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Early circulating erythroid progenitors (BFU-E) in sickle cell anemia

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Abstract. Sickle cell anemia (SS) patients can be divided into two sub-populations according to peripheral HbF levels. Patients with low ($<9\%$) HbF levels (LFSS) are characterized by an increased number of circulating BFU-E in active DNA synthesis, and release of burst promoting activity (BPA) by unstimulated low density (LD) adherent cells. In contrast, circulating BFU-E from SS patients with high ($>9\%$) HbF levels (HFSS) are normal in number, largely in resting phase, and their LD cells do not release BPA-like activity.

More recently further heterogeneity has been found among these two groups. In LFSS patients GM-CSF is constitutively produced by unstimulated monocytes. In contrast, HFSS patients' adherent cell depletion increases cycling of BFU-E in culture. CM from HFSS patients inhibits BFU-E expression in culture. Hence, LD adherent cells from HFSS patients may release an inhibitory factor(s). The nature of this factor has to be determined.

In addition, there are distinct subpopulations of BFU-E responsiveness to growth factor (GM-CSF, IL-3): a) LFSS patients have a homogeneous BFU-E population, equally responsive to GM-CSF and IL-3; b) HFSS patients, in addition to this subpopulation, have a subset of BFU-E dependent exclusively on IL-3 which is 20 to 40% of the total number of circulating BFU-E. This is similar to BFU-E from normal individuals. Hence, LFSS BFU-E represent an actively proliferating population, equally responsive to GM-CSF and IL-3, controlled by at least constitutively produced GM-CSF and possibly other factors.

These observations suggest a significant modification in BFU-E behavior in the subset of SS patients with low HbF levels and high hemopoietic stress. The heterogenous regulation of BFU-E in SS disease seems to be an epiphenomenon of HbF levels, and not vice-versa.

Key words. SS disease; sickle cell anemia; BFU-E; GM-CSF; cytokines; hemoglobin F.

General background and past work in the field

The clinical manifestations of sickle cell anemia (SS) are highly variable among carriers in spite of the presence of the same single amino acid substitution. While haplotypes linked to the β^S gene[50], co-presence of α-thalassemia[25,30] and X-linked factors[21] might determine in part the HbF response, and account for some of the genetically-determined variations in the severity of SS disease, much remains to be defined. The modification of erythropoietic regulation under the permanent stress of the hemolysis observed in sickle cell anemia, or the genetically-determined BFU-E heterogeneity may additionally contribute to the phenotypic variability among SS patients.

Surprisingly, only limited and contradictory data were available on this subject until recently. Studies on four SS patients by Ogawa et al.[49] suggested that circulating erythroid progenitors were increased in number and were insensitive to kill by ^{3}H-dT. Nathan et al.[47] have reported that the erythropoietin (Epo) requirement of SS erythroid progenitors is higher than those of normal, while Lutton et al.[38] and Pennathur-Das et al.[51] have suggested that bone marrow late erythroid progenitors (CFU-E) have an increased sensitivity to Epo. This is in agreement with recent observations by Johnson et al.[34] that BFU-E from β-thalassemic individuals are more sensitive to Epo than controls. Increased BFU-E sensitivity to Epo in some SS patients and evidence that SS erythroid progenitors represent a population distinct from fetal, neonatal and adult normal BFU-E has also been reported by Weinberg et al.[58].

The process of hemopoiesis is regulated by a family of glycoproteins (hemopoietic growth factors), which were initially described as BPA-like activites[2,4,28,31,32,36,43,46,59,61,63–67]. In 1983, Ihle and his coworkers[32] demonstrated that homogeneous IL-3 is identical to the growth factor found in WEH1-3 CM and Goldwasser et al.[28] showed that IL-3 has biological properties identical to that of BPA insofar as its ability to support BFU-E proliferation. Metcalf's group[43] reported that purified GM-CSF (granulocyte macrophage colony-stimulating factor) supports BFU-E proliferation and, consequently, may be responsible for BPA-like activity. More recently, GM-CSF, IL-3, IL-1, IL-4, IL-6, SCF (stem cell factor), and others were purified and cloned[4,31,40,59,61–64].

