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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Key words: sickle cell disease, hydroxyurea, endothelin-1.

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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

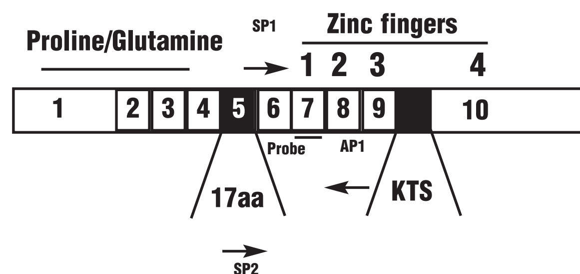

Figure 1. Position of the primers and probe of total *WT1* gene and WT1+17AA isoforms. SP1: sense primer of total *WT1* gene, located on exon 6; AP1: antisense primer of total *WT1* gene, located on exon 7; SP2: sense primer of WT1+17AA isoforms located on exon 5; the probe was designed to hybridize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

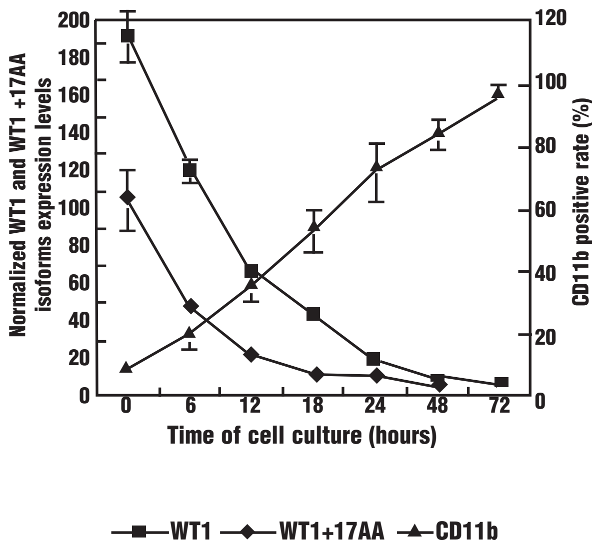

Figure 2. A comparison of the time courses of *WT1* and WT1+17AA isoforms expression and CD11b positive rates during the ATRA-induced differentiation of NB4 cells. The red square, blue prism and yellow triangle represent $WT1_N$, $WT1_N$ +17AA and CD11b expression level respectively. The expression levels of *WT1* gene and WT1+17AA isoforms rapidly decrease during the dffer-entiation of NB4 cells induced by ATRA; in contrast, the rate of CD11b expression increases gradually.

