

Impact of the reactive microenvironment on the bone marrow involvement of follicular lymphoma

Doctoral theses

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I. INTRODUCTION

Follicular lymphoma (FL) is an indolent B-cell non-Hodgkin's lymphoma (NHL) which comprises about 20% of all NHLs in Europe and North America. Approximately 85-90% of FLs carry the t(14;18)(q32;q21) chromosomal translocation, juxtaposing the BCL-2 gene with the immunoglobulin heavy chain (IGH) gene, resulting in constitutive expression of the antiapoptotic protein BCL-2. The morphological, immunophenotypic and molecular features of FL are reminiscent of those of normal germinal centers (GC) of secondary lymphoid follicles, indicating the GC B-cell origin of the tumor. The neoplastic follicles contain numerous reactive, non-neoplastic cells including T-lymphocyte subsets, macrophages and follicular dendritic cells (FDC). Lymphoma cells require cellular interactions with T-cells and FDCs in the GC-like microenvironment for proliferation. Furthermore, the interactions of FL cells with the supportive stroma provide growth- and drug resistance signals for the neoplastic cells. The importance of tumor microenvironment in the behavior of FL has also been emphasized by gene expression profiling studies: expression of genes which are enriched in T-cells and macrophages was associated with a favorable outcome, whereas genes which derived from macrophages and FDCs were associated with inferior outcome. Based on these observations several immunohistochemical studies, often with conflicting results, have defined the immune microenvironment as predictor of overall survival in FL patients.

Connexin 43, the most widely distributed gap junction protein in the lymphoid organs. Connexins, the proteins assembled into gap junction channels, are present in high numbers in secondary follicles and provide direct intercellular communication between follicular dendritic cell meshwork and between B-lymphocytes and FDCs. The direct cell-cell

communication through gap junctions are able to influence the proliferation, and migration of B-lymphocytes.

In 40-70% of FL cases the bone marrow (BM) is also involved at diagnosis and is associated with poorer outcome. BM involvement is mostly associated with paratrabecular aggregates composed of centrocytes expressing different antigens, including CD10, BCL-2 and BCL-6. Although lymph node (LN) and BM compartments of FL are related to the same neoplastic clone, several morphologic, phenotypic and genotypic differences have been reported. The cytological grade of the tumor is usually lower in the BM than in the LN; FL cells of the BM frequently lose the expression of BCL-6 and CD10 and the mutation pattern of IgH variable region genes of FL cells in the two compartments show many differences. Although these findings suggest that there must be corresponding differences in the microenvironment this has not been tested experimentally. In this study, we set out to determine the extent of this variability in a unique series of cases where LN and BM microenvironment could be examined simultaneously.

To test whether the LN and BM microenvironment of FL are different; we compared the reactive cell composition and cell-cell interactions of the microenvironment of FL between LN and BM. Furthermore to analyze whether the LN microenvironment can predict the BM involvement of FL we compared the microenvironment of FL cases that involve the BM to those that do not involve the BM. The usage of a new software tool, automated image analysis of whole digital slides of tissue microarray samples allowed the precise localization and accurate counting of reactive T cell populations and macrophages within LN and BM samples. Our results showed significant differences in the microenvironment of FL in BM and LN and we found that the significant reduction in CD8⁺ cytotoxic T-lymphocytes and CD68⁺ macrophages at LN localization may contribute to the BM involvement of FL.

II. OBJECTIVES

The importance of tumor microenvironment in the behavior of FL has been emphasized by gene expression profiling and immunohistochemical studies.

Our main questions were as follows:

- Characterization and comparison of the tumor cells localized to the lymph nodes and bone marrow.
- To test whether the LN and BM microenvironment of FL are different; we compared the reactive cell composition and cell-cell interactions of the microenvironment of FL between LN and BM.
- To analyze whether the LN microenvironment can predict the BM involvement of FL we compared the microenvironment of FL cases that involve the BM to those that do not involve the BM
- Analysis of Connexin 43 expression in FL

III. MATERIALS AND METHODS

Pathological samples

LN and BM biopsies from 35 untreated patients with FL were selected for this study, based on the availability of formalin-fixed, paraffin-embedded (FFPE) tissue blocks for the immunohistochemical analysis. Of the 35 patients tested, 20 displayed BM involvement at diagnosis. Diagnoses were based on histopathologic, immunophenotypic and molecular analyses according to the World Health Organization (WHO) classification of lymphoid tumours. The relevant clinical characteristics of patients are summarized in Table 1. In all the cases analyzed, less than 2 weeks elapsed between samplings from the LN and the BM. Six reactive LN samples and BM samples from the 15 FL patients without BM manifestation were used as controls.

