Circulating tumor cells in multiple myeloma: immunophenotypic characteristics in a complicated course of the disease

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Abstract.

The purpose of the study was to compare the immunophenotypic profile of tumor plasma cells of the bone marrow (PlC BM) and circulating tumor plasma cells (CTC MM) in patients with multiple myeloma (MM), depending on the characteristics of the course of the disease.

Methods. Parallel immunophenotypic study of bone marrow aspirate and peripheral blood samples from 34 patients with MM was carried out by 4-color laser flow cytometry (FACSCalibur flow cytometer (Becton Dickinson, USA). PlC gate was isolated by expression of CD38+bright/SSC, analysis of expression of CD138, CD45, CD56, CD11c, CD28, CD117, CD33, CD79b, CD19, CD20.

Results. Patients with newly diagnosed MM were found to have two tumor subclones - bone marrow and circulating, differing in immunophenotypic characteristics and quantitative values in uncomplicated and complicated course of the disease. A direct correlation was found between the number of PlCs determined by cytological (myelogram) and cytometric methods for detecting myeloma cells in BM and PB. Immunophenotypic differences between the BM and CTC MM subclones of tumor plasma cells are most clearly presented in the groups of patients with uncomplicated MM, in MM with kidney damage, and in MM complicated by plasmacytomas, these differences are less pronounced. Associations of the expression of various immunophenotypic markers of tumor plasma cells in the BM and PB compartments with the massiveness of tumor lesions in the bone marrow, with different course of the disease were revealed, and immunophenotypic prognostic criteria for the development of local tumor growth were proposed.

Keywords: multiple myeloma, circulating tumor cells, flow cytometry.

Multiple myeloma (MM) is one of the most common (10% of all hematological malignancies) and a complex pathogenic malignant disease of the blood system. Tumor plasma cells are the substrate of the disease.

MM is a unique model for studying the mechanisms that determine the transition from a pre-malignant state to a malignant tumor, from an early and benign phase (monoclonal gammopathy of undetermined significance) to an intermediate phase (smoldering multiple myeloma) and the final advanced stage (symptomatic or refractory multiple myeloma).

Despite the advances achieved in recent years in the study of the pathogenesis of MM and the improvement of methods of immunochemotherapy, answers to some key questions have not yet been found: why does the "sleeping" clone become aggressive in some patients, but remains stable in others? What are the mechanisms causing refractoriness to therapy? In the scientific literature, there are data on the existence of two subclones of tumor plasma cells in MM: bone marrow and circulating in the peripheral blood. The latter, according to some authors [1], are present in 80% of MM cases. Circulating tumor cells (CTC) in MM are able to leave the bone marrow, move through the bloodstream and cause the spread of the disease, as well as the development of extramedullary relapses of the disease. To confirm this position, it is highly relevant to study the immunophenotypic characteristics of CTC, which will reveal the biological characteristics of this subclone, its contribution to the pathogenesis and prognosis of MM. This will make it possible to more actively influence this population of tumor cells with the help of chemotherapeutic and innovative drugs, as well as optimize supportive therapy.

Purpose of the study: To assess the immunophenotypic profile of tumor plasma cells of the bone marrow (PlC BM) and circulating tumor cells in patients with newly diagnosed MM (CTC MM) in comparison with the characteristics of the course of the disease.

Materials and methods. A parallel immunophenotypic study of BM and PB tumor plasma cells by flow cytometry was performed in 34 patients (16 women, 18 men) with newly diagnosed MM. The median age of the patients was 59 years (ranging from 30 to 85 years). All patients had Durie-Salmon stage 3 disease at the time of examination. Fifteen (44.1%) patients had Gk paraproteinemia, another 13 (38.2%) had Gλ. Paraprotein Ak in serum was detected in 2 (5.9%) patients, in 1 (2.9%) patient - paraprotein Aλ. Non-secreting MM was detected in 3 (8.9%) patients. Uncomplicated MM was diagnosed in 16 (45.7%) patients. 10 (29.4%) patients had plasmacytomas of various localization (8 patients - bone, 2 - extramedullary). In 9 (25.7%) patients, the disease was complicated by the development of CRF, of which 4 patients required renal replacement therapy. One patient had a combined lesion - CKD and plasmacytoma of the 6th thoracic vertebra. The average level of plasma cells in the bone marrow according to myelogram data was 27%.

