ERYTHROPOIESIS
Regulatory Mechanisms and Developmental Aspects

Edited by Yehuda Matoth

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CONTENTS

PREFACE

Regulation of erythropoiesis by a humoral factor was first suggested by Carnot and Deflandre at the beginning of the century. Almost fifty years elapsed, however, before this hypothesis was placed on a firm scientific basis with the help of modern methods of research and critical evaluation. Renewed interest has resulted in an exponential proliferation of the literature on erythropoietin and the regulation of erythropoiesis during the past 20 years. Indeed, in a recent review of the subject (1), 1,418 references are cited. Conferences on erythropoiesis have been held at regular intervals in the past and their published proceedings (2, 3) have served as a useful means of orientation in this rapidly expanding field of research.

Topics of current interest and under active study were reflected in the presentations and discussions at the Tel Aviv University Conference. A more sensitive, accurate and rapid assay method for erythropoietin, than the mouse bioassay currently available, is of crucial importance for further progress in this field. Our understanding of the mechanism of erythropoietin production has been greatly advanced by the description of a renal erythropoietic factor or erythrogenin. Evidence for an inhibitor of erythropoiesis has raised the question of its possible role in the regulatory mechanism and challenged the concept of erythropoietin as the sole regulator. In addition, the fascinating subject of fetal erythropoiesis and its regulation received special attention and stimulated lively discussion.

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Y. MATOTH

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THE STEM CELL PROBLEM IN THE FETUS

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The stem cell problem in the fetus presents a number of distinctive features. Granulopoiesis is not very active throughout the greater part of fetal life, and in man the level of the blood granulocytes is quite low until the last month or so of pregnancy (1). This, presumably, is because the fetus does not as a rule have contact with bacterial antigens, which it only encounters after birth. In contrast, erythropoiesis is extremely active throughout fetal life, since it has to keep pace with the rapid increase in blood volume as well as to replace cells destroyed in the normal wear and tear of the circulation. It is not surprising therefore that the liver, the chief site of hemopoiesis during a large part of fetal life, is essentially an erythropoietic organ, in which granulopoiesis is rarely in evidence, if at all (2–5). It is not altogether clear whether this is because the hepatic stem cells are unipotential and can only develop into erythroid cells, or whether they are pluripotential, but do not develop into granulocytes because local metabolic conditions do not favor this line of development. The latter view is supported by observations such as those of Bridges et al. (6), who administered a suspension of fetal liver cells to a patient suffering from pancytopenia, and subsequently noted a pronounced granulocytosis.

The fetal liver contains singularly few lymphocytes in its parenchyma, though some may be seen in the circulating blood in the lumen of the sinusoids (3). If hepatic stem cells are pluripotent, it is surprising that lethally irradiated animals are frequently not protected by the transfusion of even considerable numbers of fetal liver cells, and it is particularly noteworthy that the lymphoid tissues fail to regenerate in many of these animals. Some of these problems are discussed more fully by Micklem and Loutit (7) and Van Bekkum and de Vries (8).

Hemopoietic sites in the fetus. Four main hemopoietic sites have been described in the fetus, namely the yolk sac, the liver, the connective tissues and the bone marrow. Furthermore, one frequently finds many developing erythroid cells and granulocytes in the blood. In addition, there may be considerable numbers of hemopoietic stem cells in the bloodstream, and it would not be surprising if a few of these escaped occasionally from the blood into various tissues. In our experience, fetal hemopoiesis in other situations, such as spleen, thymus and lymph nodes, has not been found to be very active, though it does occur.

Migrating stem cells. It is now generally accepted that, even in postnatal life, hemopoietic stem cells are to be found in the blood stream. In the fetus, the number of circulating stem cells appears to be very much greater than in the adult. Barnes et al. (9) found that

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in lethally irradiated mice transfused with blood leukocytes to confer protection, 10^7 leukocytes were required if taken from the blood of adult mice, whereas if taken from fetal blood only 10^4 to 10^5 were needed. In other words, the fetal blood which they obtained contained more than 100 times as many stem cells as that of adult animals. A difference of this order raises a number of questions.

