Granulocytes

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HEMATOPOIETIC-SPECIFIC LYN-SUBSTRATE 1 (HCLS) IS DEACETYLATED BY NAMPT/NAD+/SIRT1 PATHWAY

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Background: Recently we identified HCLS1 protein to be an essential player in the G-CSFR signalling pathway in myeloid cells (Skokowa et al., 2012). We demonstrated that upon G-CSF stimulation, phosphorylated and activated HCLS1 together with its binding partner HAX1 binds to LEF-1 transcription factor leading to LEF-1 activation and subsequently myeloid differentiation. In patients with severe congenital neutropenia (CN) harbouring HAX1 mutations, HCLS1 expression and functions are severely downregulated, leading to "maturation arrest" of myelopoiesis. We also described a new G-CSFR signalling through activation of Nicotinamide phosphoribosyltransferase (NAMPT)/NAD^+/Sirtuins in healthy individuals and in CN patients. We found that G-CSF-triggered deacetylation of myeloid specific factors is essential for myeloid differentiation (Skokowa *et al*., 2009).
Aims: In the present study we aimed to investigate whether HCLS1 is regulated by NAMPT/NAD^+/Sirtuin pathway via deacetylation.
Methods: We identified four acetyl-lysine sites in HCLS1 protein and generated rabbit polyclonal antibodies specifically recognized each of acetylated lysines for western blot. We also generated lentiviral constructs contained HCLS1 cDNA in which one of acetylated lysines of HCLS1 protein is replaced by a single amino acid.
Results: Using immunoprecipitation with anti-acetyl-lysine antibody we found that HCLS1 is acetylated in HL60 myeloid cells upon pre-treatment of cells with histone deacetylase inhibitors. We also showed that treatment of HL60 and NB4 cells with high doses of Nicotinamide but not Trichostatin A enhanced HCLS1 acetylation on lysine 123 and 241, suggesting that class III histone deacetylases (Sirtuin family) could deacetylate HCLS1 protein.
We further found that NAD^+ and NAMPT treatment of the acute myeloid leukemia cell line HL60 decreased HCLS1 acetylation on both lysines 123 and 241. Similar effects were observed in HL60 cells transduced with lentiviral construct expressing NAMPT cDNA. Inhibition of NAMPT using specific inhibitor FK866 increased HCLS1 acetylation on acetyl-lysines 123 and 241 in NB4 and on acetyl-lysine 241 in HL60 cells. To evaluate the mechanism of NAMPT-dependent deacetylation of HCLS1, we performed immunoprecipitation experiments in cell lysates of HL60 cells using anti-SIRT1 antibody and found interaction between endogenous HCLS1 and SIRT1 proteins. Moreover, interaction between HCLS1 and SIRT1 proteins were detected by immunoprecipitation in 293T cells over-expressing HCLS1 and SIRT1.
Summary / Conclusion: Taken together, we concluded that HCLS1 is deacetylated through NAMPT/NAD^+/SIRT1 pathway and that in patients with severe congenital neutropenia not only diminished expression and phosphorylation but also elevated NAMPT-triggered deacetylation may contribute to the neutropenic phenotype.