Some of these factors (IL-3, GM-CSF, etc.) act directly on progenitor cells, while the activity of others (IL-1 or TNF) are indirect[2,36,46,65,66]. Under stress conditions (e.g., infection), simulation of IL-1 or TNF release by monocytes occurs. This, in turn, greatly increases GM-CSF, G-CSF (granulocyte colony-stimulating factor) and possibly IL-3 production which, in turn, potentiates colony formation.

While hemopoietic growth factors have been extensively studied in vitro, little is known about their in vivo role, and their physiological significance. For example, in steady-state conditions, GM-CSF production is absent or too low to be detected[6]. However, under the conditions of stimulated hemopoiesis, the GM-CSF production may increase, as suggested by the fact that detectable levels of GM-CSF were found in normal neonates[22] and that BPA-like production increased during hemopoietic regeneration[56].

Finally, there is growing evidence that erythroid progenitor cells represent a heterogeneous family in terms of their responsiveness to the growth factors and surface makers[35, 54]. It has been suggested that the circulating BFU-E pool in normal individuals contains only BFU-E similar to the BPA dependent bone marrow (BM) fraction and lacks the more mature Epo responsive BFU-E[26]. It is possible that in sickle cell anemia the distribution of these subclasses of BFU-E is modified.

Recent work in the field

Our laboratory has examined the possibility that constant hemopoietic stress in sickle cell anemia leads to modification of the behavior of 14-day erythroid progenitor cells[7-18].

SS patients can be divided into two distinct populations according to the behavior of BFU-E in correlation to the peripheral level of HbF (and implicitly) the severity of anemia (fig. 1). The first group of patients with low HbF levels (<9%) is characterized by an increased number of circulating BFU-E[7, 11, 13, 16] which are engaged in active DNA synthesis as demonstrated by ^{3}H-dT suicide experiments[7, 9, 11]. Furthermore, the proportion of BFU-E in DNA synthesis is inversely correlated with the HbF levels when low density (LD) mononuclear cells were plated. Regression analysis performed on percent of BFU-E kill showed that this correlation was highly significant ($r = -0.88$; $p < 0.00003$) (figs 2, 3)[7]. The production of BPA-like factor(s) by LD mononuclear cells appear to be restricted to SS patients with HbF levels lower than 9% (fig. 4)[7].

Moreover, the proportion of BFU-E sensitive to ^{3}H-dT kill decreased when non-adherent LD mononuclear cells from these patients were plated. Addition of BPA containing SS conditioned medium (CM) raised the number and proportion of BFU-E kill to the level observed

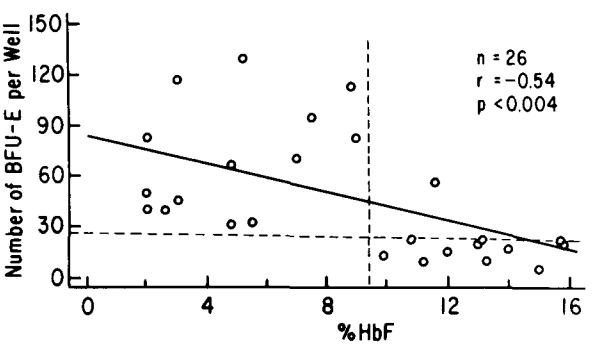

Figure 1. Correlation between the number of BFU-E per well and the peripheral HbF levels in 26 SS patients. The correlation is significant with $p < 0.004$ and the correlation coefficient is -0.54. The horizontal dashed line corresponds to the upper limit of BFU-E count in normal individuals. The vertical dashed line separates well SS patients with normal and high BFU-E counts. Notice the difference in variance between the individuals to the left of the vertical dashed line and those to the right of this line. (From Blood 75 (1990) 1010[7].)

