Leukemia Research Reports 4 (2015) 15–17

Contents lists available at ScienceDirect

Leukemia Research Reports

journal homepage: www.elsevier.com/locate/lrr

Effects of 5-azacytidine on natural killer cell activating receptor expression in patients with refractory anemia with excess of blasts

ARTICLE INFO

Keywords:
Natural killer cells
Refractory anemia with excess of blasts
Natural cytotoxicity receptors
Demethylation
5-azacytidine

ABSTRACT

Epigenetic drugs modify DNA methylation and are used in refractory anemia with excess of blasts (RAEB). These drugs may reactivate anti-oncogene expression and restore a normal phenotype instead of inducing antitumor toxicity, although they also have immunosuppressive effects on T-lymphocytes [1] In RAEB and acute myeloid leukemia, a defect in natural killer (NK) cell cytotoxicity has been shown, which relies on abnormal expression of activating receptors. Previous study has shown that 5-azacytidine impaired mRNA synthesis and induced apoptosis in NK cells [2]. In this study we investigated the effect of the demethylating drug 5-azacytidine (Vidaza®) on NK receptors with the hypothesis that demethylation of the promoters of activating NK receptor genes induces gene reactivation and thus may increase their expression.

1. Letter to editor

Despite recent progress in approach of malignant haemopathies, their prognosis frequently remains poor due to the difficulty to achieve complete remission (CR) and to the high risk of relapse. Immunotherapy could thus be of great interest in this setting. Specific immunotherapy is mainly challenged by the defect of expression of major histocompatibility complex (MHC) molecules frequently observed in cancer cells, together with the progressive selection of cancer clones that have lost their MHC molecules and thus escape from immune control by specific T-lymphocytes. In sharp contrast, natural killer (NK) cells are able to kill target cells in a MHC-independent way, i.e. these cells “sense” the absence or abnormal expression of MHC molecules to express their cytolytic capacities, provided that tumor cells express ligands for NK activating receptors (for review, [3]). Among activating NK molecules, the so-called natural cytotoxicity receptors (NCRs) NKp30/NCR3, NKp44/NCR2 and NKp46/NCR1, together with NKG2D and 2B4/CD244 play a pivotal role in NK cytotoxicity and probably in their anti-leukemia effect [4]. Since the expression of activating molecules is down-regulated in myeloid malignancies, we hypothesized that demethylating agents such as 5-azacytidine [5], used in the treatment of RAEB and AML with $<30\%$ blast infiltration in bone marrow, could contribute to the restoration of a normal phenotype of NK.

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Blood samples were obtained, before any treatment, from 6 patients with RAEB II after informed consent. The analysis of PB was done less than 4 h after blood sampling, on whole blood after red blood cell lysis (Q-Prep^{R} lysing kit, Beckman-Coulter, Villepinte, France). The NK cell population was defined as CD3-/CD56+. The following mAbs were all obtained from Beckman-Coulter: anti-CD3^{FITC} (UCHT1), anti-CD56^{PC5} (N901-NKH1), anti-NCR1/NKp46^{PE}, anti-NCR2/^{NKp44PE}, anti-NCR3/NKp30^{PE}, anti-NKG2D^{PE}, anti-CD244(2B4,p38)^{PE}. Triple staining was performed using CD3^{FITC}/CD56^{PC5} in addition with anti-NCR1/NKp46^{PE}, anti-NCR2/^{NKp44PE}, anti-NCR3/NKp30^{PE}. For cell surface staining, cells (5×10^5) were incubated for 20 min at 4 °C with 10 µL of the corresponding antibodies for 100 µL of cell preparation. Flow cytometry was performed on an Epics XL^{R} flow cytometer (Beckman-Coulter). The results are expressed as follows; percentages correspond to (% positive cells – % isotype control), while the mean fluorescent intensities (MFI) correspond to the ratio (MFI positive cells / MFI isotype control). Statistical analysis was performed using the SPSS software (SPSS User's Guide. SPSS Inc, 1993).

Regarding the expression of NKp46/NCR1 (Fig. 1A and B), both increased and decreased expression are observed depending on patients. Interestingly, the expression of the inducible molecule NKp44/NCR2 is clearly induced in 3 patients (PT1, PT2 and PT5) both regarding the percentage and the MFI. In contrast, we observed a decrease in the expression of NKp30/NCR3 in 3 patients (PT1, PT2 and PT7). Regarding NKG2D, a modification of expression was observed only in 2 patients, with 1 increase (PT4) and 1 increase (PT3) regarding the percentage of expression, while the MFI was decreased in most patients. Finally, we analyzed the expression of 2B4/CD244. This molecule is expressed in almost 100% of NK cells, and this percentage was not modified by 5-azacytidine treatment. In sharp contrast, when considering the MFI, we observed a drastic decrease from a mean MFI of 77 ± 31 versus 36 ± 15 ($p < 0.05$).

The low number of patient in our series precludes firm conclusions. Nonetheless, the 3-month treatment by the demethylating agent 5-azacitytine failed to restore or increase the expression of the NK activating molecules NCRs, NKG2D and 2B4/CD244, the expression of the later being on the contrary significantly

