THE IMPORTANCE OF INTERACTION BETWEEN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND MICROENVIRONMENT IN THE PROGRESSION OF CLL

Ph.D. THESIS

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Introduction

CLL is the most common leukemia of the adults in Western countries. In the WHO classification CLL listed among the mature B-cell neoplasms. Despite the similar cytology, immunophenotype and gene expression profile, CLL manifests itself in a variety of different clinical courses. At diagnosis, the disease burden based on clinical staging systems is the most significant prognostic factor for survival, but genetic lesions, ZAP-70, CD38 and CD49d expressions have also negative prognostic impact.

The accumulation of CLL-cells is attributed mainly to defective apoptosis, but proliferation of CLL-cells may also play a role. The microenvironment is likely to be responsible for the survival and proliferation of CLL-cells. The majority of CLL cells die *in vitro* during a short term culture in medium, but the contact with bone marrow derived stromal cells (BMSCs) or mouse fibroblast enhances the survival of CLL cells. A proliferating pool in CLL has been identified in so-called Pseudo-Follicles (PC). PCs are composed of large cycling CLL-cells, delicate follicular dendritic cell networks and activated ($CD40L^+$) T_H-cells. CD40 Ligand and some of the T-cell derived cytokines are able to induce *in vitro* CLL-cell proliferation.

Aims

1. Preparation and characterization of BMSCs cultures
2. Establishment an *in vitro* cell culture system similar to proliferation centers in CLL: investigation of effects of sCD40L and other cytokines on CLL-cells on BMSCs.
3. The exploration of different proliferation and survival capacities of CLL-cells in the *in vitro* model for PCs.
4. The investigation of impacts of CLL-cells on BMSCs.

Methods

Peripheral blood samples from twenty one CLL patients were involved in the *in vitro* experiments. Peripheral blood and bone marrow samples from 15 CLL patients were investigated toward CD49d and CD18 expressions.

BMSCs cultures were established from bone marrow aspirates exclusively from untreated patients without detectable abnormal cells.

BMSC cultures were analyzed for antigen and cytokine secretion by flow-cytometry and for mRNA expression by RT-PCR.

CLL-cells were sorted out of peripheral blood mononuclear cells (PBMC) by FACSAria Cell Sorter.

The following CLL-cell co-culture conditions were applied: 1) BMSCs 2) BMSCs and soluble CD40 Ligand (sCD40L); 3) BMSCs, CD40L, and IL-4; 4) BMSCs, CD40L, IL-2, and IL-10; 5) only medium; 6) medium and CD40L; 7) medium, CD40L, and IL-4; 8) medium, CD40L, IL-2, and IL-10. The CLL cells were harvested after 84 hours.

Before and after the breeding of CLL-cells, we investigated the immunophenotype by flow cytometry, gene expressions by RT-PCR, proliferation by multiparameter flow-cytometric analysis and survival by cell counting and measurement of apoptosis.

Results

Preparation and characterization of BMSCs cultures

Isolated bone marrow mononuclear cells were bred in DMEM supplemented with 20% fetal calf serum (FCS) and reached mature, confluent monolayer on the average

after 6 weeks. BMSCs-yields from bone marrow mononuclear cells of younger donors were higher. The mRNA expressions of the adhesion molecules VCAM-1, ICAM1, that of cytokines IL6, IL8 and the chemokine CXCL12 in BMSCs were at very high levels. The mRNA expression of CD40, IL-1β and TNFα was detectable, but to a lower extent. Multiplex bead assay of the culture supernatants confirmed the strong secretion of the cytokines IL-6, IL-8 accordingly to the observed mRNA expression.

Effects of sCD40L and other cytokines on CLL-cells on BMSCs cultures

CLL-cells upregulated the majority of the investigated antigens by the end of the co-culture with BMSCs, especially the activation antigens such as CD23, CD69, co-receptors HLA-DR, B7-2 (CD86), and adhesion molecules CD54 (ICAM1), CD49d. The expression of PAX5 in CLL, a gene critical for B-cell commitment, was decreased by BMSCs.

CD49d expression on peripheral blood and bone marrow CLL-cells was compared in order to confirm our *in vitro* results. In the majority of investigated cases the normalized CD49d expression was higher in the bone marrow than in peripheral blood.