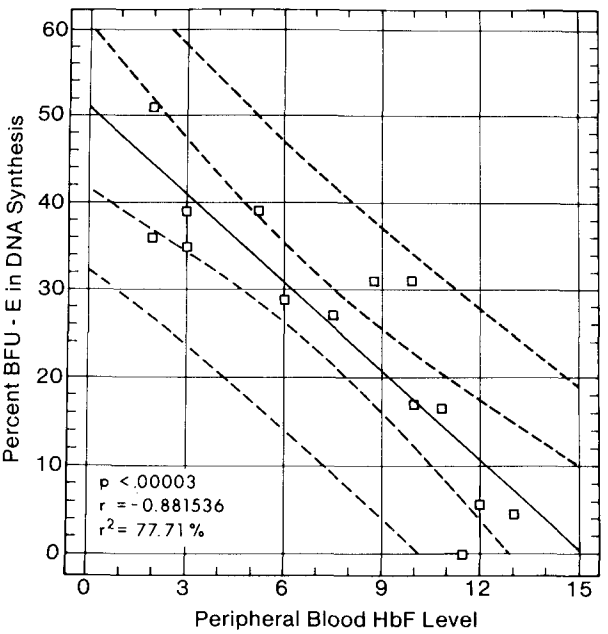

Figure 2. Correlation between percent BFU-E in DNA synthesis and peripheral blood HbF levels of 14 SS patients. Regression analysis performed on percent BFU-E kill in the group cultured in the presence of 1.5 U Epo/ml showed a strong inverse correlation between HbF level and BFU-E kill ($p < 0.00003$; $n = 14$).

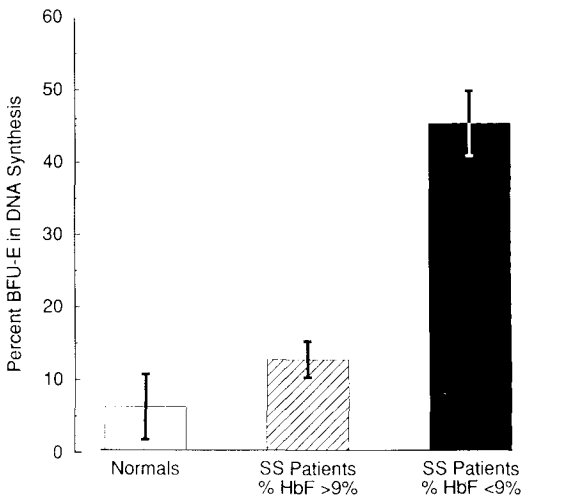

Figure 3. Comparison of percent BFU-E in DNA synthesis in normal individuals, SS patients with HbF levels >9% and SS patients with the HbF levels <9%.

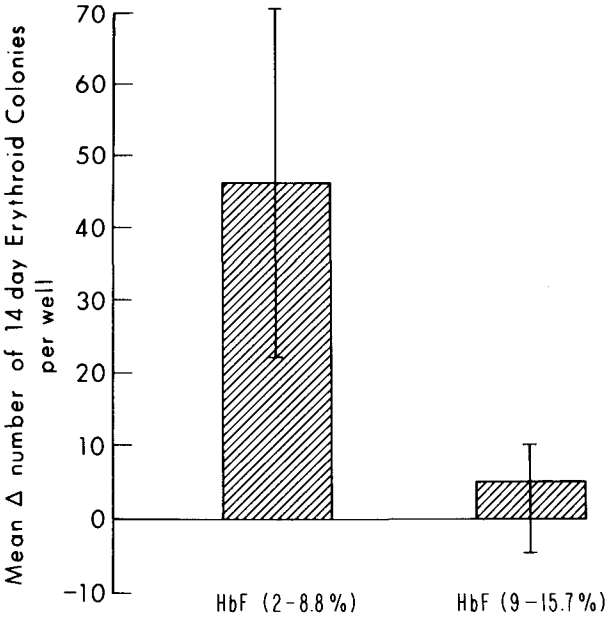

Figure 4. Comparison of BPA-like activity production by LD cells of SS patients with high and low peripheral HbF. LD non-adherent cells were plated in the absence or presence of 20% autologous CM and 0.75 U Epo per ml, of culture. The number of colonies is defined as the difference between the number of colonies produced in the presence or absence of autologous CM ($n = 20$; $p < .05$). (From Blood 75 (1990) 1006–1010[7].)

