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THE SALVAGE TREATMENT WITH CYCLOSPORIN A IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Backgrounds. Physicians face therapeutic dilemas when patients became resistant to known treatment in life-threatening conditions. A review of the literature shows a lack of comprehensive information on the clinical use of Cyclosporin A in the treatment of idiopathic thrombocytopenic purpura (ITP). *Aims.* To verify the usefulness of Cyclosporin A therapy in refractory ITP. *Method*. Study was carried out on long-term treatment (median 36 months) with Cyclosporin A (3 mg/Kg/day) in 14 selected adult patients with chronic, severe ITP resistant to all the usual therapies. *Results.* A median follow-up of 26,2 months shows that Cyclosporin A treatment obtained an improvement in 10 out of 14 patients (71%): 9 patients (64%) achieved a complete response and one (7%) a partial response. Four patients (28%) were unresponsive. In 5 patients (50%) the discontinuation of Cyclosporin A was successful, maintaining the remission over time, without further therapy. In the remaining responsive patients, the remission was dependent on continued of drug. The Cyclosporin A intolerance was slight in spite of long-term treatment and no nefrotoxicity occured. Conclusions. Our study shows the safety and efficacy of Cyclosporin A therapy in resistant ITP. Because the potential role in second neoplasia appearance and the well known teratogenic role of this immunosuppressor, cyclosporin A will be done only in resistant ITP cases (dramatic clinical cases).

Myelodysplastic syndromes II

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EFFECT OF CA2 ANTI-TUMOR NECROSIS FACTOR (TNF) α ANTIBODY THERAPY ON HAEMOPOIESIS OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Backgrounds. TNFα plays a prominent role in the pathophysiology of myelodysplastic syndromes (MDS) by inducing apoptotic death of bone marrow (BM) haemopoietic cells directly and/or indirectly by upregulating Fas antigen expression. *Aims.* To explore the biological and immunoregulatory effect of the treatment with the anti-TNFα monoclonal antibody cA2 on BM progenitor/precursor and stromal cells and lymphocyte subsets as well as the clinical response in MDS patients. *Methods.* Ten low-intermediate risk MDS patients received intravenously cA2 (3 mg/kg) at weeks 0, 2, 6 and 12. At baseline and end of the treatment, we evaluated: (a) The BM stem/progenitor cell reserve and function using a limiting dilution assay for the enumeration of the long-term culture initiating cells (LTC-ICs) in the $CD34^+$ cell fraction, clonogenic assays for the quantification of the colony-forming cells (CFCs) in the BM mononuclear (BMMCs) and $CD34^+$ cell fraction, and flow-cytometry for the evaluation of the percentages of $CD34^+$ cell subpopulations and the proportion of apoptotic (7-aminoactinomycin-D positive; 7-AAD^+) and Fas^+ cells in the $CD34^+$ cell fraction. (b) The activation status of BM and peripheral blood (PB) lymphocytes using flow-cytometry. (c) The BM stromal cell function to sustain the autologous and normal haemopoiesis using standard long-term BM cultures (LTBMCs) or irradiated LTBMCs recharged with normal $CD34^+$ cells. Clinical responses were evaluated according to standardized criteria. *Results.* The number of LTC-ICs cells did not change significantly following treatment compared to baseline. Of the CD34+ cell subpopulations, a significant increase was obtained in the proportion of $CD34^+/CD33^+$ myeloid progenitor cells compared to baseline (p=0.0192). The proportions of $CD34^+/CD61^+$ megakaryocytic and $CD34^+/CD71^+$ erythroid progenitor cells and the percentage of $GlycoA^+$ erythroid precursor cells did not change significantly. The number of CFCs obtained by BMMCs and $CD34^+$ cells increased significantly following treatment compared to baseline (p=0.0399 and p=0.0304, respectively). This increase was due to the improvement of CFU-GM (granulocyte-macrophage colony forming units) and CFU-Meg (megakaryocytic colony forming units) numbers in the BMMCs (p=0.0298 and p=0.016, respectively) and $CD34^+$ cells (p=0.0441 and p=0.002, respectively) post-treatment. The proportion of apoptotic (7AAD+) cells and the percentage of Fas^+ cells in the $CD34^+$ cell fraction decreased significantly post-therapy compared to baseline (p=0.0215 and p=0.0344, respectively). The proportions of activated BM and PB T-cells decreased significantly after treatment as was indicated by the percentage of Fas+ $HLA\text{-}DR^+$, $CD25^+$, $CD38^+$ and $CD69^+$ cells in the $CD3^+$ cell fraction. Treatment with cA2 reduced significantly TNFa levels in LTBMC supernatants (p=0.0043) and improved significantly the haemopoiesis supporting capacity of LTBMC adherent cells. Two patients displayed minor haematologic responses while the remaining displayed stable disease with no disease progression. *Summary-Conclusions.* Treatment with cA2 down-regulates the Fas-mediated apoptotic depletion of BM $CD34^+$ cells, increases the clonogenic potential of haemopoietic progenitor cells, ameliorates the hemopoiesis supporting capacity of BM stroma and decreases the proportion of activated T-lymphocytes in both BM and PB. The encouraging biological insights from cA2 administration may appear useful in conducting further clinical trials using cA2 for selected MDS patients particularly those with evidence of immune-mediated inhibition of haemopoiesis.

