this could be related to the predominance of adipocytes in the femoral samples. Indeed, it has been shown that VEGF mRNA is upregulated during the conversion of 3T3 preadipocytes to adipocytes.[7] The VEGFR-1, VEGFR-2 and NRP-1 were measured in the same series of samples. VEGFR-1 mRNA levels were quite variable from case to case. VEGFR-1 mRNA was either absent in iliac crest and femoral bone marrow or expressed at the same level in both tissues or expressed only in femoral bone marrow or was expressed at higher level in femoral bone marrow than in iliac crest bone marrow (Figure 1B). Values for VEGFR-2 were available in five samples only: there was no significant difference between femoral and iliac crest marrow (Figure 1C).

VEGFR-2 is essential for the development of hematopoietic stem cells during early embryonic development, it may be redundant in adult bone marrows. Since activation of VEGFR-1 is fully sufficient to rescue hematopoietic stem cell survival *in vitro* and hematopoietic repopulation *in vivo*,[8] the presence of VEGFR-2 may be related to the maintenance of bone marrow vasculature. NRP-1 was expressed at higher level in femoral bone marrow than in iliac crest in each donor (Figure 1D) and it seems to be inversely correlated with the hematopoietic activity. The cellular origin of NRP-1 was assessed on isolated cell populations by a floatation/sedimentation procedure. By contrast to sedimented cells (hematopoietic and stromal cells), high levels of NRP-1 mRNA were detected in the adipocytic population (Figure 2A). This was confirmed by *in situ* hybridization (not shown) and at the protein level by immunohistochemistry (Figure 2B). This is the first report demonstrating neuropilin-1 expression in bone marrow *in vivo*. The capacity of adipocytes to produce NRP-1, previously suspected to play an interactive role with hematopoietic cells[9] suggests that adipocytes may contribute to the regulation of hematopoiesis and/or that NRP-1 may be a novel regulator of adipocyte activity in the bone marrow, possibly as a receptor for VEGF. Although this study does not provide a definitive link between NRP-1, adipocyte function and hematopoiesis, such a relationship may exist and deserves further studies.

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Red Cell Disorders

Decreased plasma endothelin-1 levels in children with sickle cell disease treated with hydroxyurea

Plasma endothelin-1 (ET-1) is elevated in patients with sickle cell disease (SCD). Hydroxyurea (HU) is the only drug with demonstrated clinical efficacy in SCD. Here we show that treatment with HU results in a decreased concentration of circulating ET-1 which is not correlated with the HU-induced increase in HbF level. Blunting of the ET-1 vasoconstrictive stimulus could contribute to the beneficial effects of HU.

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Sickle cell disease (SCD) is characterized by unpredictable painful crises resulting from vaso-occlusion by rigid, sickled red blood cells (RBC). Still, factor(s) initiating vasoocclusive crises (VOC) remain largely unknown. In SCD, the vascular endothelium is chronically activated and expresses various adhesion molecules on its surface. Exacerbation of this activation, in particular within an inflammatory context, is believed to be (one of) the major triggering factor(s) of VOC by promoting the abnormal adhesion of RBC and other circulating cells to the endothelium.[1] The concentration of endothelin-1 is elevated in the plasma of SCD patients, especially during bouts of acute chest syndrome (ACS) and other complications of VOC.[2] Given that it is a powerful vasoconstrictor and pro-inflammatory agonist, ET-1 might be also play an important role in VOC.

Hydroxyurea (HU) significantly reduces the incidence of VOC and ACS, as well as global morbidity and mortality.[3,4] Its initial intended use was to induce fetal hemoglobin (HbF). However, the increment in HbF levels is not constant and it appears that effects of HU are the results of multi-targeted actions.[5] We recently demonstrated that HU down-regulates *ET-1* gene expression by endothelial cells in culture both in basal conditions and after stimulation with pro-inflammatory cytokines.[6] The aim of the

Table 1. Endothelin-1 (ET-1) plasma levels in children with sickle cell disease treated or not with hydroxyurea (HU) compared to levels in healthy AA controls.