The first and most obvious question concerns the identity of these circulating stem cells. If they are present in such greatly increased numbers, is it possible to identify them? Though definite identification is not yet possible, there are some points of interest which may have a bearing on the problem. If human fetal blood may be taken to be representative of mammals as a whole, a striking feature of the blood leukocytes in man is that throughout a large part of fetal life they consist predominantly of lymphocytes (1) Winter et al. (10) paid special attention to the morphology of the fetal blood lymphocytes, and concluded that in midfetal life "virtually all circulating lymphocytes are of the transitional type with a high N:C ratio and a leptochromatic nucleus." Fig. 1 illustrates a random selection of such cells from the blood of an 18-week human fetus. Of the various groups of lymphocytes present in blood postnatally, two cell types are relatively infrequent in the fetus, namely the pachychromatic small lymphocyte, and certain of the larger basophilic cells. Both may be found in fetal blood, but in relatively small numbers when compared with the blood after birth.

A full discussion of the various types of lymphocytes present in the blood in postnatal life will be found elsewhere (11). There seems little doubt that in the adult the great majority of the larger basophilic cells are plasmablasts in varying stages of development, entering the blood for the most part through the thoracic and right lymph ducts. The pachychromatic small lymphocytes in postnatal life consist, to an appreciable extent, of conditioned small lymphocytes, the so-called "memory" cells, capable of responding only to a secondary antigenic stimulus. It would not be surprising, therefore, that during fetal life, when the organism receives hardly any antigenic stimulation, neither of these cell types is present in the blood.

The fact that the blood contains large numbers of both transitional cells and hemopoietic stem cells during fetal life may be purely coincidental. But in the light of the bone marrow findings, discussed elsewhere in this volume, it is not inconsistent with the view that the hemopoietic stem cells are to be found in the lymphocyte-transitional group. In such a case the association of increased numbers of transitional cells with hemopoietic stem cells would be more than a chance occurrence. At any rate, there is no other obvious alternative for the stem cell. Monocytes are few and far between.

Negative implications of the composition of the blood leukocytes. The composition of the blood leukocytes in the fetus has what might be termed an important negative implication in relation to the stem cell problem. Maximow who summarized his views in 1927 (12), maintained that throughout the greater part of fetal life the hemopoietic stem cells were mainly the primitive cells of the mesenchyme, just as in postnatal life the essential stem cell was the so-called reticular cell. The precise relationship between these two cell types was never quite clear, but they were not generally thought to be capable of migration through the bloodstream. It follows, therefore, that if these cells really are the essential stem cells one must postulate either that they become capable of migration through the blood—in which case they must presumably undergo a considerable change in morphology which renders them unrecognizable while they are in the circulation—or that there exist other types of stem cell, with migratory properties.

As far as migratory properties are concerned, lymphocytes and transitional cells would obviously meet the situation, but Maximow had, at times, a somewhat ambivalent approach to these cells. Though he maintained repeatedly that small lymphocytes were capable of growth and differentiation (13), he often seemed to ignore them when propounding schemes for blood cell development. Thus, one of the schemes of blood cell development (in postnatal life) most frequently reproduced (14) begins with the hemocytoblast, the large basophilic blast cell. In the fetus the starting point, as noted, is the primitive mesenchymal cell (12).

The yolk sac as a source of stem cells. The first site of blood formation in the fetus is the yolk sac, and here the stem cells are presumably of local origin. They have generally been thought to arise from the primitive mesenchyme (See ref. 12 for further discussion), though an entodermal origin has also been suggested (15). Whatever the origin of the cells, they are characteristically large and basophilic.