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RESULTS OF CLONALITY ASSAY AND MEASUREMENT OF APOPTOTIC RATE AND TELOMERE LENGTH SUPPORT USEFULNESS OF SEPARATION OF REFRACTORY CYTOPENIA FROM REFRACTORY ANEMIA AS A DISTINCT SUBTYPE OF EARLY MDS

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Background and aim of the study. The degree of clonality, telomere length

and the rate of apoptosis represent laboratory markers that may be related to the progression of pathological clone in patients with myelodysplastic syndrome (MDS). In this study we investigated these markers in patients with different subtypes of MDS. Methods. X-chromosome inactivation pattern clonality assay based on PCR amplification of polymorphic short tandem repeats of the human androgen receptor (HUMARA) gene was performed in granulocyte, CD14+ and CD3+ cell subpopulations isolated from bone marrow and peripheral blood of 58 females with primary MDS and 20 healthy controls. The results were compared with measurement ot the telomere length by Terminal Repeat Fragment (TRF) method and with apoptotic rate of CD34+ and GlyA+ subpopulations assessed by flow cytometry (Annexin V. and TUNEL methods). Results. In 19 patients with advanced MDS (RAEB,RAEB-T,CMML according to the FAB classification) clonal granulocyte and CD14+ cell subpopulations (allele ratio ≥ 9:1) were present in bone marrow and peripheral blood of 74% and 87% of patients, respectively. Shortened telomere length (TRF < 7,5 kbp) and low rate of apoptosis of CD34+ bone marrow cell subpopulation were present in all patients with advanced MDS. In patients with early MDS, clonal patterns of hematopoiesis were present only in 2 out of 17 patients (12%) with RA, RARS or 5q- syndrome according to the WHO classification. On the other hand, clonal granulocyte or CD14+ cell subpopulations were present in bone marrow or peripheral blood of 20 out of 22 patients (90%) with RCMD (according to WHO criteria). In accordance with these results, 80% of patients with early MDS and clonal granulocyte cell subpopulations exhibited low apoptotic rate of CD34+ bone marrow cells (5-12%). On the contrary, 80% of patients with non-clonal cells had increased apoptotic rate of CD34+ cells (30-80%). Reduced telomere length was found in 71% patients with clonal cell subpopulations v.s. 45% patients with non-clonal cells. Median survival of patients with early MDS and clonal cells was 62,5 months v.s. 47,8 months in those with non-clonal cells (p=0.05) and 65,7 months in RA patients v.s. 50,0 months in RCMD patients (p=0.05). Conclusions. The results confirm our preliminary observations suggesting that RCMD represents a separate clinical and laboratory entity with adverse prognosis which is distinct from RA and support hypothesis of multistep pathogenesis of MDS, where dysplasia limited to erythropoiesis may represent an early step and multilineage dysplasia is a subsequent step reflecting progression of pathological clone.

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INHIBITION OF THE MKK3-P38 MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY IS REQUIRED FOR NEUTROPHIL DIFFERENTIATION OF HUMAN CORD BLOOD DERIVED CD34+ CELLS