Tissue microarrays

Tissue microarrays (TMA) were constructed with a computer-driven semi-automated instrument (TMA Master, 3D HISTECH Ltd, Budapest, Hungary) by selecting representative tumor areas based on hematoxylin-eosin stained slides. At least duplicate cores of 2 mm diameter were arrayed from the LN samples into the recipient blocks. In case of the BM samples 1 or 2 core samples were collected into TMAs.

Immunohistochemical analysis

TMA blocks were cut to 3 μm sections and applied to silane-coated adhesive microscope slides. Immunohistochemical and fluorescent immunohistochemical staining was performed on dewaxed sections.

Automated counting of immunostained cell types

Immunostained TMA slides were scanned at 20X magnification using a Panoramic scan instrument (3D HISTECH) equipped with Carl Zeiss objective (NA=0.83; Carl Zeiss MicroImaging Inc, Jena, Germany). The number of positively stained cells was determined using the HistoQuant software imaging system of the same company under visual supervision of two pathologists to avoid any technical bias. Minimal number of 6 follicular areas or minimum of a 1mm² area were evaluated. The follicular and interfollicular areas and the infiltration of the BM were selected by a pathologist using permanent annotations on the digital slides, whereas the software collected the data automatically following the setting up of the threshold intensity levels for positivity. In some cases it was difficult for the software to identify the individual cells in cell groups, mostly in cases of cytoplasmic or membrane staining. Therefore, these subsets were quantified as the fraction of antibody positive area divided by the sum of antibody positive and antibody negative cellular area.

Scoring of stromal cell and FDC markers, and connexin 43 expression

Immunoreactions for stromal cell and FDC markers, and Cx43 were evaluated using a 4-scale scoring system in the lymph nodes with the TMA module software (3DHISTECH) by considering the positively stained area. In bone marrow we evaluated the presence and pattern of stromal cells, FDC cells and CX43 expression.

Statistical analysis

The frequencies and association between the different clinicopathological and immune parameters were estimated and compared using the Student's t-test for independent variables

and Student's t-test for dependent variables in case of the normally distributed variables. The Mann-Whitney U-test and Wilcoxon matched pair test was used to compare the not-normally distributed variables. Categorical data were compared using Fisher's exact test. A p-value of <0.05 was considered as statistically significant. All statistical analyses were performed using the Statistica 9.1 software (StatSoft. Inc., Tulsa, OK, USA).

IV. RESULTS

Different phenotype of malignant cells in LN and BM samples

The cytological grade, proliferation index and CD10 expression of tumor cells in the LNs and in the complementary BM samples of 20 untreated patients with FL were compared according to the WHO criteria. In 12 cases there was a downgrading between LN and corresponding BM sample. In one case, the cytological grade of FL cells was grade 1 in both compartments. In 16 cases, tumor cells in the LNs showed grade 2, and the respective BM samples displayed either grade 1 (9 cases) or grade 2 (9 cases). In 3 cases, lymphoma cells in the LN samples revealed grade 3, but all the corresponding BM infiltrates had lower grades (grade 2 in 1 cases; grade 1 in 2 cases). There was no case in which the grade of the BM infiltration was higher than that of the corresponding LN sample. The tumor cells of all LN and corresponding BM samples showed BCL-2 and CD10 positivity. Ki67 staining revealed positivity in fewer tumor cells in the BM than in the matching LN samples ($6.23\pm 4.61\%$ versus $21.08\pm 6.19\%$ $p < 0.0001$).

Differences in the reactive cell compartment of the LN and BM infiltration of FL

We next set out to test the microenvironmental differences between LN and corresponding BM samples by examining the distribution of reactive cell subsets using computerized image analysis. The microenvironment of the BM infiltrates was considerably different compared to the microenvironment of the malignant follicles in the LNs. We observed significantly lower number of PD1⁺ T-lymphocytes ($5.89\pm 3.15\%$ vs $18.59\pm 8.64\%$; $p = 0.002$), and significantly higher number of CD8⁺ T-lymphocytes ($11.02\pm 7.61\%$ vs $5.51\pm 2.41\%$; Wilcoxon matched pair test; $p = 0.004$), CD68⁺ macrophages (131.41 ± 52.59 cells/ 0.1mm^2 vs 38.77 ± 15.28

cells/ 0.1mm^2 ; Wilcoxon matched pair test; $p<0.0001$) and FOXP3⁺ regulatory T-lymphocytes (T_{reg}-lymphocytes) (151.00 ± 86.39 cells/ 0.1mm^2 vs 76.85 ± 39.64 cells/ 0.1mm^2 ; Student's *t*-test; $p=0.005$) in the BM metastases compared to the LN samples. The CD4⁺ T-lymphocytes and CD57⁺ T-lymphocytes showed no significant differences between these two compartments.