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PRESENCE OF PERIPHERAL BLOOD CELLS WITH PNH PHENOTYPE IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Background: Chronic idiopathic neutropenia (CIN) is a disorder of granulopoiesis characterized by increased apoptosis of the granulocytic progenitor cells and presence of activated, oligoclonal T-lymphocytes with myelosuppressive properties in the peripheral blood (PB) and bone marrow (BM). Based on the existing evidence, we have suggested that CIN represents the mild form of the spectrum of immune-mediated BM failure syndromes.
Aims: To probe the hypothesis that similar to other BM failure syndromes, i.e. aplastic anaemia and myelodysplatic syndromes, CIN patients display PB cells with PNH phenotype.
Methods: We have studied 102 patients fulfilling the diagnostic criteria for CIN and 43 age- and sex-matched healthy individuals. All patients had absolute neutrophi counts lower than 1800/μl (White) or 1500/μl /ml (Black) for more than three months, had no evidence of any underlying disease that might be associated with neutropenia, no history of exposure to irradiation, use of chemical compounds or intake of drugs to which neutropenia might be ascribed, normal BM karyotype and negative anti-neutrophil antibody activity. Flow cytometry was performed for the identification of peripheral blood erythrocytes, granulocytes and monocytes displaying PNH phenotype. Specifically, the CD59dim (type II) and CD59negative (type III) PNH red cells were evaluated within the Glycophorin A (CD235) positive cell fraction. The FLAER negative PNH granulocytes and monocytes were assessed within the CD24 and CD14 cell fraction, respectively.
Results: The proportion of type II and type III PNH red cells were significantly increased in CIN patients (0.0587±0.337 and 0.0282±0.094, respectively) compared to healthy individuals (0.0064± 0.0067 and 0.0030±0.0021, respectively) (P<0.0001 and P=0.0088, respectively). Similarly, the proportion of FLAER negative (PNH phenotype) monocytes were significantly increased in CIN patients (1.47±1.795) compared to healthy controls (0.1651±0.173) (P<0.0001). No statistically significant difference was identified between patients and controls in the proportion of PNH granulocytes .
Summary / Conclusion: Patients with CIN display increased proportion of PNH red cells and monocytes in the PB. These data support further the concept that CIN shares common pathogenetic features with immune-mediated BM failure syndromes.

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RISK OF TRANSFORMATION TO MYELODYSPLASIA/LEUKAEMIA AND SEPSIS/DEATH IN PATIENTS AFFECTED WITH SEVERE CONGENITAL NEUTROPENIA:DATA FROM THE ITALIAN NEUTROPENIA REGISTRY (INR)

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Background: Severe Congenital neutropenia (SCN) is a disease characterized by persistent absolute neutrophil count below 500/mmc, early onset of bacterial infections and block of maturation of neutrophils in the bone marrow. SCN is a premalignant disorder. Granulokine-colony-stimulating factor (G-CSF) has radically changed the prognosis of these patients conferring defence against infections, but it probably favours the process of transformation to myelodysplasia (MDS)/leukaemia (AL). The cumulative risk of sepsis/death and transformation to MDS/AL is different according to the cohort studied (Severe Congenital Neutropenia International Registry-SCNIR, French registry and the Swedish Cohort)
Aims: To describe the cumulative incidence of MDS/AL and sepsis causing death in the cohort of SCN patients enrolled in the INR.
Methods: Anagraphical and historical data (date of birth, gender, date of emergence of neutropenia, date of diagnosis, date of last follow up) as well as data on bone marrow morphology, type of molecular mutation, presence of G-CSF mutation, G-CSF dose, number and type of infections, cause of death if any, were extracted from the INR.
Results: From June 2003 to December 2012, among 306 registered in INR, 22 patients were diagnosed with SCN and considered eligible for the study. Nine out of 22 (41%) were females and 13/22 (59%) were males; median aged, at last follow up, 6,3 years (range 0,5-40,2). The patients were diagnosed with SCN at a median age of 9 months (range 0-418). All subjects had the maturative arrest at promyelocyte or myelocyte stage. In the cohort, 16/22 (72%) carried the ELANE mutation, 2/22 (9%) were HAX1 mutated and the remaining 4/22 (18%) had no known mutation. All subjects were treated with G-CSF started at a median age of 9 months (range 0,5-418). Median G-CSF dose exposure was ≤5 mcg/kg/day in 10/21(48%), 6-10 mcg/kg/day in 5/21 (24 %) , 11-19 mcg/kg/day in 3/21 (14%) and ≥20 mcg/kg/day in the remaining 3/21 (14 %). In the cohort none developed AL, while one patient/22 (4%) progressed to myelodysplasia after 4 years of G-CSF treatment at dose of 5 mcg/kg/day. Four out of 22 pts (18%) died , 2 patients for sepsis during follow-up and the other 2 for transplant related causes (infection and acute GVHD). The two sepsis/death events before transplantation were related to absent compliance to G-CSF. The cumulative incidence of MDS/AL trasformation was of 6% at 4 years after the beginning of G-CSF and the probability to develop MDS was 8% at 8 years of life. The cumulative risk of sepsis causing death was 10% after 3 years after the beginning of G-CSF ; the risk to die for sepsis was calculated as 10% at 3,5 years of life.
Summary / Conclusion: The cumulative incidence of MDS in our cohort is lower than the one calculated in the Swedish, French and in the SCNIR cohort mainly after 10-15 years of follow up. This might be due to younger and smaller Italian cohort if compared at least with the french and the SCNIR ones. The incidence of sepsis/death is almost comparable with the French and the SCNIR cohort. The explanation of the deaths might be caused mainly to

