ELSEVIER

Blood 142 (2023) 3854

The 65th ASH Annual Meeting Abstracts

POSTER ABSTRACTS

113.SICKLE CELL DISEASE, SICKLE CELL TRAIT AND OTHER HEMOGLOBINOPATHIES, EXCLUDING THALASSEMIAS: BASIC AND TRANSLATIONAL

Distinctive Roles of Nitric Oxide Synthase Isoforms in Hydroxyurea-Mediated Cytostasis of Erythroid Progenitors

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Chemotherapeutic hydroxyurea (HU) is used in the therapy of sickle cell anemia, thalassemia and myeloproliferative neoplasms according to its stimulation of fetal hemoglobin and myelosuppression. We reported that most nitric oxide (NO) in mammals is produced by the reduction of nitrate and nitrite. The NO properties of HU have been widely explored, while its enzymatic NO synthase (NOS) dependence is not completely defined. We have already demonstrated concomitant inhibition of erythroid cell proliferation by HU and NO donors. In this study, we investigated the NOS-mediation in HU regulation of proliferation, differentiation and cell cycle in HEL92.1.7 human erythroleukemic cell line and / or erythroid progenitors of Nos2- or Nos3-null mice.

HU induced a dose-dependent increase in NOS2 and NOS3 protein levels and their enzymatic activity by increasing production of NO and citrulline in erythroleukemic cells. Inhibition of the NOS enzymes by pan-specific L-NAME, NOS2 inhibitor 1400W, or NOS2/NOS3 inhibitor DPI was sufficient to prevent the HU-mediated decrease in HEL92.1.7 proliferation assessed by Ki67 immunofluorescence. HU increased the frequency of cells in S-phase (66.58±2.44 vs. 55.18±1,06%) of the cycle at the expense of G0/G1 (17.07±1.67 vs. 28.55±1.01%) due to blocked DNA synthesis. The combined treatment of HU with 10μM DPI decreased the number of cells in G0/G1 (3.34±0.98 vs. 17.07±1.67%) and increased in S (72.3±0.96 vs. 66.58±%) and G2/M (24.36±0.19 vs. 16.35±2.28%) compared with HU alone. Moreover, NOS3 knock-down by shRNA decreased the percentage of cells in S phase from 69,19±2.68 to 34.23±1.07% ($p<0.001$) and increased in G2/M from 15.62±2.17 to 34.71±3.76 ($p<0.001$). HU-induced apoptosis was prevented by NOS2 (1400W) inhibition, determined by annexin V, decreasing the number of cells in early and late apoptosis in erythroleukemic cells. In accordance, NOS3 knock-down was not able to rescue HU-induced apoptosis. Furthermore, by performing *in vitro* enzymatic assay we showed that regulation of NOS2 activity seems to be achieved by direct interaction with HU leading to a dose- and time-dependent increase in NO and citrulline products.

HU increased the expression of Nos1/2 isoforms by 20%, and Nos3 by 10% in mouse erythroid progenitors (mERP) of wild-type mice. Bone marrow cells isolated from Nos2- or Nos3-null mice treated orally with HU showed decreased protein nitrosylation in bone marrow compared to HU-treated wild-type mice. In *ex vivo* colony formation assay, both Nos2-/- and Nos3-/- bone marrow cells formed significantly more late erythroid colonies (CFU-E, 34- and 24-fold, respectively, $p<0.001$), and Nos2-/- also yielded more of the early erythroid (BFU-E, 9- and 7-fold, $p<0.01$) and granulocyte/macrophage (CFU-GM, 14- and 15-fold, $p<0.01$) colonies compared to wild-type mice untreated or HU-treated. HU treatment of mERP, of Nos2-/- and Nos3-/- origin, demonstrated decreased frequency of S-phase cells to levels comparable to untreated wild-type mice. Nos2 and Nos3 deficiency was able to rescue Caspase-3 expression in the presence of HU in mERP. Nevertheless, HU increased the quantity of late apoptotic cells in both Nos2 and Nos3 deficient mice.

Our results demonstrated that HU induces the enzymatic activity of NOS proteins that mediate the cytostatic effect. NOS2/3 enzymes are involved in HU-inhibition of proliferation of human erythroleukemic cells and ex vivo differentiation of mERP. The observed NOS enzymes were fully and partially involved in HU-induced apoptosis of HEL92.1.7 cells and mERP, respectively. Uncovering all aspects of HU mechanism of action may help to increase the responsiveness or reduce the negative effects of cytotoxic chemotherapy.

Disclosures No relevant conflicts of interest to declare.

https://doi.org/10.1182/blood-2023-178579