Stromal cells and follicular dendritic cells and CX43 expression in the BM

We analyzed the stromal cells both in normal and infiltrated bone marrow. In the infiltrated BM we observed a hyperplastic LNGFR⁺ stromal cell meshwork compared to the normal BM. All infiltrated BM samples showed CD21⁺ or CD23⁺, and CXCL13 expressing FDC meshwork. We observed the most prominent CX43 expression on the CD21⁺ FDC in the BM.

The microenvironment of LNs in FL shows significant differences between cases with and without BM infiltration

To determine whether the total number or percent of reactive immune cells infiltrating the tumor could have a specific relationship with BM infiltration, we performed a quantitative analysis of the various immune cells. We preferred to use BM involvement as the grouping variable, as it is a poor prognostic factor and it is independent from the clinical therapy. The number of the infiltrating CD8⁺ T-cells ($5.57\pm 2.36\%$ vs $10.02\pm 9.6\%$; Mann-Whitney *U* test; $p=0.04$) and CD68⁺ macrophages (38.15 ± 15.03 cells/ 0.1mm^2 vs 62.59 ± 30.62 cells/ 0.1mm^2 ; $p=0.004$) was significantly lower in FL cases with BM involvement, compared to the cases without BM manifestation. None of the other immune cells showed any significant associations with the BM involvement. There were no considerable differences between the interfollicular areas of the two groups.

Stromal cells and follicular dendritic cells and CX43 expression in the LN

The LNGFR+ stromal cells, CD21+, CD23+ FDC and the CXCL13 expression didn't reveal any differences in FL cases with BM involvement, compared to the cases without BM manifestation. We couldn't prove any association between the CX43 expression and the progression and grade of FL.

V. DISCUSSION

The tumor microenvironment plays a critical role in forming the tumor cell phenotype and in determining the tumor cell propagation. In this study we have demonstrated distinct difference in the reactive microenvironment of the LN and corresponding BM in FL, and we also observed considerable differences in the microenvironment of LNs in cases with and without BM infiltration.

The comparative analysis of the LN and BM showed differences as well as similarities between the non-malignant microenvironment of these two compartments of FLs. The amount of CD4⁺ T-lymphocytes and CD57⁺ T-lymphocytes were in the same range in the LNs and BMs, and all of the BM samples revealed a CD21⁺ and CD23⁺ FDC meshwork, which were present in the LN, but absent in the normal BM. These findings are highly consistent with the ‘importing’ hypothesis of Ghia et al, suggesting that the malignant cells in the BM are importing components from the LN necessary to create a microenvironment that permits the growth and survival of the tumor cells in the BM. The importance of the non-malignant microenvironment in the survival of neoplastic B-cells is also emphasized by in vitro studies, which concluded that FL cells were unable to proliferate in the absence of stromal cells and without CD40 receptor or cocultured CD4⁺ T-lymphocytes. In the microenvironment of FL the FDCs provide anti-apoptotic signals for B-lymphocytes, whereas the T-lymphocytes support B-cell differentiation by providing CD40L and cytokines.

The comparative analysis of LN and BM also revealed differences in the non-neoplastic as well as in the neoplastic compartment. Significantly more CD8⁺ cytotoxic T-lymphocytes, FOXP3⁺ regulatory T-lymphocytes, CD68⁺ macrophages and less PD1⁺ T-lymphocytes were detected in the BM compared to the matching LN sample. Furthermore, our results

confirmed previous findings that the malignant B-lymphocytes in the BM have lower grade and proliferation compared to the malignant cells in the LN. These findings could be explained by the different composition of the microenvironment in the BM, which may have an impact by selecting and eliminating the malignant B-lymphocytes with higher Ki67 index and immature phenotype: both CD8+ cytotoxic T-lymphocytes and CD68+ macrophages can eliminate malignant B-cells via direct antitumoral responses and chemokines. The higher number of Treg-lymphocytes and the higher Treg- and PD1+ T-lymphocyte ratio in the BM also suggests the selective force of the microenvironment, namely that Treg-lymphocytes can suppress B-cell proliferation leading to increased cell death. On the other hand, Treg-lymphocytes have the ability to downregulate B-cell proliferation, as they suppress PD1+ T-lymphocytes and their function causing reduced proliferation rate of the malignant cells in the BM.

Alternatively, the differences found between microenvironment in LN and BM could be the result of the partially different tumor cell populations of these two compartments. We and others previously showed that besides the LN-experienced FL cells there is a population of malignant cells in the BM which seems to arise from 'LN-inexperienced' clones. This observation suggests the possibility that the subsets of malignant cells originating from 'LN-inexperienced' clones may require different microenvironment and activate other types of the immune cells. This hypothesis is also supported by the lower amount of the PD1+ T-lymphocytes in the BM, which are important components of germinal centers providing signals for B-cells to differentiate into Ig-secreting cells.