low/absent compliance to G-CSF therapy. Early diagnosis, amelioration of compliance to therapy and very strict monitoring of clinical status and bone marrow features might reduce these complications in SCN population.

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ACETYLATION OF LEF-1 TRANSCRIPTION FACTOR REGULATING HEMATOPOIETIC DIFFERENTIATION

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Results: Previously we identified that NAMPT is essential for the G-CSF –triggered granulocytic differentiation via a NAD^+- sirtuin-1-dependent pathway. Sirtuins are NAD+-dependent protein deacetylases and we found that myeloid-specific transcription factors C/EBPaand C/EBPß are deacetylated and activated by SIRT1. We and others demonstrated, that lymphoid enhancer-binding factor 1 (LEF-1) transcription factor is crucial for neutrophil granulocytopoiesis and lymphopoiesis. LEF-1 mediates the proliferation, survival and differentiation of granulocyte and lymphocyte progenitor cells by binding and activation of cell type-specific transcription factors. LEF-1 expression was severely diminished in myeloid progenitor cells of severe congenital neutropenia patients, who show "maturation arrest" of granulopoiesis. NAMPT, NAD+ and SIRT1 levels were elevated in myeloid cells and in plasma of severe congenital neutropenia patients, demonstrating hyperactivation of this pathway. To evaluate the mechanism of NAMPT-triggered granulocytic differentiation, we analysed whether LEF-1 could be de-/acetylated. Immunoprecipitation with acetylated-Lysine Ab in Jurkat cells showed acetylated form of LEF-1 protein. Moreover, by means of immunoprecipitation with SIRT1 antibody we found interaction between LEF-1 and SIRT1. We further identified lysine residues in LEF-1 protein, which could be acetylated and generated specific rabbit polyclonal antibody specifically recognized acetyl-Lys of the LEF-1 protein. Western blot experiments using these antibody confirmed acetylation of LEF-1 protein on the particular lysine residue. To study the involvement of NAMPT in the deacetylation of LEF-1 protein we treated Jurkat cells with human recombinant NAMPT for 24 hr. Indeed, we found significantly decreased levels of acetylated LEF-1 protein and elevated mRNA expression of LEF-1 after treatment with NAMPT. At the same time, inhibition of NAMPT using specificinhibitor FK866 resulted in increased levels of acetylated LEF-1 protein after 24 hr of FK866 treatment. This was in line with elevated mRNA expression levels of LEF1 (2 folds) and its target genes Survivin (3 folds), c-myc (2 folds), HCLS1 (1,5 folds) and Cyclin D1 (1,5 folds) after 48 hours of FK866 treatment. Further we analysed whether NAMPT effects on LEF-1 trans-activating functions are through direct acetylation of LEF1 protein itself. We made LEF-1 mutant with a single amino acid substitution, which removes lysine acetylation site in the LEF1 protein and performed reporter gene assay using TOP promoter construct. TOP promoter construct contains six LEF-1/TCFs binding sites and luciferase promoter. We found that if acetylation is removed, LEF-1 protein activated TOP promoter 17- 26 fold stronger, as compared to WT LEF-1. This data shows that LEF-1 binding and activation at LEF-1/TCFs binding sites of regulatory DNA is due to direct changes of acetylation of the LEF1 protein itself. Hence, deacetylation of LEF-1 might be crucial process in the mechanism of NAMPT-triggered granulocytic differentiation and might be an important part of the pathomechanism of neutropenia. In conclusion, in patients with congenital neutropenia, LEF-1 is severely downregulated and the remaining LEF-1 protein is deacetylated. LEF-1 is therefore physically and functionally missing.