The large avian yolk sac contains a substantial amount of hemopoietic tissue, whereas the mammalian yolk sac, relatively vestigial and present for only a short time, does not appear to play such an important part in fetal blood formation. However, despite its small size, Moore and Metcalf (16) have recently maintained that the mouse yolk sac is the primary source of hemopoietic stem cells, which then migrate to the liver and subsequently to other hemopoietic tissues. This concept may be correct, but it is not fully substantiated by the available evidence. Moore and Metcalf (16) cultured entire mouse embryos, with intact yolk sacs, for two days. The cultures could not be continued for a longer period, since after two days "the growth of the embryo became disorganised due to a breakdown in the normal pattern of development..." In view of this deterioration *in vitro* it seems not improbable that even during the two days of culture there was abnormal development, since it was only in some embryos that they found "evidence of early liver development." In embryos in which the yolk sac was first removed, development did not seem to be very different, but "histologically there was no evidence of hemopoietic cells of any type, either in vascular channels or in the region of the developing liver." It is not clear to what extent the absence of these cells in the circulation may have contributed to the unsatisfactory growth of the embryo. But quite apart from this, even in perfectly normal mice, according to Rifkind et al. (17), the first sign of erythropoiesis in the liver only appears in 10½-day-old fetuses. It is not surprising, therefore, that in embryo cultures, either with or without removal of the yolk sac, there was no hepatic erythropoiesis by the ninth day.

Hepatic stem cells. As far back as 1853, von Kolliker (18) first drew attention to the hemopoietic role of the fetal liver, and ever since then, the origin of the hepatic hemocytoblasts has been the subject of controversy. According to Saxer (19) and Maximow (20, 21), the hemocytoblasts of the fetal liver arise through the differentiation of mesenchymal cells which infiltrate the hepatic trabeculae as they grow into the septum transversum. Maximow's views were later summarized (12) in an extensive review of the hemopoietic tissues. Neuman (22), Schmidt (23) and Schridde (24) believed that the hepatic hemocytoblasts were derived from the endothelium of the hepatic sinusoids, but this view has now fallen into disfavor. Another origin was suggested by Van der Stricht (25, 26), who thought that the hepatic hemocytoblasts were derived from circulating stem cells which readily settle in the hepatic sinusoids, where the circulation is slow, and which subsequently proliferate in close proximity to the hepatic cells. Yet another origin for the stem cells was first suggested by Toldt and Zuckerkandl (27) who

described a series of cells intermediate in morphology between the irregularly shaped, more or less polygonal liver cells and the basophilic round hemocytoblasts. Thomas and Yoffey (3) also came to this conclusion, namely that the hepatic hemocytoblasts are derived from the undifferentiated liver cells of entodermal origin in the hepatic trabeculae. It should be emphasized that this is definitely a minority view, and one to which Maximow (12) strongly objected. In accordance with his general concept of the role of mesenchyme in hemopoiesis, Maximow supported the view that hemocytoblasts in the liver were derived from primitive mesenchymal cells. With the great weight of Maximow's authority behind it, this view seems to have gained general and almost unquestioned acceptance. However, if one examines the evidence carefully it does not appear to be as convincing as is generally believed.

Schridde (24) was unable to find, in fetal liver, the wandering mesenchymal cells described by Maximow, while Thomas and Yoffey (3) were also unable to observe them throughout most of the liver parenchyma, though they could be seen in the vicinity of the larger blood vessels. If one examines Maximow's own illustrations (see for example ref. 12, Fig. 94, p. 477) one can see a striking resemblance between some of the cells which he designates as hemocytoblasts and those which he considers to be hepatic cells. The smaller mesenchymal cells are quite distinctive, but the larger ones are not. Furthermore, the mesenchymal cells depicted in the fetal liver seem to differ appreciably from mesenchymal cells illustrated in other parts of the embryo (compare for example with ref. 12, Fig. 91, p. 473).

