole), 4% mannose (12 residues/mole), 4% glucosamine (12 residues/mole), and 2% glucose (6 residues/mole). Microdetermination of the amino acid composition has not yet been carried out satisfactorily, but, within the limit of detection, there appears to be no methionine or cysteine (less than 2-3 residues/mole) and only small amounts of arginine, tyrosine and valine. The absorbance maximum in the middle ultraviolet range is 276 nm and the $A^{1\%}_{1cm}$ at that wavelength is 7.4.

The molecular weight of the iodinated erythropoietin determined by electrophoresis in gels containing sodium dodecyl sulfate (Weber and Osborn, 1969) is 46000 (Goldwasser and Kung, 1972).

The effects of chemical and enzymic probes, of known specificities, on the biological activity of erythropoietin can give us some information about the chemical nature of the hormone. A requirement for tyrosine or histidine was deduced from the inactivation by fluoroscein isothiocyanate (Lowy and Borsook, 1962) and by iodination (Goldwasser and Kung, unpublished); that of arginine or lysine, from the inactivation by trypsin. Treatment of erythropoetin with hydroxynitrobenzylbromide, which is specific for tryptophan, also caused loss of activity (Lowy and Keighly, 1968). Reaction with p-chloromercuribenzoate, on the other hand, did not inactivate, nor did esterification of primary alochol groups with anhydrous formic acid (Lowy, Keighly and Borsook, 1960).

We have found that removal of the terminal sialic acids by either acid or sialidase treatment causes complete loss of activity whether the in vivo assay is done in fasted rats or plethoric mice. When the assay was done in vitro, no loss of activity could be detected. Speculations about this seeming

paradox suggested that the erythropoietin molecule itself contained the sialic acid that was required for stability in vivo, but not for action on the target cells of the marrow (Goldwasser, 1966). The work of Ashwell and his collaborators makes it clear that this is the case generally for glycoproteins (Morell, Gregoriadis, Schemberg, Hickman, and Ashwell, 1971), and that removal of the penultimate galactose or its oxidation can restore the clearance rate of the asialoagalactosyl glycoprotein to a near normal value (Morell, Irvine, Sternlieb, Schemberg, and Ashwell, 1968). The same may be true of erythropoietin activity; therefore we are now testing the biological activity of desialated erythropoietin that has had any terminal galactose modified. We have found, however, that pretreatment of the assay rats with either asialo-orosomucoid or with stachyose allows asialoerythropoietin to exert a small, but significant effect in vivo (Goldwasser et al., unpublished).

A more complete chemical characterization of erythropoietin, as well as testing of its clinical efficacy, will have to wait until more material becomes available for study. When one considers that, even in plasma from very anemic animals (containing one unit per ml), erythropoietin represents between 0.0001% and 0.0002% of the protein, the task of isolation of milligram quantities in pure form can be seen as a formidable one.

One alternative source of erythropoietin is the urine of severely anemic patients. There are data (Espada and Gutnisky, 1970) showing that a high degree of purification from this source is possible, so that, if enough raw material were available, pure human erythropoietin might be produced in large quantity. Erythropoietin might also be derived from kidney cell cultures. Some evidence Osawa, 1967; McDonald, Martin, Simmons, and Lange, 1969) has been published suggesting that this is feasible, but no large-scale preparation has been described to date.

Mode of Action of Erythropoietin

It is convenient to consider the process of erythroid cell differentiation as the sum of three separate, but interrelated processes (Goldwasser, 1966). The first of these, sensitization, refers to the target cell becoming competent to be induced by erythropoietin. There is a paucity of information on this process, which was postulated to involve the transitory appearance of a specific receptor for the hormone during the cell cycle. Recently we have found evidence consistent with the existence of such a receptor on some cells of the bone marrow (Chang and Goldwasser, unpublished).

Before the hormone can have an 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 whether erythropoietin penetrates the cells has not been answered, because the ^{125}I-labeled pure hormone (Goldwasser and Kung, 1971) is inactive. We must find a way to label minute amounts of purified erythropoietin without causing inactivation; such experiments are now being carried out.

The second process in establishing the differentiated state, induction, refers to the primary biochemical step that occurs as a result of the specific interaction of erythropoietin (or a secondary chemical signal) with the target cell nucleus.