The sCD40L slightly altered the immmunophenotype and gene expression of sorted CLL-cells induced in the co-culture with BMSCs. The expression of CD11c, CD18, CD40, CD54 and HLADR was induced, whereas those of CD20 and CD184 decreased.

IL4 stimulation of CLL-cells cultured on BMSCs with sCD40L dramatically upregulated several antigens including CD23, CD40, CD86, CD54, but lowered the CD45, the B-cell specific CD19, CD20, adhesion antigens CD49d, CD44, chemokin

receptor CD184 (CXCR4) and left unchanged CD138. IL4 significantly increased the expression of IRF4 mRNA and protein in CLL cells, although PAX5 and Blimp1 did not change.

IL2 and IL10 induced the majority of the investigated adhesion molecules including CD18, CD11c, CD49d, CD54, CD44 and decreased the receptor of SDF-1 (CD184).

The different proliferation and survival capacities of CLL-cells in the in vitro model for PCs

No dividing CLL-cells were detectable in the peripheral blood of the 21 cases studied. Only the combination of sCD40L, IL2 and IL10 induced cell division of CLL-cells co-cultured with BMSCs in 8 cases. CLL-cells from all thrombocytopenic cases were able to proliferate. We could also induce cell division in two cases with platelet counts around the minimum normal level (140x and 166x 10^9/l). The proportion of cells in S and G2/M phase varied between 0.7 and 7.8% at the end of the co-culture period. A couple of adhesion antigens and co-receptor for T-cells were expressed by the proliferating cells at a high level, but the immunophenotype of the gated S+G2/M and G0/G1 cells always overlapped. Therefore, it was not possible to gate the cycling population by means of fluorescence intensities of these antigens.

BMSCs protected 50-70% of CLL-cells from spontaneous apoptosis *in vitro.* In medium, CLL-cells from those cases with proliferation capacity or the group of non-proliferating cases with unmutated IgV_H and/or peripheral blood lymphocytosis above 50,000/μL also showed significantly higher survival rates in medium than cells from indolent cases with mutated IgV_H and lower lymphocyte counts. Different cytokines (sCD40L, IL2, IL10) alone or in combination exerted very heterogeneous effects on the survival of CLL-cells on both BMSCs and medium.

The investigation of impacts of CLL-cells on BMSCs

In the presence of CLL cells, BMSCs significantly up-regulated the mRNA expression of ICAM-1 and CD40, whereas the expression of VCAM-1 and CXCL12 remained unchanged. In the co-culture of CLL cells and BMSCs significantly raised the cytokine secretion of IL1β, IL8, and TNFα. The secretion of IL6 was also strongly elevated, but this difference failed to reach statistical significance. By the determination of cytokine mRNA expression in BMSCs and CLL cells we showed that IL-6, IL-8, and IL1β derived mostly from the BMSCs and the increased TNFα levels originate from the CLL cells.

Conclusions

1. BMSC cultures can be established from bone marrow mononuclear cells during on average 6 weeks that is suitable for *in vitro* breeding of hematopoietic cells. Significantly more BMSCs grow from bone marrow aspirates of younger donors.
2. BMSCs induced mostly the expression of activation and adhesion antigens on the surface of CLL-cells.
3. The bone marrow microenvironment upregulated also *in vivo* the CD49d expression on CLL-cells.
4. Soluble CD40L exerts only a marginal effect on antigen and gene expressions of CLL-cells.
5. IL4 gives rise to a unique immunophenotype and raising IRF4 expression in CLL-cells, which may refer to plasma cell differentiation.
6. IL2 and IL10 induced the majority of the investigated molecules independently from proliferation capacity of CLL-cells.
7. We were not able to detect proliferating CLL-cells in the peripheral blood.

8. Only the combination of sCD40L, IL2 and IL10 induced *in vitro* cell division of CLL-cells.
9. A low platelet count indicated most sensitively the ability of CLL-cells to proliferate.
10. A proliferation specific immunophenotype were not found on the surface of CLL-cells.
11. BMSCs did not protect completely the CLL-cells from apoptosis.
12. CLL-cells from poor prognostic cases showed better survival in medium.
13. The cytokines did not improve the survival of CLL-cells in vitro.
14. CLL-cells induced the antiapopototic features of BMSCs by raising the expression of ICAM1 and secretion of IL8, IL6, IL1β.

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