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THYROID DISEASE-ASSOCIATED NEUTROPENIA: FREQUENCY, CLINICAL, LABORATORY AND IMMUNOPHENOTYPICAL FINDINGS

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Background: Granulopoiesis abnormalities have been described in association with thyroid disease. However data regarding systematic evaluation of adult neutropenia and concurrent or prior thyroid disease are scarce.
Aims: To investigate the frequency of thyroid disorders among patients presenting with neutropenia, to evaluate possible differences in peripheral lymphocyte subsets in neutropenic patients with thyroid diseases, as well as their immunological profile.
Methods: We retrospectively analyzed the clinical and laboratory findings of 218 consecutive patients who presented with neutropenia to the outpatient Hematology Clinic of our Department between 2005 and 2008. Laboratory evaluation included CBC, blood smear, chemistries, folate, vitamin B12 and iron status, serology for HBV, HCV, HIV, EBV and toxoplasmosis, ANA, anti-DNA Abs, RF, complement factors C3 and C4, thyroid function tests (FT3, FT4, TSH, anti-TPO Abs, anti-TG Abs, TRAbs), and antineutrophil antibodies (anti-PMN Abs) by GIFT, GAT and LIFT methodology. Furthermore immunophenotype of blood lymphocytes was performed by three-color flow cytometry.
Results: Among 218 patients with neutropenia, 95 had thyroid disease (43.5%), 65 chronic immunologic neutropenia, 33 T-large granular lymphocytosis, 11 autoimmune disorders and 14 other diagnoses. Patients with thyroid disorders had an increased frequency of recurrent infections compared to other patients. (P=0.04). The following significant correlations were found: a. negative correlation between FT3 levels and absolute neutrophil counts (ANC) (r=-0.274, P=0.007), b. negative correlation between T4 levels and absolute CD4 counts (r=-0.274, P=0.02), c. positive correlation between TSH levels and absolute CD4 counts (r=-0.16, P=0.05), d: negative correlations between TPO-Abs and TG-Abs levels and C4 levels (r=0.16, P=0.05; r=0.266, P=0.001). In addition CD2+ and CD4+ lymphocyte subsets were significantly higher in patients with thyroid disease compared to patients with normal thyroid function (P=0.05, P=0.002). Among the 95 neutropenic patients with thyroid disease, 51 had Hashimoto thyroiditis (HT, 23.4%), 9 Graves' disease (GD, 4.1%), 6 total thyreoidectomy associated with nodules (TTM, 2.8%), 18 non-toxic multinodular goiter (NTMG, 8.2%) and 11 hypothyroidism (HP, 5%). Patients with autoimmune thyroid disease had an increased frequency of concomitant autoimmune cutaneous or systemic manifestations (P=0.03). Among the various thyroid disorders there was a statistically significant difference in the distribution of CD20+ lymphocytes (P=0.038): HT patients had an increase in the percentage of B-lymphocytes, while the opposite was evident for the TTM-subgroup. In patients with GD an increase of the proportion of NK cells and a decrease in the percentage of TCRγδ+ lymphocytes were noted (P=0.05, P=0.005). Patients with NTMG had significantly higher ANC (P=0.004) compared to other thyroidopathies. Anti-PMN Abs were found in 37.2% of thyroid disorders. A positive correlation between anti-PMN Abs titers and anti-TPO levels was evident (P<0.05).
Summary / Conclusion: The frequency of thyroid disease among neutropenic patients may be higher than previously reported. The existence of anti-PMN antibodies, as well as the different distribution of lymphocyte subsets among patients with different thyroid diseases indicates both humoral and cellular mechanisms in the pathophysiology of thyroid disease-associated neutropenia.