To respond or not to respond to hydroxyurea in thalassemia: a matter of stress adaptation?

Antonella Ronchi and Sergio Ottolenghi

Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Milano, Italy

E-mail: antonella.ronchi@unimib.it; sergio.ottolenghi@unimib.it *doi:10.3324/haematol.2013.084392*

In hereditary hemoglobinopathies, such as sickle cell anemia (SCA) and beta-thalassemia (BT), the absolute level of fetal hemoglobin (HbF) production is a critical predictor of severity. High HbF is protective against sickling and vaso-occlusive complications in SCA, and high levels of gamma-globin (the non-alpha globin component of HbF) compensate for beta-globin deficiency in BT, reducing the alpha/non-alpha-globin imbalance.[1]

Several chemical agents have been found to induce high levels of HbF in patients. Among these, hydroxyurea (HU) treatment has gained wide acceptance for the treatment of SCA, and has more recently been evaluated in trials in beta-thalassemia intermedia (BTI).[2-4] In SCA, HU substantially increases HbF, induces erythrocyte macrocytosis, with increased corpuscular hemoglobin and better hydration, and diminishes sickling and hemolysis, leading to overall increased hemoglobin and to transfusion independence. In BTI, HU similarly improves hematologic parameters, leading to transfusion independence. Unfortunately, there are limitations to a wider therapeutic use of HU. In fact, the hematologic response to HU varies among patients. This is linked to dosage (higher dosage being more effective) and correlates with bone marrow toxicity. Variability in toxicity and response among patients means that, in many patients, the threshold for acceptable toxicity may be reached before a favorable hematologic response has been obtained and therapy must thus be discontinued. Differences in pharmacokinetics and pharmacodynamics, as well as in genetic background among patients, have been investigated.[2,3]

Pourfarzad and colleagues[5] took a direct approach to the identification of genes involved in the favorable hematologic response to HU in BTI. Following a trial of HU administration, they selected patients who responded to the treatment (R-patients), achieving transfusion independence, and patients who did not respond (NR-patients). They then expanded erythroid progenitors from these patients *in vitro* for 10-15 days, and hematologic parameters from cultures from R- and NR-patients were compared before and after *in vitro* HU treatment. Finally, they analyzed changes in the transcriptome in these cells, finding important differences between cells from R- *versus* NR-patients. This study, therefore, links intrinsic erythroid biochemical pathways to the response to HU.

HU addition to erythroid cultures from normal individuals, in proliferation conditions, decreased cell proliferation and more than doubled both HbA and HbF levels (HbF levels were raised slightly more), indicating induction of initial differentiation. When the experiment was repeated with cells from patients, NR-cultures showed a much stronger growth inhibition upon HU treatment than R-cells, and greater cell death. The basal total Hb level in R-cells was much higher (approx. 10-fold) than the level in NR-cells. After HU treatment, the final Hb level was approximately 6-fold higher in R-cells than in NR-cells, reflecting the Hb changes observed in patients. Intriguingly, although the HU-induced HbF level was approximately 8-fold higher in R- than in NR-cells, the fold increase of HbF was much more pronounced in NR-cells. Overall, these data indicate that: i) R-cells are more receptive towards better hemoglobinization even before HU administration; ii) they are also more protected from HU-induced cell death. How does this happen? The analysis of the transcriptome identifies approximately 650 genes differentially expressed between untreated R- and NR-cells. In R-cells, gene expression is only moderately affected by HU (with changes in less than 300 genes), whereas in NR-cells almost 2000 genes (including most of those affected in R-cells) show changes in expression. Interestingly, following HU treatment, NR cells tend to resemble R-cells more closely.