Immunophenotypic study of bone marrow aspirate and peripheral blood samples was carried out by 4-color laser flow cytometry (2-laser flow cytometer FACSCalibur (Becton Dickinson, USA). The bone marrow aspirate was preliminarily filtered and washed twice with a phosphate-buffered saline (PBS) solution, then erythrocytes were lysed (PharmLyse lysis solution (Becton Dickinson, USA), then washed twice with PBS solution. The peripheral blood samples were pre-poured into several portions in volume of 500-1000 μl, erythrocytes were lysed, washed twice with PBS solution, and the sediment was concentrated in one test tube at 1500 rpm for 5 min. Further, the bone marrow and PB samples were incubated at room temperature for 30 min in the dark with monoclonal antibodies (MABs) of a certain specificity, conjugated with fluorescent dyes in various combinations. Used the following MABs: anti-CD38-FITC, anti-CD138-PE; anti-CD45-PerCP, APC; anti-CD56-PE, APC; anti-CD11c-PE, anti-CD28-PE, anti-CD79b-PE, anti-CD117-PerCP, anti-CD19-PerCP-Cy5.5; APC; anti-CD20-APC, anti-CD33-APC (Becton Dickinson, USA). The gating strategy and the selection of immunophenotypic markers were carried out in accordance with the recommendations of the European Consortium for Flow Cytometry (Euro-Flow, 2012), adapted for a 4-color flow cytometer. CD38, CD45 were used as "framework" markers for identification and quantitative assessment of plasma cells, along with the characteristics of light scattering. The main stages of the strategy of gating plasma cells in patients with MM were the isolation of a gate of CD38-positive cells with a high expression intensity - (bright) against the lateral light scattering channel (SSC), followed by assessment of the content of positive cells within this gate for antigens of interest. The criterion for positivity was the presence of antigen expression on the surface of more than 20% of tumor cells. The analysis included at least 500,000 events. Normal plasma cells BM and PB were excluded by the CD45 + (bright) CD19 + CD56- phenotype. The quantitative assessment of PlC was carried out according to the content of CD38 + (bright) cells in terms of all nucleated cells (NC) BM and PB.

Statistical processing of the results was carried out using the Statistica v. 8.0. with the calculation of the mean and squared error of the mean (M±m), the median, indicating the minimum and maximum values. The shape of the distribution of quantitative variables was analyzed using the Shapiro-Wilk test. To determine the statistical significance of differences in the mean values of quantitative indicators, the Student's t-test was used.

Results. Depending on the median level of BM tumor plasma cells (27% according to myelogram data), patients were divided into 2 groups: group 1 with a plasmacytosis level of less than 27% and group 2 with a plasmacytosis level of more than 27%. In these groups of patients, we characterized the immunophenotypic features of BM and CTC MM plasmocytes (Table 1).

Table 1. Immunophenotypic features of PlC BM and CTC MM depending on the level of plasmacytosis in BM according to myelogram data (% of positive cells).

Investigated parameters	PlC BM Less than 27% N=17	PlC BM More than 27% N=17	**P**	CTC MM Less than 27% N=17	CTC MM More than 27% N=17	P
The number of tumor cells (CD38+Bright), % of NC Of them:	**7.53±1.7**	**18.37±5.33**	**0.03**	**0.75±0.20**	**2.76±1.38**	0.08
CD138+	69.95±8.48	81.3±3.41	0.11	28.31±7.28	28.84±7.39	0.48
CD56+	65.09±7.74	59.45±8.57	0.31	38.24±4.69	41.36±7.53	0.36
CD117+	6.75±1.76	8.76±4.16	0.32	3.85±1.26	3.94±1.22	0.48
CD11c+	45.25±8.82	30.18±6.10	0.08	45.49±5.34	36.6±6.12	0.13
CD33+	**23.01±5.86**	**6.25±1.6**	**0.01**	16.20±3.46	11.76±3.09	0.17
CD28+	41.85±10.41	49.08±7.09	0.28	**19.5±4.40**	**30.12±7.78**	**0.02**
CD19+	**19.58±5.70**	**6.11±3.02**	**0.02**	**20.22±5.51**	**7.76±1.82**	**0.02**
CD20+	**3.92±1.08**	**19.97±7.27**	**0.02**	7.54±1.46	7.09±2.17	0.43
CD79b+	33.60±8.49	28.34±6.34	0.30	12.75±2.99	18.73±3.81	0.11