Ultrastructural studies have not as yet thrown much light on the problem. Thus Jones (28) follows Maximow, and asserts categorically that "the youngest definitive erythroblasts arise extravascularly from mesenchymal derivatives...," but it is not clear by what criteria these derivatives are identified. Sorenson (29), working with rabbit fetuses, emphasized the close relationship between the erythroblasts in the liver and the cells of hepatic trabeculae. Grasso et al. (30), in a combined electron and light microscope study, concentrated primarily on the definitive hemocytoblast and later stages. Zamboni (31) studied the livers of nine human fetuses, 7 to 20 weeks of age. He illustrates a stem cell in his Fig. 11 which is not unlike his description of the liver cell. Though he does not illustrate a primitive mesenchymal cell, he nonetheless states: "Thus, derivation of the hepatic hemocytoblasts from mesenchymal cells of the septum transversum caught between ingrowing entodermal cells appears much more likely."

Rifkind et al. (17) endeavored to throw further light on the problem in an ultrastructural study of fetal hepatic erythropoiesis in mice. At 10 days, the entodermal hepatic diverticulum is separated by a basement membrane from the mesenchymal cells which it is invading. Their Fig. 3 illustrates the two cell groups and a cleft between them. The morphological differences between hepatic and mesenchymal cells are not very marked, as the authors themselves point out. Unfortunately, definitive erythropoiesis does not commence until $10\frac{1}{2}$ to 11 days, by which time the hepatic and mesenchymal cells are inextricably intermingled, so that it is not possible to decide whether it is hepatic or mesenchymal cells which differentiate into erythroid cells. However, it is evident from their account that they take Maximow's view as their starting point: "In both the yolk-sac and the liver it is believed that the erythropoietic tissue develops in an embryonal mesenchyme closely approximated to an entodermal derivative, the yolk and endoderm on the one hand, and the hepatic epithelium derived from the gut on the other." As evidence for this they cite

Bloom (32), who adhered closely to Maximow's views.

Though Rifkind et al. (17) found that the morphological differences between hepatic and mesenchymal cells were not very clear-cut, it is of interest that they drew a sharp distinction between erythroblasts of yolk sac and hepatic origin. Yolk sac erythroblasts are found only in the lumen of the blood vessels, and can readily be distinguished on morphological grounds from the endogenous hepatic erythroid cells; they do not appear to be precursors for hepatic erythropoiesis. "The earliest recognizable endogenous hepatic hematopoietic cells appear extravascularly..." The view that hemopoietic cells of vitelline origin differ from their hepatic counterparts is further supported by the marked contrast to their response to erythropoietin. Vitelline hemopoiesis does not seem to be affected by the addition of erythropoietin (33), whereas hepatic erythropoiesis appears to be definitely enhanced by this substance (34).

It is possible that a histochemical approach may throw fresh light on the problem. Rosen (35) studied the distribution of glucose-6-phosphatase hydrolyzing enzyme in neonatal rat liver. He found it to be localized in the hepatocytes, nucleated red cells and heterophil granulocytes and stated that "in each of the cell types observed the activity was restricted to the nuclear envelope and the endoplasmic reticulum." The presence of this enzyme in both hepatocytes and red cells does not of course prove the origin of the latter from the former, but equally it is not incompatible with such an origin. It is pertinent at this point to note that the yolk sac contains fetal hemoglobin, whereas the liver contains the adult variety (36), though it has been suggested that cells of yolk sac origin can produce adult hemoglobin in the liver (37).

Hemopoietic activity of primitive mesenchymal cells. Maximow (12) has been the foremost advocate of the concept that the primitive mesenchymal cells are capable of developing into basophilic hemocytoblasts, and he has produced *inter alia* illustrations of areas of hemopoiesis in the loose mesenchyme of the head region. It is not clear from Maximow's account how frequently he found these areas of hemopoiesis in the connective tissues, but those which he describes do not seem to be either very extensive or particularly active. He emphasizes that granulocytes are mostly seen in pairs only, while he also notes that the occasional groups of erythroblasts consist typically of cells which are all at the same stage of development. While this appearance may be due to the proliferation of a hemocytoblast resulting from the transformation of a primitive mesenchymal cell, it could also be due to a circulating stem cell which had escaped from the bloodstream and then underwent several divisions. From what we now know about cellular migration streams through the connective tissues in postnatal life (11), this is an explanation which must be taken into consideration.