Which categories of genes are differentially expressed in R- *versus* NR-cells upon HU treatment? Pourfarzad *et al.* detect important differences in genes linked to proliferation, apoptosis and stress erythropoiesis, but surprisingly little difference in genes related to gamma-globin (i.e. HbF) regulation. The involvement of stress erythropoiesis in the activation of HbF overexpression in adults has a long history. Some 30 years ago it was proposed that conditions forcing regeneration of erythroid marrow, such as recovery from anemia or bone marrow posttransplantion phases, increase HbF production via shortening of differentiation time and/or cell cycle duration of erythroid progenitors.[6,7] This would selectively amplify early maturation stages in which HbF is synthesized, even in adult hematopoiesis. Similarly, HU preferential toxicity for fast-cycling late progenitors would increase, during regeneration, the proportion of erythroid cells derived directly from spared immature progenitors. Later research into mechanisms of *in vitro* induction of HbF by a variety of conditions and drugs (including HU) led to the proposal of a further 'cell stress signaling model', not incompatible with the previous one (reviewed by Mabaera[8]). According to this model, DNA damage, oxidative stress with reactive oxygen species (ROS) generation, and nitric oxide (NO) generation, all converge into the stimulation of the p38 MAPK pathway, leading to the activation of transcription factors (CREB1, ATF2[9,10]) affecting gamma-globin production. Significantly, changes in gene expression detected in R-cells after HU administration involve genes associated with terminal erythroid differentiation, adaptation to stress, prevention/repair of oxidative damage, and protection from stress-induced apoptosis. Hence, the beneficial effects of HU in R-, but not in NR-patients, may depend on the 'optimal' balance between improved differentiation, HbF production and overall hemoglobinization, dependent on HU-induced stress, and simultaneous appropriate protection from concomitant toxic effects of HU. This might partially bypass the inefficient erythropoiesis that is typical of BT.

The gene expression changes detected in this work may

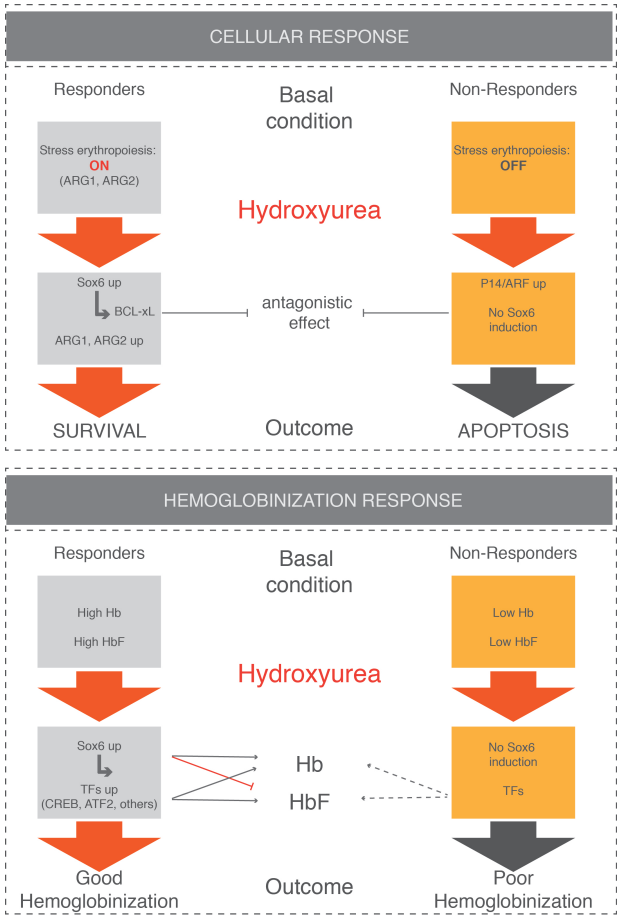

Figure 1. Schematic representation of potential antagonistic mechanisms affecting cellular and hemoglobinizarion responses of R- and NR–cells. Bold arrows: strong effects; dashed arrows: weak effects.