shown in the reference.[3] The sense primer specific for WT1+17AA isoforms (referred to as SP2: 5'-AAT GGA CAG AAG GGC AGA GC -3') was located on exon 5 and the corresponding antisense primer and probe were the same as for total *WT1*. The PCR product size for WT1+17AA was 169 bp overlapping the 86 bp total *WT1* product. All sample analyses were performed in duplicate. Normalized *WT1* expression level ($WT1_N$) was determined as the ratio of *WT1* to *GAPDH*$\times 10^4$, as was the WT1+17AA isoforms ($WT1_N$+17AA). The expression of CD11b was simultaneously determined with a Becton Dickinson FACSCalibur flow cytometry. The NBT reduction test was also performed. Statistical analysis of oneway ANOVA and coefficient correlations were performed with SPSS software (version 10.0). p values <0.05 were considered to be statistically significant. The NBT reduction rates of NB4 cells prior to and 24, 48 and 72 hours after exposure to ATRA were 8.1%±2.0%, 53.7%±9.2%, 72.8%±14.7% and 90.2%±14.2%, respectively, with a statistically significant increment (p<0.05) for each time point. However no statistical changes of NBT reduction rates were found before 24 hours of exposure to ATRA. Figure 2 shows that the CD11b positive rates increased statistically as the cells were exposed to ATRA from time to time for longer culture (p<0.05) with a rapid decrease of the expression level of both *WT1* and WT1+17AA isoforms. The $WT1_N$ level was 191.11, 121.17, 66.72, 43.47, 18.29, 4.04 and 3.79 respectively, prior to and 6, 12, 18, 24, 48 and 72 hours after exposure to ATRA. The decrease became visible 6 hours after exposure to ATRA, and there was 10-fold decrease (191.11 to 18.29) by 24 hours of induction and an almost 50-fold decrease (191.11 to 4.04) at 48 hours after induction. Simultaneously, the $WT1_N$+17AA level was 105.12, 46.89, 20.50, 10.38, 8.85, 2.16 and 1.92, respectively, at the above time points with a 10-fold (105.12 to 10.38) decrease at 24 hours and a 50-fold (105.12 to 2.16) decrease at 48 hours. Both *WT1* and WT1+17AA expression changed inversely to the dynamic changes of CD11b positive rates ($\gamma 1$=-0.65, p<0.01; $\gamma 2$=-0.77, p<0.01. *WT1* gene is overexpressed in almost all leukemic blasts and many leukemic cell lines,[4] whereas normal blood cells and $CD34^+$ hematopoietic progenitors have been found to express *WT1* at a far lower level[5] or not at all.[6] Several large series of leukemia cell lines (e.g. the K562 cell line and HL-60 cell line) have demonstrated a statistically significant decrease of *WT1* expression level during induced differentiation, indicating that *WT1* expression may functionally block blood cell differentiation.[7] Here our results showed that the levels of expression of *WT1* gene and WT1+17AA isoforms rapidly decrease during the differentiation of NB4 cells induced by ATRA, which is in accordance with our previous competitive RT-PCR results.[8] During the induced differentiation of NB4 cells, the events of down-regulated *WT1* gene expression and up-regulated CD11b antigen expression occurred much earlier than the reduction of NBT rate, suggesting that *WT1* down-regulation may be a relatively early event which blocks NB4 cell differentiation. Which of the four isoforms contributes predominantly to blockade of NB4 leukemic cell differentiation needs to be further investigated.

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Key words: WT1 expression, WT1 isoforms, ATRA, differentiation.

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Chronic Myeloproliferative Disorders

Expression of polycythemia rubra vera-1 decreases the dependency of cells on growth factors for proliferation

An increase in the level of polycythemia rubra vera -1 (PRV-1) mRNA has been reported in some myeloproliferative disorders. We have studied the effects of PRV-1 on cell proliferation and cell survival. In cell growth assays, the number of heterologous cells expressing PRV-1 increased faster than sham-transfected cells, a difference that was more pronounced in serum-free media. Even after 5 days of exposure to serum-free media, cells expressing PRV-1 continued to proliferate, whereas the control cells ceased to proliferate. We conclude that PRV-1 is a pro-proliferation molecule, and hypothesize that its overexpression may have a role in the pathogenesis of myeloproliferative disorders.

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Polycythemia rubra vera-1 (PRV-1, also known as CD177 or NB1) is a member of Ly-6/uPAR protein family, whose members have been shown to play a role in cell proliferation.[1] In normal subjects, PRV-1 is expressed on a subpopulation of neutrophils, on myelocytes and on metamyelocytes. An increase in the level of mRNA encoding PRV-1 has been reported in neutrophils from patients with polycythemia vera (PV) and essential thrombocythemia (ET);[2-6] however, the function of PRV-1 is unknown. We have conducted *in vitro* studies investigating the function of PRV-1 in a heterologous cell line stably expressing this molecule. After transfecting either PRV-1 cDNA or empty plasmid to Chinese hamster ovary (CHO) cells, we established a stable CHO cell line expressing PRV-1 (CHO-PRV-1) and a control cell line (CHO-Sham). Flow cytometry studies with MEM-166, a monoclonal antibody to CD177 (BD Pharmingen, San Diego, CA, USA), showed that CHO-PRV-1 cells, but not CHO-Sham cells, expressed PRV-1 (*data not shown*). In the cell growth assay, the number of CHO-PRV-1 cells increased faster than CHO-Sham cells in the presence of 10% fetal bovine serum (FBS, Sigma-Aldrich) in the growth media (Figure 1A).