	Age range (years)	*ET-1 pg/mL (mean±SEM)*	*Statistical significance*
SS children with clinical events HU-treated (n=8) and untreated (n=10)	2.9–13.2	1.32 ± 0.17	NS
SS children in steady state untreated (n=17)	3.0–14.9	0.65±0.11	NS
SS children in steady state HU-treated (n=16)	3.4–15.1	0.37±0.05*	*p*=0.03
Healthy AA controls (n=26)	2.6–15.8	0.65±0.07	NS

All the children in the HU-treated group had been treated for at least a year. Plasma was prepared and stored at –20°C. ET-1 was measured by ELISA (Quanti Glo, R&D system, Abingdon, UK). Intra- and inter-assay differences were 2.2 and 4.0%, respectively. Statistical analysis was performed with an unpaired t test using GraphPad Prism (Graph Pad Software, San Diego, CA, USA). A difference between groups was considered statistically significant when p< 0.05. NS: not significant.

present study was to test whether this observation *in vitro* had any clinical relevance *in vivo*. To address this issue, ET-1 plasma levels were measured in 80 homozygous SS children, treated or not with HU. These children were recruited from among the 800 SCD children followed at the Sickle Cell Center of the Robert Debré pediatric hospital in Paris. The results are shown in Table 1. Circulating ET-1 levels were not different in SS children in steady state than in an age and race-matched group of healthy AA controls. As previously reported, children with VOC, bacterial or viral acute infection, had indeed significantly higher levels of circulating ET-1, independently of their therapeutic status (HU-treated or not). However, the striking observation was that the levels of circulating ET-1 in children treated with HU were almost two times lower than those in untreated children in a steady state or in controls. Of course this decrease in ET-1 levels might not result directly from the effect of HU on endothelial cells but could simply be the indirect consequence of the HU-induced HbF increase. However, three lines of evidence argue against this single possibility. First, we compared ET-1 and HbF levels in the SS children at steady state, treated or not with HU, and found no correlation between these two parameters. Second, levels of circulating ET-1 were followed over 14 and 16 months from the onset of therapy in 2 children (Figures 1A-1B). In both cases the levels of circulating ET-1 underwent a similar sharp decrease within the first 2 months of treatment and then stabilized. In the first child, HbF increased steadily during the whole follow-up period from 11.9% to 14.5% at 2 months, and to 20.8% at 14 months (Figure 1A). However, in the second child, HbF had not changed from its initial 2.7% value after 2 months when ET-1 had already dropped by 60%; it then increased to 5.1% at 4 months and plateaued around this value. Thus, ET-1 level does not always parallel the HU-induced changes in HbF level. Finally, to evaluate the potential relationship between circulating ET-1 and HbF further, we measured these two parameters in SS children aged from 1.2 months to 2 years, i.e. the period of the physiological

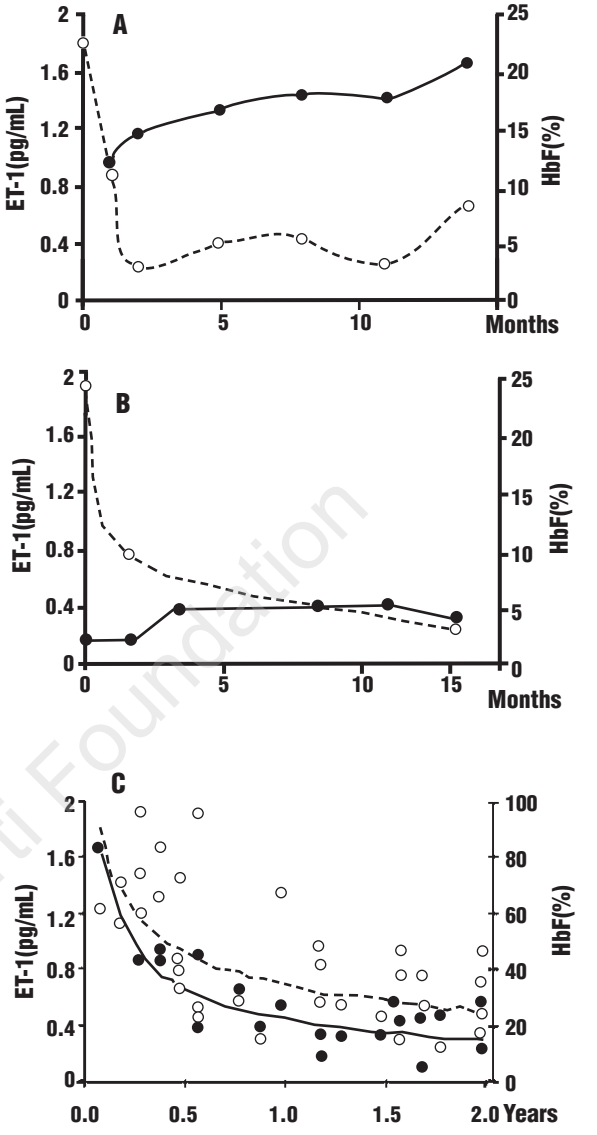

Figure 1. Comparison of HbF and plasma ET-1 levels. A and B: longitudinal follow-up of two 8-year old SS girls for 14 and 16 months, after the onset of HU treatment. ET-1 plasma levels (○ open circles; dashed line) and HbF levels (●full circles; full line). C: ET-1 plasma levels (○ dashed line) and HbF levels (● full line) in 29 SS infants aged from 1.2 months to 2 years.