allow complex gene interaction networks to be defined, and these could explain the observed biochemical and biological changes. Some of the observed effects are, at first sight, paradoxical. For example, in normal cells, HU seems to be able to induce all types of hemoglobins, rather than simply HbF. The differential effect on HbF *versus* HbA induction in samples from normal individuals is, in fact, modest as compared to the stronger HbF induction in cells from patients, in particular NR-cells. Presumably, selection for differentiating cells with better alpha/non-alpha globin ratios (hence, cells with increased HbF) might account for this effect. In addition, the fold-increase of HbF in NR-cells, upon HU treatment, is much larger than in R-cells. This may reflect the fact that R-cells already express part of the HU-dependent gene expression program and thus start from a higher baseline. However, interestingly, of the several factors that are known to control gamma-globin regulation (Bcl11a, KLF1, MYB, PRMT5, SOX6), only the last one is deregulated. Loss of function experiments showed that SOX6 is a gamma-globin repressor.[11,12] When over-expressed, however, it stimulates cell differentiation and the expression of all globin genes, although the gamma-globin gene is considerably less activated, presumably due to the specific repressive effect of SOX6.[13] In R-cells, SOX6 is over-expressed, particularly after HU administration. This may contribute to explain the increased propensity to differentiation and hemoglobinization of R-cells, together with the blunted HbF stimulation.

SOX6 overexpression in HU-treated R-cells may also be related to their protection from apoptosis relative to NR-cells. NR-cells (but not R-cells) activate the trigger of mitochondrial apoptosis p14/ARF, the expression of which induces loss of the antiapoptotic protein BCL-xL.[14] Sox6 indeed activates the BCL-xL gene,[12,13] and in agreement with this, Pourfazad *et al.* show that R-cells have higher levels of BCL-xL than NR-cells and that HU is a strong inducer of BCL-xL. Thus, R-cells are protected from apoptosis due both to increased BCL-xL antiapoptotic activity and to the absence of significant induction of p14/ARF (Figure 1).

The dissection of the regulatory circuits of R-cells reported by Pourfarzad *et al.* may provide a rational basis for designing molecules able to activate the stress response and thus to improve hemoglobinization and, in particular, HbF production, while possibly reducing toxicity and other undesirable effects of HU. This may, ideally, make NR-cells responsive to HU as R-cells, widening the therapeutic use of this drug. An interesting therapeutic target could be to blunt the HbF response, possibly due to SOX6 overexpression, as proposed above. Another target could be the differential expression of pro-apoptotic and anti-apoptotic molecules (Ink4A, BCL-xL). A further promising direction for research is represented by association studies in patients to identify genetic elements predisposing to the beneficial HU effects. So far, the best predictor is a high background HbF level which is, in turn, related to Bcl11a polymorphisms or to the Xmn I restriction site polymorphism of the gamma-globin promoter.[3] Other HU-response related polymorphisms have been mapped in the HU-induced Sar gene, controlling protein trafficking, the overexpression of which activates the gamma-globin gene.[15] The availability of a list of genes over-expressed in R-cells offers an opportunity for a search for polymorphisms which might underlie the responder phenotype *in vivo*. Interestingly, the ARG1 and ARG 2 (Arginase 1 and 2) genes, which are strongly activated in R-cells before and after HU administration, were previously associated with HbF increase in response to HU.[3] They act as scavengers of nitric oxide, a by-product of HU treatment and an inducer of apoptosis, as well as a mediator of P38-dependent stress response. Finally, extensive genome wide association studies (GWAS)[16] recently identified 75 genetic loci accounting for part of the variation in the population of red blood cell phenotypes. Some of these genes encode for cell cycle regulators, transcription factors and cell signaling molecules. Combining the information from the analysis presented by Pourfarzad *et al.* with data from GWAS may provide further important information.

Antonella Ronchi and Sergio Ottolenghi are Professors of Genetics at the Università di Milano-Bicocca, Milano, Italy.

Acknowledgements: AR is supported by Fondazione Cariplo, grant n. 2012-0517

Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

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