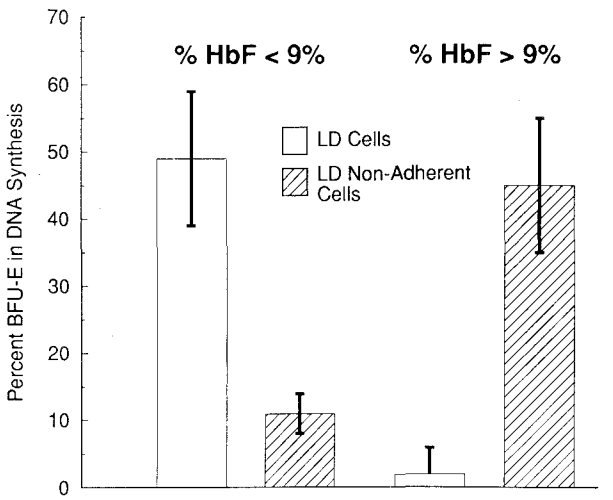

Figure 5. The effect of light density adherent cells depletion on proliferating BFU-E expression in cultures from SS patients with %HbF < 9% and > 9%.

when LD cells were plated. These results strongly suggested that BPA-like factor(s) present in CM from SS patients with low HbF are implicated in the regulation of BFU-E (figs 5, 6).

These results led to the analysis of the role of the accessory cells and their product(s) in the regulation of SS patients' BFU-E growth since other laboratories have reported that adherent cells stimulate[29,68] suppress[50,51] or have no effect on hemopoiesis[37].

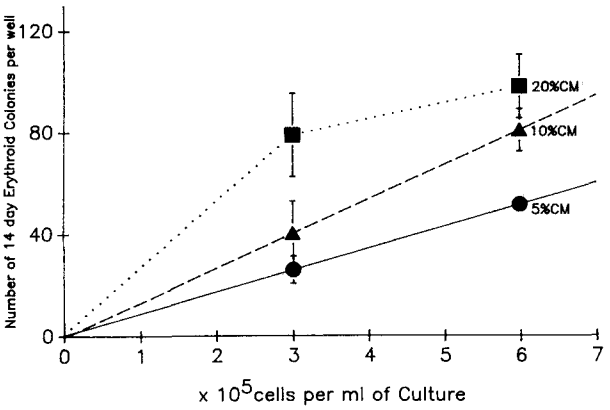

Figure 6. The effect of different concentration of CM from SS patients with %HbF < 9% on the erythroid colony formation in culture.

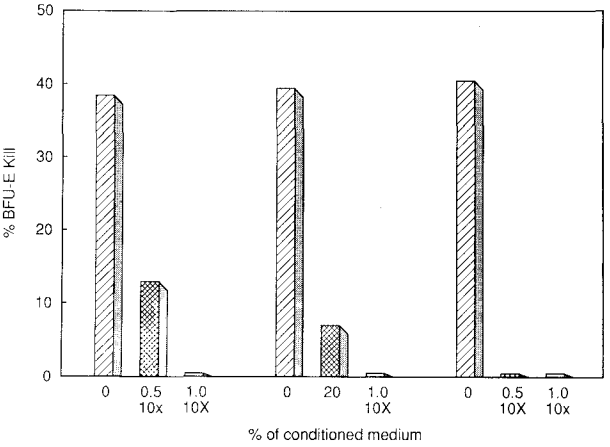

Figure 7. The effect of three different batches of CM from SS patients with %HbF > 9% on cycling BFU-E expression in culture, except for one group the CM was 10 × concentration.