The early development of the bone marrow presents special features. Hammar (38) described the "primary" marrow as consisting of loose connective tissue between dilated thin-walled vessels. This is followed by infiltration with lymphoid cells and the formation of red marrow, frequently in cords surrounding thin-walled arteries (39) and large veins. Maximow (12) described the occurrence of hemocytoblasts in the deeper layers of the periosteum, from which they presumably then make their way into the marrow cavity. Yoffey and Thomas (40) studied the development of the marrow in a series of 50 normal human fetuses delivered by hysterotomy and ranging in age from 8 to 28 weeks. Marrow was obtained from femur, humerus, clavicle, ribs, vertebrae and sternum and study of the sections confirmed the findings of Hammar (38), that the primary marrow is loose connective tissue among dilated vessels. However, in

none of the many sections examined were hemocytoblasts ever seen in the periosteum.

One final point should perhaps be made in considering the origin of blood cells from mesenchyme. The literature contains abundant references to primitive mesenchymal cells but their precise characterization leaves much to be desired. The types of rounded mesenchymal cells described by Maximow in the fetal liver have a very different morphology from cells similarly named in loose connective tissue.

Myeloid stem cells. Unlike the hemopoiesis in yolk sac and liver, myeloid hemopoiesis is distinguished by the conspicuous presence of lymphocytes and transitional cells, at a time when they are presumably not discharging any immunological function. In 50 healthy human fetuses, ranging in age from 8 to 28 weeks, Yoffey and Thomas (40) found that femoral marrow was first clearly in evidence at 11 to 12 weeks. From this time onwards, lymphocytes and transitional cells form about 25% of the total nucleated cells of the marrow (range 10 to 45%), and about 15% of the lymphocyte group are transitionals. This high lymphocyte-transitional content is of the same order as one finds in the smaller laboratory animals, and for reasons which have been fully discussed elsewhere (41) the number in man undergoes a steady decrease in postnatal life, and in the human adult it is well below this figure. The transitional cells in human fetal marrow are quite typical, and have been illustrated in photomicrographs (42). The composition of fetal marrow suggests that one is dealing here with a fundamentally different hemopoietic pattern from that which is found in yolk sac and liver. The myeloid pattern, once established in the fetus, persists throughout life subject to the variations consequent upon changing stem cell requirements (41) which depend upon a number of factors, including the rate of growth, and the effect on the bone marrow of a host of stimuli from the changing external environment. The postnatal marrow is a great deal more variable than is the marrow of the fetus in its sheltered environment.

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DISCUSSION

DR. J. M. YOFFEY (*Israel*): (answering question from Dr. M. Feldman): Moore and Metcalf's studies do not altogether fit in with the clinical findings. If the liver cells are pluripotential, they ought to give more effective protection to irradiated animals. Some have claimed that fetal liver transfusion stimulates granulopoietic regeneration, but as far as I know this is not the general experience. Van Bekkum and others have failed to obtain satisfactory regeneration of lymphoid tissue after fetal liver transfusion.

In man, fetal liver transfusion seemed at one time to hold out great promise as a means of replacing damaged hemopoietic tissue, but it seems to have fallen into disfavor. Ambitious projects for elaborate banks of fetal liver do not seem to have fulfilled their earlier promise.

One other point should be noted about the paper by Moore and Metcalf to which you have referred. They cultured embryonic mice from the seventh to the ninth day. In some of these cultures they removed the yolk sacs at the outset, while

in others they left it in place. They examined the cultured embryos on the ninth day and found no hemopoiesis in the fetal liver. They seem to have regarded this as conclusive proof that hemopoietic stem cells were migrating from yolk sacs to liver. However, hepatic hemopoiesis in the fetal mouse does not seem to get under way until the tenth to eleventh day of fetal development, as shown by Rifkind and his associates at Columbia. Thus, unless there were very radical species differences, one would not expect to find hepatic hemopoiesis by day 9 whether the yolk sac is removed on day 7 or not.