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Patients with myelodysplasic syndromes (MDS) suffer from recurrent bacterial infections as a result of differentiation defects of the neutrophil lineage. While limited number of genetic defects of MDS progenitor cells has been described, the defective intracellular signal transduction pathways modulating these developmental defects remain undefined. Mitogen-activated protein (MAP) kinase cascades play a key role in regulating a plethora of cellular processes. They typically are organized in a three-kinase architecture consisting of a MAPK, MAPK activator (MKK or MAPK kinase), and a MKK activator (MAPK kinase kinase). The p38 MAPK pathway mediates a wide variety of cellular processes in response to extracellular stimuli such as UV light, osmotic shock, inflammatory cytokines and growth factors and it has been shown that MKK3 and MKK6 are the main MKKs activating p38. Although p38 has been demonstrated to regulate differentiation in several cell types, its role in regulating neutrophil development in both normal as well as in defective MDS granulopoiesis remains to be investigated. *Aims*. The aim of this study was to investigate the role of the p38 MAPK signalling module in neutrophil differentiation and to determine whether p38 MAPK signalling may play a role in aberrant neutrophil development in MDS Mononuclear cells were isolated from umbilical cord blood using a ficoll-paque solution and MACS immunomagnetic cell separation was used to isolate CD34+ cells. Cells were cultured in IMDM supplemented with 9% serum and differentiation towards neutrophils was induced upon addition of SCF, FLT-3, GM-CSF, IL-3 and G-CSF. After 6 days of culture, only G-CSF was added. The specific p38 pharmacological inhibitor SB203580 was freshly added every 3 - 4 days during culture. Retroviral transduction experiments were performed at day 2 and 3 to ectopically express constitutively-active MKK3. During neutropoiesis cells were counted every 3 days and the percentage of apoptotic cells was determined by analyzing Annexin-V positive cells. The morphology of differentiating neutrophils was analyzed by May-Grunwald Giemsa staining. Neutrophil differentiation was also analyzed by intracellular staining of lactoferrin. *Results*. Inhibition of p38 during neutrophil differentiation by SB203580 resulted in an approximately 30% increase in proliferation, which was not due to enhanced survival. In addition, after 17 days of neutrophil differentiation, approximately 45% of SB203580 treated cells consisted of banded or segmented nuclei, whereas only 25% of the control cells were characterized as mature neutrophils. Conversely, ectopic expression of constitutively-active MKK3 resulted in a 40% reduction in proliferation compared to eGFP alone, which was not due to an increase in apoptosis. Transduction of cells with eGFP resulted in approximately 35% mature neutrophils after 17 days of culture, whereas ectopic expression of constitutively-active MKK3 resulted in an almost complete block in neutrophil differentiation. *Conclusions*. These results demonstrate that regulating p38 activity is critical for neutrophil development. Inhibition of p38 activity is necessary for terminal differentiation of CD34+ progenitor cells. Since neutrophil development is blocked in patients with MDS and downregulation of p38 activation is required for normal neutrophil differentiation, it can be hypothesized that deregulation of p38 activation might be involved in aberrant neutrophil maturation in MDS.

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MITOCHONDRIAL INVOLVEMENT IN 5-AZACYTIDINE-INDUCED APOPTOSIS

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Backgrounds. Although 5-azacytidine is the only drug approved by the FDA for high-risk MDS, its mode of action has not been well characterized. Aim: The aim of the study was to investigate the mechanisms of 5-azacytidine-induced cytotoxicity in myeloid P39 cells in order to optimize the use of this drug. *Methods.* Cells were incubated with 0.1-2 μM of 5-azacytidine for 4-48 hours. Nuclear apoptotic morphology was assessed by light microscopy. Processing of caspases, cytochrome-c release into the cytosol, cleavage of PARP, expression and cleavage of Bcl-2 family proteins were analyzed by Western blot. Loss of mitochondrial membrane potential was estimated by FACS analysis using the fluorescent dye TMRE. *Results.* 5-azacytidine induced apoptosis in P39 myeloid cells. Dose-dependent activation of PARP as well as processing and activation of caspase-2 were observed. Furthermore cleavage of Bcl-2-family proteins, namely, Bcl-2, Bax and Bid was documented. Mitochondrial involvement, characterized by loss of mitochondrial membrane potential and release of cytochrome-c into the cytosol was detected. Although caspase-3 activation occurred, various inhibitors of caspase-3 (DEVD-fmk), -2 (VDVAD-fmk), -8 (IETD-fmk), -9 (LEHD-fmk) and pan-caspase inhibitor (zVAD-fmk) did not block apoptosis. Moreover, inhibitors of the poly (ADP-ribose)polymerase, PARP, (nicotinamide and 3-aminobenzamide) only partially block apoptosis (25-41% and 31-43% decrease, respectively). *Conclusion*. 5-azacytidine activates PARP, which in turn induces mitochondrial dysfunction. At the mitochondrial level this compound suppresses anti-apoptotic properties (cleavage of Bcl-2) and increases pro-apoptotic activities (cleavage of Bax and Bid). These events result in the loss of mitochondrial membrane potential and release of cytochrome-c into the cytosol. As PARP inhibitors only partially block loss of mitochondrial membrane potential, and caspase inhibitors did not have any effect on any of apoptosis manifestations, we conclude that 5-azacytidine induces cell death via activation of caspase-independent pathway. It seems that caspase activation plays a secondary role in this process.

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CLINICAL CHARACTERISTICS AND TREATMENT OF 217 NEW MDS PATIENTS DURING THE YEAR 2005 IN A TERTIARY REFERRAL CENTER

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Backgrounds. Heterogeneity of myelodysplastic syndromes not only relates to the biology of the disease but also to the spectrum of patients