Significant (p=0.03) differences in the amount of PlC BM, determined cytometrically, were revealed: with plasmacytosis of more than 27%, according to myelogram data, the level of PlC BM was 2.5 times higher than with less (less than 27%) plasmacytosis. The same trend was observed when analyzing the content of tumor cells in PB: a more pronounced plasmacytosis according to myelogram data corresponded to a greater number of CTC MM detected cytometrically, and vice versa, a lower amount of CTC MM corresponded to a lower level of plasmacytosis.

Noteworthy is the higher expression of the adhesive molecule CD33 on PlC BM in the group of patients with plasmacytosis of less than 27%. No differences in the level of expression of this antigen on CTC MM were found in both groups. Expression of CD28 on CTC MM was detected in a significantly higher number of tumor cells in the group of patients with plasmacytosis in BM of more than 27%. There was a higher expression of CD19 on both PlC BM and CTC in the group of patients with plasmacytosis less than the median. At the same time, the expression of CD20 was significantly higher on tumor PlC BM in the group of patients with plasmacytosis of more than 27%. On CTC MM, the expression of this marker was approximately at the same level in both groups.

Thus, firstly, we have revealed a direct correlation between the number of PlCs determined by cytological (myelogram) and cytometric methods for detecting myeloma cells in BM and PB.

Second, we revealed some associations between the level of plasmacytosis and immunophenotypic features of BM and PB tumor cells: positive expression of CD33 on BM PlC was associated with a low level of plasmacytosis in BM; positive expression of CD20 on PlC BM was associated with a higher level of plasmacytosis; positive expression of CD19 on both PlC BM and CTC was associated with a low level of plasmacytosis. Positive expression of CD28 was expressed on PlC BM and did not depend on the level of plasmacytosis, while on CTC MM it was associated with a higher level.

Table 2. Immunophenotypic features of PlC BM and CTC MM in patients with uncomplicated course (% of positive cells).

Investigated parameters	PlC BM N=16	CTC MM N=16	**P**
The number of tumor cells (CD38+Bright), % of NC **Of them:**	**11.007±2.54**	**0.461±0.169**	**0.0003**
CD138+	**78.0 ±6.47**	**29.51±8.649**	**0.0000**
CD56+	**66.58±8.58**	**32.77±5.83**	**0.0012**
CD117+	5.21±1.71	3.982±1.349	0.282

CD11c+	40.96±7.01	40.09±5.82	0.461
CD33+	20.89±7.75	13.81±3.6	0.2006
CD28+	**49.29±7.44**	**26.38±5.9**	**0.0098**
CD19+	7.393±3.114	11.29±2.81	0.172
CD20+	15.41±6.39	8.44±2.3	0.151
CD79b+	34.59±7.06	22.774±4.115	0.074

It was found that in uncomplicated MM the number of BM tumor plasma cells was significantly higher (11.01±2.54%) than in PB (0.46±0.17) (P=0.0003). CTC MM was characterized by a significant decrease in the expression of CD138, CD56, CD28 compared to PlC BM.

At the time of the onset of the disease, 10 patients had plasmacytomas of various localization (8 - bone, 2 - extramedullary). Comparative characteristics of PlC BM and CTC MM in patients with MM complicated by plasmacytomas are presented in Table 3.

Table 3. Immunophenotypic features of PlC BM and CTC MM in patients with MM complicated by plasmacytomas (% of positive cells).