neonatal decrease of HbF (Figure 1C). Unexpectedly, levels of circulating ET-1 decreased with age from 1.90 pg/mL to the normal 0.65 pg/mL value[2] in 2 years. Although this result is surprising as the opposite might have been expected, i.e. an increase in ET-1 concurrent with the HbF decrease and the onset of the disease, it does show the absence of a relation between circulating ET-1 and HbF levels. No data have been reported regarding neonatal changes in circulating ET-1 in SCD or healthy children and, at this stage, the significance of our observation remains unclear.

Whatever the mechanism, treatment with HU is accom-

panied by a reduction in circulating ET-1 and, as a consequence, by the blunting of a vasoconstrictive stimulus which could partly account for the beneficial effects of HU. The decrease of circulating ET-1 is consistent with the HU-induced decreased expression of the *ET-1* gene in endothelial cells in culture.[6] However, it has also been shown that the number of pro-adhesive RBC[7,8] and the expression of adhesion molecules on lymphocytes, monocytes and neutrophils[9,10] are decreased in patients treated with HU. Thus, it is probable that all these pleiotropic effects of HU concur to reduce the aggressiveness of circulating cells towards the endothelium, and thereby ET-1 production, thus conferring the clinical benefits.

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Molecular Hematopoiesis

Changes in expression of *WT1* isoforms during induced differentiation of the NB4 cell line

The levels of expression of *WT1* gene and WT1+17AA isoforms rapidly decreased during the differentiation of NB4 cells induced by all-trans retinoic acid; this decrease was conversely related to the dynamic changes of CD11b positive cells, indicating that the abnormally high expression of *WT1* gene and WT1+17AA isoforms was associated with a block of NB4 cell differentiation.

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The Wilms' tumor gene (*WT1*) produces four major distinct isoforms due to two alternative splicing events within this gene. The first alternative splice corresponding to exon 5 causes the presence or absence of a 17-amino acid insertion between the trans-regulatory and ZF domain while a second splice results in gain or loss of a 9 bp insertion encoding 3 amino acids (KTS) between the third and fourth ZF DNA-binding domain; this produces four distinct isoforms designated as -17AA/-KTS, +17AA/-KTS, -17AA/+KTS and +17AA/+KTS.[1] WT1 isoforms are proposed to have distinct functions.[2] The changes in the ratio of these four isoforms in cells is thus thought to render different phenotypes.

In this study, a real-time quantitative reverse transcription polymerase chain reaction (RQ-RT-PCR) method was established for detecting the expression levels of *WT1* gene, WT1+17AA isoforms and *GAPDH* in NB4 cells induced by all-trans retinoic acid (ATRA 0.5 uM) using LightCycler. RNA extraction, cDNA conversion, standard preparation for RQ-RT-PCR, and the composition and condition of the PCR reaction mixture were as described previously.[3] All primers and the TaqMan probe were designed by Primer Priemer software (version 5.0) and their positions referred to the *WT1* sequence are shown in Figure 1. Detailed sequences of the sense (SP1), antisense primers (AP1) and fluorescent probe of total *WT1* were

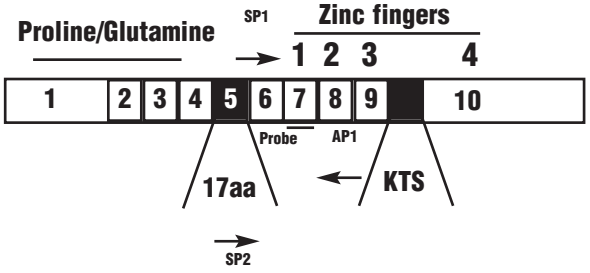

Figure 1. Position of the primers and probe of total *WT1* gene and WT1+17AA isoforms. SP1: sense primer of total *WT1* gene, located on exon 6; AP1: antisense primer of total *WT1* gene, located on exon 7; SP2: sense primer of WT1+17AA isoforms located on exon 5; the probe was designed to hybridize at the sense strand of exon 6/7.