Our data demonstrated that unstimulated adherent cells from low HbF SS patients produced a stimulatory factor, while unstimulated adherent cells from high HbF patients produce an inhibitory factor (figs 6, 7). In the normal control, in contrast with both groups of SS patients, the presence or absence of adherent cells had no effect on erythroid colony formation. Only adherent cells account for the stimulatory or inhibitory activity found in SS patients because LD non-adherent cells alone had no effect on colony formation.

In low HbF SS patients, adherent mononuclear cell depletion leads to the consistent decrease of erythroid colony numbers and reduction in the apparent BFU-E cycling (fig. 5). The addition of fresh or 10 × CM to the culture from low HbF SS patients restored the initial pattern of colony formation and allowed cycling BFU-E to generate colonies (fig. 6).

Since the proportion of adherent cells in LD cells from low HbF and high HbF SS patients are similar the previous findings argue against the possibility that the decrease of cycling BFU-E in culture is due to the

selective loss of a cycling subpopulation by adherence. These results imply that adherent mononuclear cells of low HbF SS patients constitutively release a growth factor(s) responsible for cycling BFU-E expression in culture, and also confirm that the BFU-E grown in the absence of the adherent cells capable of generating BPA retain the capacity to respond to the stimulatory factor. Furthermore, the observation[48] that BPA-like activity can be detected in the serum of patients with acquired aplastic anemia suggests that BPA is constitutively released under stress conditions, strengthens the argument.

Since monocytes[5, 27, 42] are the most likely source of GM-CSF production, and this cytokine is one of the factors involved with Epo in BFU-E regulation, we used antibody neutralization analysis to test its presence in low HbF SS patients' CM.

The potentiating effect of CM on erythroid colony formation by non-adherent cells was totally abrogated by 1:30 dilution of the anti-rhGM-CSF antibody as demonstrated by a decrease in colony number, compared to that generated in the presence of CM + Epo alone. We find that the neutralization of the stimulating factor present in SS CM is concentration dependent (fig. 8).

Hence, GM-CSF is at least one factor *constitutively* released by monocytes of low HbF SS patients which is implicated in the circulating BFU-E regulation. This is a novel situation, since GM-CSF secretion has not been detected in unstimulated adult accessory cells[60], except one report where proliferation of highly purified progenitor was assayed in culture in the presence of different CM[45].

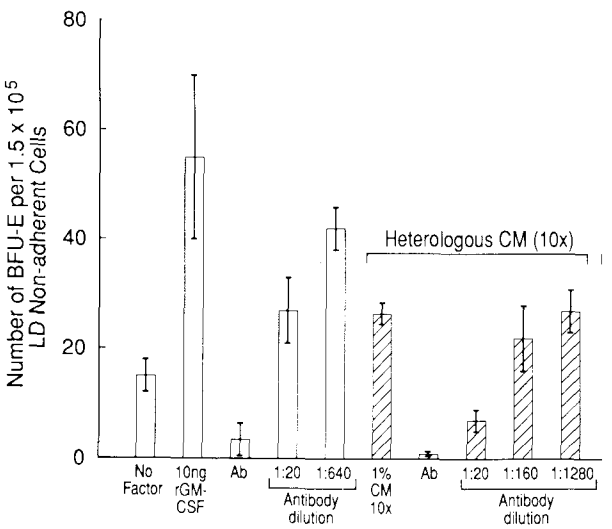

Figure 8. Effect of anti-rhGM-CSF on BPA-like activity present in heterologous 10 × SS CM. A neutralization experiment was conducted using 1% 10 × heterologous SS CM (diagonal stripes), and 10 ng of rhGM-CSF (plain bar) on the same LD non-adherent target cells (SS patients HbF = 3.9). The abscissa in figure 8 is labelled with the indicated factors and antibody dilutions. Label 'Ab' indicates that the undiluted antibodies were used for neutralization experiments in this group.

