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Total expression of HLA-G and TLR-9 in chronic lymphocytic leukemia patients

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ABSTRACT

Suppressed immune status facilitates immune escape mechanisms that allow chronic lymphocytic leukemia cells to proliferate and expand. The expression of HLA-G could effectively inhibit the immune response. In immune response inhibitory signals follow activation of immune system which might be occur during bacterial or viral infection in CLL patients. In the current study we characterized two components of immune system, inhibitory molecule HLA-G with its receptor – CD85j and Toll-like receptor 9.

The material was obtained from 41 CLL patients and 41 HV with similar median age. In CLL patients expression of intracellular and surface HLA-G and soluble HLA-G levels were significantly higher than in HV. We found higher expression of CD85j compared to HV and the positive correlation between expression of HLA-G and CD85j. All the CLL cells expressed TLR-9, and the level of expression positively correlated with expression of HLA-G and CD85j. Patients with higher expression of intracellular expression of TLR-9 have significantly longer treatment-free survival than patients with low expression of TLR-9 (57 months vs. 8 months, respectively).

Summarizing in CLL we characterized activatory and inhibitory components of immune system that might be connected functionally. Analysis of TLR-9 expression might have additional prognostic value for CLL patients.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in western countries. An accumulation of leukemic cells in peripheral blood of patients might be caused by apoptosis derangement as well as an increase of CLL cells proliferation in germinal centers. The etiology of the disease is still unknown, however there is increase evidence on possible bacterial stimulation or activation of auto-reactive lymphocytes initiate leukomogenesis [1]. Moreover CLL patients suffer from recurrent infections which has been known to be major cause of morbidity and mortality of CLL patients over many years. In CLL patients immune escape mechanisms allows leukemia cells to proliferate and expand. Several recent studies showed that the expression of HLA-G might represent one of the most powerful agents to damage the cellular immune response [2,3]. HLA-G is non-classical major histocompatibility complex (MHC) class I molecules for the first time described in 1990 by Kovats et al. [4] on cytotrophoblast cells. HLA-G molecule plays an important role during pregnancy protecting fetus tissue from immune recognition by maternal immune system. The expression of HLA-G can be induced in pathological conditions. Various studies have reported increased expression of HLA-G in malignant diseases. Enhanced HLA-G surface expression has been described in most of lymphoproliferative disorders [5–9]. HLA-G has direct inhibitory effects on natural killer cells (NK), T cells, dendritic cells (DC) and has indirect effects by inducing regulatory T cells (Treg) [10]. Tregs are critical for the regulation of immune responses to foreign and self-antigens but are also hypothesized to be responsible for promoting cancer development via suppression

Abbreviations: CLL, chronic lymphocytic leukemia; MHC, major histocompatibility complex; NK, natural killer cells; DC, dendritic cells; Treg, regulatory T cells; TLRs, Toll-like receptors; PAMPs, pathogen-associated molecular patterns; TLR-9, Toll-like receptor 9; HV, healthy volunteers; ELISA, enzyme-linked immunosorbent assay; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; FBS, fetal bovine serum; sHLA-G, soluble HLA-G; MFI, Mean Fluorescence Intensity; TFS, treatment-free survival; CpG ODN, CpG oligodeoxynucleotides; IL, interleukin; TNF, tumor necrosis factor.

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of antitumor immune responses. Recently, the subpopulation of Treg induced by HLA-G was described [3,11,12]. Moreover increased frequencies of Treg were observed in CLL patients [13].

In CLL patients who suffer from recurrent infections the nonspecific immune response could be continuously stimulated. The response of the innate immune system could eliminate infectious agents and is crucial for developing pathogen-specific adaptive immunity mediated by B and T cells. Toll-like receptors (TLRs) are responsible for host defense against pathogens by recognizing a wide variety of pathogen-associated molecular patterns (PAMPs). These receptors regulate innate immunity and determine the polarization and function of adaptive immunity. TLRs signaling might take also part in the regulation of B-cell differentiation process [14]. Triggering TLRs results in increased expression of inflammatory genes, which then play a protective role against various infections. Functional TLRs, in particular TLR-9, are expressed in a most of tumors and suggest that activation of tumoral TLRs induces the releasing of proinflammatory as well as immunosuppressive factors. This process increases the resistance of tumor cells to cytotoxic lymphocyte assault and further results their escape from immune surveillance [15]. However, it has been suggested that the stimulation of TLRs expressed on CLL cells could increase immunogenicity of tumor cells and thus potentially contribute to the induction of tumor-specific immune response [14,16]. Therefore, in current study we characterized two components of immune system, inhibitory molecule HLA-G with its receptor – CD85j and Toll-like receptor 9 (TLR-9).