http://dx.doi.org/10.1016/j.lrr.2014.09.004

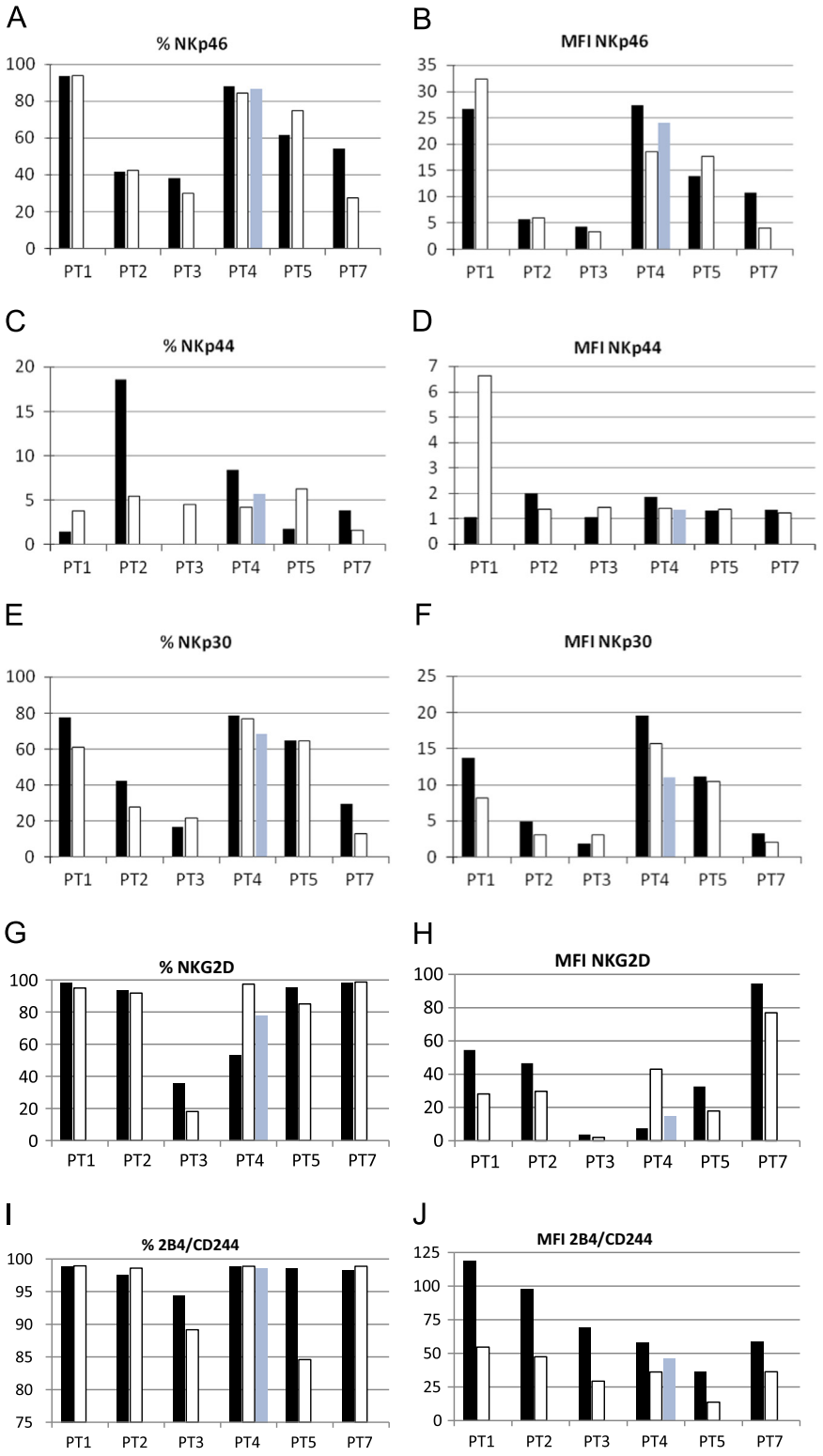

Fig. 1. Expression of activating receptors on Natural Killer cells expressed as percentage of positive cells (left panels) and mean fluorescence intensity (MFI, right panels) for NKp46 (A and B), NKp44 (C,D), NKp30 (E and F) NKG2D (G and H) and 2B4/CD344 (I and J). Data are expressed as the percentage of positive cells or of MFI after subtraction of the negative isotype control. Black bars correspond to analysis before treatment, white bars after 3 cycles of 5-azacytidine treatment, and gray bars (only patient PT4) analysis after 6 cycles of 5-azacytidine. PT: patient.

down-regulated (decreased MFI in 6 out of 6 patients tested). Such a drastic down-regulation of 2B4/CD244 has also been observed in multiple myeloma but interestingly in this case only in bone marrow but not in peripheral blood [6]. We did not check NK cells phenotype in bone marrow of our RAEB patients since this analyze was not part of standard treatment follow-up. On the other hand, we failed to detect any increase in the inhibitory receptors we tested (CD158a, CD158b, CD158e, CD158i, CD159a, data not shown). Since the 2B4/CD244 is an important co-activating molecule for the NK, via its interaction with the EBV-inducible

CD48 molecule, we can speculate that 5-azacytidine treatment could also contribute to immune deficiency via the inhibition of this co-activating pathway, although the rate of infection observed under 5-azacytidine did not increase in comparison with the other therapeutic options available for these patients [7] and was at least in part dependent on myelodysplasia characteristics, such as unfavorable cytogenetics [8]. In addition, these data could suggest that the methylation status is probably not the central mechanisms for the NCRs and NKG2D transcriptional regulation, although the exact pattern of demethylation induced by 5-azacytidine is not entirely analyzed and cannot infer drug efficiency. Finally, our data also argue for the use of concomitant immune-stimulatory approaches such as lenalidomide [9] or other IMIDs class drugs in the treatment of RAEB or AML, in order to associate the supposed differentiation effects of demethylating agents with the development of an efficient anti-leukemic innate immune response.

Authors contribution

RTC, AL, ThLT, CS, DM and contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting of the article or revising it critically for important intellectual content and for final approval of the version to be submitted.

Acknowledgment

This work was funded in part by the Association pour le DEveloppement de la REcherche Médicale (A.DE.RE.M).

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Received 8 July 2014
2 September 2014
15 September 2014

Available online 15 January 2015

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