However, because we have worked with mixed cell populations, the possibility that the GM-CSF stimulatory effect on BFU-E is operating via stimulation of other cells to produce factors that, in turn, are the direct stimulators of these erythroid progenitors, cannot be excluded at this time.

As mentioned before, erythropoiesis in the bone marrow microenvironment is under the control of positive regulatory growth factors as GM-CSF, IL-3 (or multi-CSF), IL-4, etc.[4, 31, 40, 41, 59, 61–64], in addition to erythropoietin. These positive stimuli are balanced, most likely, by inhibitory factors such as TGFβ, interferons, inhibin, or NRP[1, 19, 44, 55]. The role of adherent cells in the control of sickle cell BFU-E behavior appears to be an example of the type of dual control system.

In contrast to the circulating BFU-E from low HbF SS patients that appeared to be under constitutive positive regulatory control, BFU-E from high HbF SS patients appear to be under constitutive (since the adherent cells were not stimulated) negative control. This statement is based on the observation that adherent cell depletion prior to suicide experiments in high HbF SS patients results in an increase of erythroid colony formation, and an increase of apparent BFU-E kill, which may reflect a release of BFU-E from a quiescent state due to the loss of inhibitory regulation by monocytes or their products (fig. 5).

The fact that high HbF SS patients' CM has an inhibitory effect on erythroid colony formation and apparent BFU-E cycling, and thus restores the growth pattern observed in the presence of adherent cells, is in favor of the hypothesis that a soluble BFU-E inhibitor is produced by high HbF SS patients' monocytes. However, the exact nature of this inhibitory molecule remains to be determined.

Cycling of circulating BFU-E in normal individuals is low[7, 49, 53] and adherent cell depletion prior to ^{3}H-dT kill has no major effect on final BFU-E expression in culture. However, addition of 1% 10 × CM from low HbF SS patients to the cultures set up with non-adherent cells from normal idividuals increases the cycling BFU-E expression in culture, as can be expected in the presence of GM-CSF. These results further support the notion that GM-CSF sensitive circulating BFU-E are present in normal individuals. The addition of autologous CM from normal individuals had no significant effect on circulating BFU-E expression in culture. Hence, unstimulated accessory cells from normal individuals do not release constitutively stimulatory or inhibitory factors detectable in our bioassay.

The results presented suggest that the circulating BFU-E in low and high HbF SS patients represent different subpopulations. Subsets of BFU-E of increasing maturity and decreasing self-renewal capacity can be

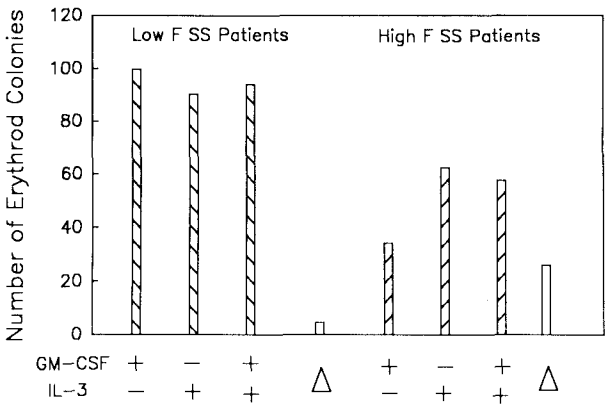

Figure 9. SS patients' circulating BFU-E response to rGM-CSF; rIL-3 and rGM-CSF + IL-3 in the cultures with delayed addition of Epo. The abscissa in figure 9 is labelled with the symbol +; —indicating the presence or absence of growth factors from Day 0 of the culture.

distinguished by characteristic changes in their properties[23]. One of these properties is the sensitivity to the regulatory factor Epo, burst promoting activity (BPA), GM-CSF, IL-3 and others. Subsets of hemopoietic progenitors can be defined on the basis of their selective response to the regulatory factors[3, 17, 24, 29, 54]. Recent reports[3, 17] suggest that responsiveness of circulating BFU-E to growth factor varies among SS patients; thus, the sensitivity to cytokine may be a useful way of further defining the phenotypic characteristics of BFU-E subsets in low and high HbF SS patients.