The difference in the number of cells was statistically significant at each time-point, except at 0 and 24 hr, and persisted throughout the 5-day follow-up. This difference in growth rates between CHO-PRV-1 and CHO-Sham cells was further increased when the cells were grown in the absence of FBS. The growth curves diverged further with time, and the difference in the cell number was most prominent on the fifth day (Figure 1B).

We studied cell proliferation by measuring the percentage of bromodeoxyuridine (BrdU)-incorporating cells. On the first day after exposure to serum-free media, the percentage of BrdU (+) cells was similar between CHO-PRV-1 and CHO-Sham cells; however, starting on the 4th day, there was a significantly higher percentage of proliferating cells among CHO-PRV-1 cells than among control cells (8.8±0.8% vs 0.8±0.1%, respectively; p=0.02) (Figure 2A). Additionally, we compared the rate of apoptosis and necrosis between the two cell lines by measuring binding of annexin V and incorporation of SYTOX green stain, using the Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR, USA). We found that although apoptosis was not different between CHO-PRV-1 and CHO-sham cells (Figure 2B), the percentage of necrotic cells was higher in sham-transfected cells than in PRV-1-expressing cells (Figure 2C).

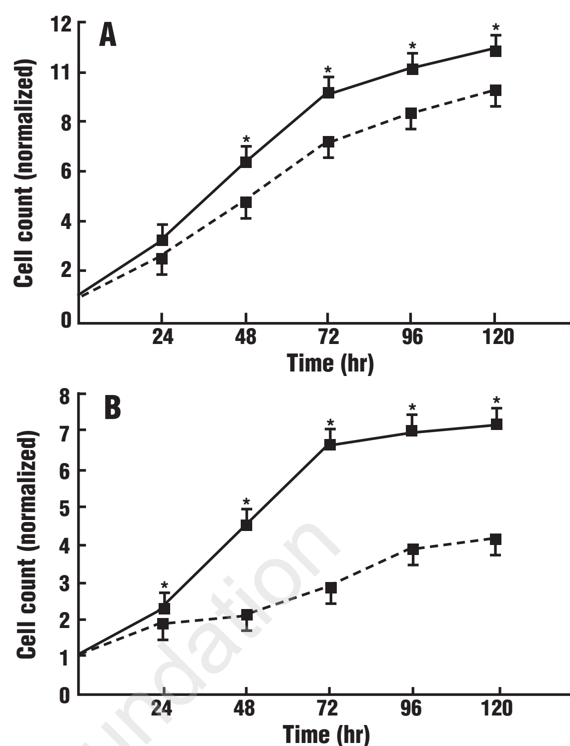

Figure 1. Cell growth and expression of PRV-1: 1×10^9 CHO-PRV-1 (-■-) or CHO-Sham cells (--■--) were seeded in 6–well plates. Four to 6 hours after seeding (time 0), growth media were replaced with DMEM (Invitrogen) with either 10% FBS (a) or no FBS (b). At 24-hour intervals, cells were detached from the plates and counted with a hemocytometer. The figure summarizes the results of 3 separate experiments. The number of cells at any time point was normalized to the initial cell count and is shown on the Y-axis (Cell count at each time-point/Cell count at 0 time-point). *Shows statistically significant differences in the cell counts ($p<0.05$, Student's t-test).

There are clinical observations that support a pro-proliferative role for PRV-1. Clinical settings that are associated with elevated PRV-1 expression, such as administration of granulocyte colony-stimulating factor, sepsis, pregnancy, and polycythemia vera are also known to be associated with higher neutrophil counts.[7-9]

Another finding of our studies is that expression of PRV-1 reduces the dependency of cell proliferation on growth factors present in serum. Interestingly, in a recent study, only progenitor cells of ET patients whose neutrophils overexpressed PRV-1 were capable of forming *in vitro* colonies in the absence of erythropoietin.[10] Several important questions on the expression of PRV-1 are unanswered. Do early hematopoietic progenitor cells

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