DR. G. PERACH (*Israel*): We are trying to approach this problem of stem cells in fetal liver by using the Till and McCulloch method, and I have observed the formation of granuloid colonies after injecting the irradiated animals with fetal liver of different ages—thirteen, fifteen and up to nineteen days. This means that even *in vivo*, although the recipient is an adult animal, the potential for forming the granuloid colonies exists and the actual formation takes place.

DR. YOFFEY: Yes, I know, and you have published something about that already, haven't you? That is why I was particularly interested in raising this other point. If that is the case, why does fetal liver not give more effective and more constant protection. Dr. Feldman, do you get adequate or full protection in other animals?

DR. M. FELDMAN (*Israel*): Well, the question of full protection depends on the definition. Not only can one get protection with fetal liver, but one can get protection across genetic barriers, because in the absence of lymphoid cells you do not get the graft vs. host response, and in fact this was one of the earliest approaches to overcome the gap. Now, indeed, the protection in many cases is not that which one gets perhaps with isologous or syngeneic bone marrow cells, but the reason for this is that although erythroid and granuloid cells do differentiate, the lymphoid cell differentiation is not the same as in the case of bone marrow. So the position is slightly different with regard to the function of lymphoid cells.

DR. YOFFEY: Well, what is the nature of this lymphoid? If it is a pluri-potential cell...

DR. FELDMAN: Oh no, I did not say that. I said only that it is an oligo-potential cell in the sense that the same stem cell can give rise to granulocytes, erythroid cells and megakaryocytes. We do not know whether or not the same cell can give rise to lymphocytes.

DR. N. A. SHORE (*USA*): If indeed these liver cells give rise to the early erythroid or oligo-potential cells, how many liver cells have to become stem cells?

DR. YOFFEY: I don't know. We looked solely at morphology, and we asked the same question when we looked at the liver cords. If all the liver cells are, in fact, transforming into erythroid cells, as we certainly thought some were on morphological grounds, then one might well wonder why the fetal liver persists at all. Why does it not disintegrate and disappear? Presumably, however, the primitive entodermal cells undergo two lines of development. Some become differentiated into specialized liver cells, while others, which have not yet undergone specialization, can function as hemopoietic stem cells. In any case, whatever the stem cell, the histological appearances suggest that some liver cords, packed with developing red cells, might well disintegrate. We could not satisfy ourselves of the existence of any other precursor than what we thought was a fetal liver cell. If you can produce more convincing evidence than Maximow's of a mesenchymal or other stem cell, I personally would be very glad to accept it, since obviously the liver cell as such raises a number of basic problems. But despite all the arguments against the idea that some primitive hepatic cells act as stem cells, the pictures I have shown give the morphological background which made us decide that our interpretation is the only one which seemed to meet the situation.

There is one general problem which I would like to raise. We talk about stem cells, and tend to entertain the idea that there is just a single stem cell. But is this necessarily so? Or can any undifferentiated cell, whatever its situation, provided it has not yet undergone its specific functional differentiation, develop along hemopoietic lines if given the appropriate stimulus?

DR. SHORE: May I ask another question? Were the fetuses you obtained—since they were products of abortions—septic or infected *in utero*?

DR. YOFFEY: They were not from abortions, but were all removed by hysterotomy. They were ob-

tained absolutely fresh, and the material was perfect. I'm only sorry we did not examine it by electron microscopy, but we were not doing any ultrastructural work at that time. The material was absolutely healthy.

DR. A. ASCHKENASY (*France*): I would like to ask Dr. Yoffey whether he has performed some experiments *in vitro* or *in vivo* concerning a possible effect of erythropoietin on the erythropoietic differentiation of hepatic cells?

DR. YOFFEY: No we haven't actually, and obviously, one should, but we haven't done it as yet. Hasn't the Rehovot group worked with erythropoietin on fetal liver?

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