The comparison of the microenvironment of LNs of FLs with and without BM infiltration showed significant differences between the two groups. Significantly more CD8⁺ and CD68⁺ cells were present in the biopsies of FLs lacking BM manifestation suggesting that the microenvironment may influence the migration and invasion of neoplastic cells to the BM. This finding is in line with results of several recent studies showing that CD8⁺ T-lymphocytes are predictive for a favorable survival, protecting from the progression of the disease through either direct cytotoxicity or cytokines, such as IFN- γ . The migration and adhesion of immature B-cells is regulated by IFN- γ , as it is interfering with the chemokine receptor-signaling pathway (IFN- γ – PI3K – PKC α), which results in inhibition of cytoskeleton rearrangement (actin polymerization), adhesion and B-cell migration.

The number of macrophages was significantly different between the cases with and without BM involvement. However, the role of macrophages in the prognosis and progression of FL is highly contradictory, our results indicate that the high amount of macrophages in the LN prevents disease progression and BM infiltration. According to Dave and colleagues, genes that are highly and preferentially expressed in macrophages were present in both of the immune response 1 and immune response 2 signatures, which predicted a favorable and unfavorable outcome in FL patients. Farinha et al suggest that CD68⁺ macrophages are independent predictors of survival and portend a worse outcome. In opposite of this findings other studies showed that CD68⁺ macrophages are associated with favorable time to progression and they are a component of the immunosurveillance pattern in follicular lymphoma. The highly conflicting results on the prognostic role of macrophages could be explained with different treatment protocols used in the studies. Possible mechanism of preventing from tumour progression is the release of reactive oxygen or nitrogen (nitrogen-monoxid, NO) intermediates which also contributes to the antitumoral activity of

macrophages. Moreover high NO concentrations also impair the activity of matrix metalloproteinases (MMPs), which play important roles in matrix remodeling and metastatic process. The results our comparative analysis of the microenvironment of LNs in cases with and without BM infiltration suggest that cytotoxic T-lymphocytes and macrophages play a relevant role in the prevention of tumor cell propagation and migration and that their reduced numbers in FL lymph nodes can be a negative prognostic maker to predict the BM involvement in FL.

The follicular dendritic cell (FDC) meshwork in secondary lymphoid follicles play important roles in the affinity maturation of activated B lymphocytes by selecting the highest affinity B cell clones for survival upon binding to antigens presented by FDC. The role of the FDC in the progression of FL was suggested in the first gene-expression profiling study, where FDC related genes appeared in the “Immune response 2” group which predicted an inferior outcome of the disease. Disappearance of the CD21+ FDC meshwork was observed in FL cases with transformation into DLBCL. The comparison of the FDC meshwork of LNs of FLs with and without BM infiltration revealed no considerable differences.

Among interaction through cell-cell adherence and cytokines gap junction direct cell-cell communication channels made up of connexin 43 (Cx43) protein couple FDC meshwork for a functional syncitium. Cx43 channels are present between FDC and some activated B lymphocytes. We observed a hyperplastic CX43 expression in the bone marrow infiltration of FL, which was prominent on the CD21+ FDC meshwork. In the lymph nodes the expression of CX43 showed correlation with the follicular dendritic cells.

VI. CONCLUSIONS

Original findings of the dissertation are:

- The grade and proliferation index of the tumor cells was significantly lower in the bone marrow compared to the lymph node.
- Tumor cells infiltrating the bone marrow create a microenvironment, and cell-cell interactions, which provides signals for the survival of the tumor cells.
- The results our comparative analysis of the microenvironment of LNs in cases with and without BM infiltration suggest that cytotoxic T-lymphocytes and macrophages play a relevant role in the prevention of tumor cell propagation and migration and that their reduced numbers in FL lymph nodes can be a negative prognostic maker to predict the BM involvement in FL.
- Presence of gap junctions, made up of connexin 43 (Cx43) protein showed no correlation with the tumor progression, and the grade of FL.

VII. PUBLICATION RECORD

VII. I. Publications in the subject of the dissertation

Rajnai H, Bodor C, Balogh Z, Gagy E, Csomor J, Krenacs T, Toth E, Matolcsy A
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IF: 2.857

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IF: 2.580

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A, Szepesi A. The Density of CD8+ T-Cell Infiltration And Expression of BCL2 Predicts
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10.1007/s00428-013-1519-9

IF: 2.676

VII. II. Publications in different subject

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IF: 1.272

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