Using a delayed addition of Epo to eliminate exclusively Epo dependent BFU-E[20], we demonstrated that populations of circulating BFU-E in patients with low and high HbF had a different sensitivity to GM-CSF and IL-3, and that the pattern of the BFU-E response to the growth factors is characteristic and different in

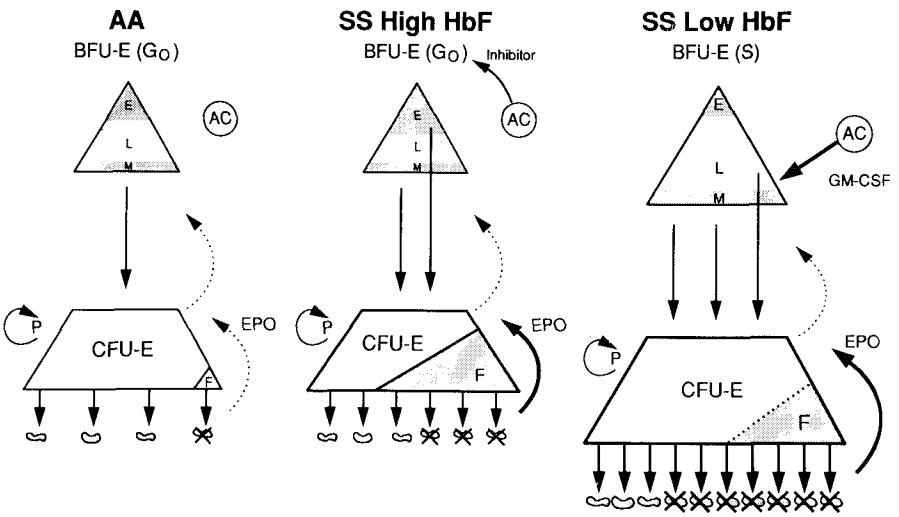

Figure 10. Working model that describes the inhomogeneity of circulating BFU-E regulation in SS patients with high and low HbF, as compared to AA individuals.

Abbreviations and symbols used in the diagram:

AC = Accessory Cell; E = Early BFU-E; L = Late BFU-E; M = Mature BFU-E; P = Proliferation; F = CFU expressing HbF; G_0 = Quiescent or Low Proliferation; → = Constitutive release; X = RBC destruction; and Epo = Erythropoietin. The BFU-E compartment is represented as a triangle and the CFU-E compartment as a trapezoid. The triangular and trapezoid model of the BFU-E and CFU-E pools emphasizes the hierarchical nature of the compartments and sequential differentiation. In AA individuals, the most primitive BFU-E (early BFU-E-'E') shown in the apex of triangles gives rise to more mature progenitors (late BFU-E-'L') which, in turn, generates mature BFU-E (mature BFU-E-'M') seen on the base of the triangle.

We postulate that early BFU-E are IL-3 responsive, while 'L' BFU-E are equally responsive to GM-CSF or IL-3 and mature BFU-E are Epo dependent. In steady state, BFU-E are mainly quiescent or slowly proliferating due to a regulated release of growth factors (GM-CSF, IL-3). Mature BFU-E leave the compartment in a stochastic way, giving rise to highly proliferating Epo dependent CFU-E. The CFU-E finally differentiates to erythroblasts and ultimately red cells.