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 (Krantz and Goldwasser, 1965; Gross and Goldwasser, 1969). Within five minutes, adult rat marrow cells in vitro exposed to erythropoietin synthesize a small amount of a type of RNA not detected in control cells. The 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 the RNA is not reduced by pronase, DNase, 8 M urea, or 0.01 M 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, an RNA-DNA complex, or an RNA-RNA complex held together by magnesium ions or double-stranded RNA. We have no evidence yet regarding the function of this RNA, but we do know that it is confined to the nuclei of the marrow cells, 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 transfer occurs to a small extent not detectable by present methods. For instance, if the 150S RNA were a "unit of transcription" (Scherer and Marcaud, 1968; Georgiev, Ryskov, Coutelle, Mantieva, and Avalcyan, 1972) and contained the nuclear precursor of the cytoplasmic 9S globin messenger RNA, the messenger 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 150S RNA synthesis, and the fact that it seems to be found only in the nuclei of erythropoietin-treated cells, argue 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.

Very shortly after the synthesis of 150S RNA is induced, another species of large, rapidly labeled, heterogeneous nuclear RNA is formed as a result of erythropoietin action (Gross and Goldwasser, 1969). This RNA, with a range of sedimentation coefficients from around 60 to 70S, is also found in control cells of the marrow and has been described in other eukaryotic cells. Its function, too, is not established, but is assumed to be concerned with the unit of transcription. Whether any relationship between 150S RNA and marrow cell 60S RNA

exists is unknown. However, our 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 compositions are different, suggest that the two are not related as precursor and product.

The available evidence strongly suggests that the initial nuclear response of susceptible cells to erythropoietin involves transcription, and that all the events described below are dependent on this new synthesis of RNA that may be closely associated with the inductive mechanism.

The third phase, specialization, refers to the complex of molecular changes which occurs when the induced cells acquire new functions distinguishing them as having become differentiated. The cells also lose some of their old functions during specialization; at present we have no information, however, on the mechanisms underlying these losses.

Since the ultimate function of the differentiated erythroid cells is to make large amounts of hemoglobin, it is not surprising that one of the early events is the synthesis of new ribosomal RNA starting about 15 minutes after erythropoietin is added to the cells (Gross and Goldwasser, 1969). 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 cellular function (Gross and Goldwasser, 1970a), and by 9S RNA which is probably the globin messenger.

Our studies of the effect of erythropoietin on synthesis of 9S RNA (Gross and Goldwasser, 1971) have shown that some 9S RNA is formed even under conditions of totally suppressed hemoglobin synthesis. We think that this RNA may represent the message for a protein not involved in erythropoiesis. When marrow cells from polycythemic animals are given erythropoietin, they synthesize

increased amounts of 9S RNA some nine to ten hours before hemoglobin synthesis starts. The transposition of RNA from marrow cell nuclei to cytoplasm takes about three hours; this leaves about six to seven hours during which the presumed globin messenger may be 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.

About two hours after erythropoietin is added to cultures, an increase in DNA synthesis occurs (Paul and Hunter, 1969; Gross and Goldwasser, 1970b). 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 increase in cell division. This is also borne out by our finding that erythropoietin-induced DNA synthesis is completely 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-sensitive 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 (Hrinda and Goldwasser, 1969). The effect of erythropoietin on cellular uptake of glucosamine, both the formation of a glycolipid (Dukes, 1968) and of stroma (Dukes, Takaku, and Goldwasser, 1964), is not apparent for about five hours. An hour later (at about six hours), the induced (normal) cells show increased hemoglobin synthesis (Gross and Goldwasser, 1971). If cells from polycythemic animals are studied, hemoglobin synthesis is detectable only about 10 to 12 hours after the addition of erythropoietin. The main difference between normal and "polycythemic"

marrow cells lies in the complete absence of differentiated cells of the erythroid series in the latter. 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 also acts on some differentiated cells. Earlier studies with whole animals suggested the same conclusion (Matoth and Ben-Porath, 1959; Hodgson and Eskuche, 1968).

The process called specialization is really a complex of processes that is very far from being completely understood. One of the important questions related to the molecular events eventually leading 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.

Conclusions

The central problem in understanding the action of erythropoietin is that of regulation of gene expression. This is also true of embryonic development and of 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 eventually causes the cells to 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, when a sensitized cell is hit, the signal, either erythropoietin taken up by the cell or some secondary signal, acts on the nucleus to

initiate a new transcription. The function of what appears to be 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 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. Eventually, however, the observations concerning induction and specialization which have been made on normal marrow cells will have to be confirmed in cells from polycythemic animals.

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