2. Materials and methods

2.1. Study subjects

The material was obtained from 41 CLL patients diagnosed at the Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin (28 males, 13 females, mean age 67). Twenty-eight patients were classified in stage A, twelve in stage B and eight in stage C according to the Binet classification. Detailed characteristic of CLL patients is shown in Table 1. This study was approved by the Local Ethics Committee No. KE-0254/178/2006, and the patients were informed about use of their blood for scientific purposes.

2.2. Plasma collection and cells isolation

Plasma was obtained from the blood samples of 41 CLL patients and 41 healthy volunteers (HV) by centrifugation and cryopreserved at −80 °C for ELISA test. Peripheral blood mononuclear cells (PBMC) from CLL patients and HV were isolated by ficoll density gradient centrifugation and cryopreserved at −80 °C to the time of analysis. The viability of analyzed PBMC was always >95% as determined by trypan blue staining.

2.3. Flow cytometry analysis

Frozen mononuclear cells from CLL patients and HV were thawed, once washed in RPMI-1640 (Biochrom AG) with 50 μl DNA-ase (Sigma–Aldrich, concentration 5 Unit/mg) and twice in Phosphate Buffered Saline (PBS) (Biochrom AG) with 2% Fetal Bovine Serum (FBS) (Biochrom AG). The viability was counted. The staining of the surface antigens CD5, CD19, CD85j and HLA-G was preformed after the incubation with monoclonal antibody according to manufacturer's protocol (BD Biosciences, USA). Mononuclear cells were also stained with anti-CD19 MACS beads and were separated by MACS columns. The intracellular expression TLR-9 on CD5+CD19+CLL cells was performed after permeabilization using with Fix/Perm Kit (BioLegend, USA). After permeabilization cells were washed twice with Perm Buffer and incubated for 15 min. In the next step anti-TLR-9 antibodies were added. After intracellular staining cells were washed twice with PBS and analyzed by flow cytometry for the expression of TLR-9. Flow cytometry analysis was performed on a FACSCalibur and analyzed using the CellQuest (BD Bioscience) software packages. Each time 100,000 cells were acquired.

Table 1
Clinical characteristic of 41 chronic lymphocytic leukemia patients.

Characteristic	Patients (n)
Sex	
Female	13
Male	28
Age (years)	
<40	1
40–49	3
50–59	6
60–69	13
70–79	14
>80	4
Binet's classification	
A	21
B	12
C	8
WBC (G/L)	
<20	4
20–50	24
50–100	11
>100	2
ZAP-70 (cut off 20%)	
Positive	23
Negative	16
NA	2
CD38 (cut off 30%)	
Positive	9
Negative	27
NA (not available)	5
Cytogenetics	
Normal karyotype	8
Trisomy 12	4
Del 13q	12
Del 11q	2
Del 17p	2
NA (not available)	13

2.4. ELISA for the soluble HLA-G (sHLA-G)

The plasma level of sHLA-G was assessed using sHLA-G ELISA test (BioVendor, Laboratory Medicine, Czech Republic). Samples were thawed and incubated according to manufacturer's protocol. sHLA-G concentration were assessed using Microplate reader (BioRad, USA) based on the calibration curve.

2.5. Statistical analysis

All results are presented as median values with rage. The U Mann–Whitney test was used to evaluate the differences between subgroups of patients. The correlations of variables were computed with the Spearman rank correlation coefficient. The Kaplan–Meier method and the log-rank test were used to assess TFS of CLL patients.

3. Results

3.1. The surface HLA-G expression in CLL patients

The surface expression of HLA-G was assessed on magnetically-selected CD19 positive B cells and subsequently gated on CD5+CLL