In SS patients with genetically determined low HbF, the continu*ous high RBC destruction leads to the regulatory changes in the erythroid compartment. In this situation, BFU-E (mainly 'L' BFU-E, GM-CSF IL-3 responsive) are proliferating ('P') which leads to the amplification of the BFU-E compartment, as illustrated by the larger triangle. The 'L' BFU-E expansion is linked to the decrease of 'E' BFU-E shown as a smaller 'E' BFU-E compartment in the apex of the triangle. The continuous BFU-E proliferation is sustained by constitutive release of GM-CSF by accessory cells. Finally, the higher number of 'M' BFU-E, as well as 'L' BFU-E leaves the compartment to compensate for the increased output of CFU-E to proerythroblast compartment.

In SS patients with genetically determined high HbF levels, the situation is different since HbF plays a protective role and prevents the extensive RBC destruction. The BFU-E compartment retains a structure similar to that of normal individuals, except that 'E' BFU-E are more numerous and the constitutive release of inhibitor by accessory cells held BFU-E quiescent or slowly proliferating. Thus, in HbF SS patients the fine balance between inhibitory and stimulatory factor(s) allows the immediate proliferative response in case of increased peripheral stress. However, in high HbF SS patients in steady state, proliferating CFU-E are responsible for RBC production.

these two subsets of SS patients (fig. 9). The low HbF SS patients have a homogenous population of circulating BFU-E equally responsive to GM-CSF and IL-3. High HbF SS patients, in addition to this subpopulation with equal responsiveness to GM-CSF and IL-3 have a subset of BFU-E that is exclusively dependent on IL-3 which is about 40% of the total number. The circulating BFU-E from high HbF SS patients appeared to respond to the regulatory factors in a similar pattern as normal controls. Epo dependent BPA independent BFU-E were rare in all individuals, which is in agreement with previous reports.

These findings and our previous data[7, 10] allow us to characterize SS patients' circulating BFU-E as follows (fig. 10). The BFU-E of low HbF SS ($<9\%$) patients are an actively proliferating population, which is under control of positive regulatory factors produced constitutively by adherent cells (mostly monocytes) and GM-CSF appeared to be at least one of them. In contrast, circulating BFU-E from high HbF SS patients are under control of a negative regulatory factor produced by adherent cells. In the presence of adherent cells or their product, the BFU-E from high HbF SS patients generated a number of erythroid colonies in the control range, and appeared to be insensitive to ^{3}H-dT kill. Low cycling BFU-E from normal individuals may respond to the stimulatory factor (GM-CSF) present in low HbF SS CM, as low HbF SS BFU-E, and their unstimulated adherent cells do not produce any factor detectable in our bioassay. Hence, both low HbF SS and high HbF SS patients differ from normal, but in distinct ways.

We also confirmed[26] that most of the circulating BFU-E are primitive progenitors, as demonstrated by their GM-CSF and/or IL-3 requirements to generate colonies, and we further demonstrated that the numbers of circulating BFU-E responding to GM-CSF and/or IL-3 vary with the peripheral blood level of HbF. In addition to GM-CSF/IL-3 responsive BFU-E present in low HbF SS patients, these patients have a subpopulation of circulating BFU-E that is exclusively IL-3 dependent.

According to these data, sickle cell anemia may represent a naturally occurring model of unbalanced hemopoiesis leading to the constitutive production of various growth factors (GM-CSF, in low HbF SS patients) which, in turn, may selectively trigger a subset of erythroid progenitors giving rise to mature blood cells.

The present data does not establish if this pattern of BFU-E response is unique to sickle cell anemia, or whether it is also found in other hemolytic anemias. There is a recent report that at least, β-thalassemia does not conform to the circulating BFU-E patterns observed in SS patients[58]. In any case, even if the properties described above are a general response associated with the extent of erythropoietic stress, it represents a real phenomena in SS disease that might be relevant to understanding both the heterogeneity in phenotypic expression and the potential role of therapeutic interventions involving cytokines in this disease.

The challenge for the next stage of this investigation is to determine if this is a property of all erythropoietic stress situations or a specific feature of sickle disorders. It is also important to define the inter-relationship of the cytokines involved and explore potential cytokine manipulations that can ameliorate the phenotype of these patients.

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