Investigated parameters	**PlC BM N=10**	**CTC MM N=10**	**p**
The number of tumor cells (CD38 + Bright), % of NC Of them:	3.182±1.497	1.188±0.314	0. 100
CD138+	**77.99±9.16**	**19.175±6.433**	**0.000**
CD56+	40.76±7.49	35.24±7.10	0.290
CD117+	9.73±4.6	4.608±1.49	0.144
CD11c+	45.644±12.07	43.51±8.2	0.439
CD33+	10.772±2.415	16.13±4.55	0.145

CD28+	51.99±14.45	29.33±10.22	0.09
CD19+	25.56±8.12	19.816±7.583	0.29
CD20+	4.68±1.37	7.53±2.07	0.121
CD79b+	**37.98±10.43**	**10.3±3.603**	**0.011**

The number of cytometrically detected tumor cells in BM in patients with plasmacytomas was approximately 3.5 times lower than in patients with uncomplicated MM. Moreover, we found that in this group of patients the total number of tumor plasma cells in BM did not differ significantly from that in PB. In general, in this group of patients, CTC MM was characterized by a significant decrease in the expression of CD138, CD79b compared to PlC BM, which may indicate the loss of the syndecan-1 molecule upon their exit from BM.

Thus, tumor PlC BM in plasmacytomas differed phenotypically from PlC BM in uncomplicated MM and were characterized by a vivid expression of CD138, a decreased level of expression of CD56, CD33, and an increase in the level of expression of CD19. CTC MM in patients with plasmacytomas were characterized by less pronounced expression of CD33, CD56, compared with bone marrow plasma cells. Probably, such immunophenotypic features as the loss of adhesion molecules and a lower level of cell differentiation predetermine the intensive release of tumor cells from BM into the bloodstream with further formation of plasmacytes of different localization. It is worth noting the stable expression level of the CD11c integrin molecule, which carries out intercellular interactions and chemotaxis in inflammatory processes both in BM tumor cells and in PB in patients with uncomplicated MM and with plasmacytomas.

It should be noted that in patients with MM with the presence of renal pathology, the highest content of tumor plasma cells was detected in BM and PB, when compared with patients with uncomplicated MM and with the presence of plasmacytes.

When comparing the immunophenotypic features of PlC BM and CTC MM in patients with kidney damage (Table 4), we revealed a significantly higher number of tumor cells in BM compared to PB.

Table 4. Immunophenotypic features of PlC BM and CTC MM in patients with MM complicated by kidney damage (% of positive cells).

Investigated parameters	PlC BM N=9	CTC MM N=9	p
The number of tumor cells CD38+Bright, (% of NC) Of them:	18.876±7.282	4.083±2.353	0.034
CD138+	**72.77±9.19**	**35.7±9.51**	**0.004**
CD56+	77.13±9.94	57.61±8.27	0.065
CD117+	3.32±1.51	3.94±1.67	0.387
CD11c+	**17.69±5.89**	**43.20±9.07**	**0.013**
CD33+	15.213±6.66	11.14±3.73	0.290
CD28+	19.9±7.64	15.02±6.18	0.297
CD19+	7.040±5.729	12.91±6.51	0.127
CD20+	20.1±10.97	6.02±1.83	0.108
CD79b+	19.83±9.14	9.72±3.24	0.147

CD138 expression was significantly lower on CTC MM than on PlC BM. Intergrin CD11c expression was significantly increased on CTC MM compared to PlC BM. The level of malignant myeloma cells expressing CD56 remained high in both BM and PB.

We have identified a general pattern characteristic of all compared groups of patients, which consists in a significant decrease in the expression of the syndecan-1 (CD138) molecule on CTC MM, as compared to bone marrow plasmocytes. Expression of syndecan-1 is characteristic exclusively of plasma cells, both normal and myeloma. Taking into account such characteristic features of MM as low proliferative activity of tumor cells (less than 1%) and recurrent course of the disease, it can be assumed that myeloma cells are capable of self-reproduction (MM stem cells), tumor regrowth, and resistance to therapy. It has been proven that the role of myeloma stem cells can be played by plasma cells that lack expression of CD138 [2]. According to the

results of some clinical studies, it was revealed that CD138-negative cells are characterized by a higher clonogenic activity and a higher expression of the differentiating B-cell antigens CD19 and CD20 [3]. With long-term cultivation in vitro, they are able to restore the CD138-positive population of MM cells, and are also resistant to chemotherapy. Some authors consider this population of MM cells to be less mature and possessing an increased proliferative potential [4–6].

Conclusions.

1. Immunophenotypic differences between subclones of tumor plasma cells BM and CTC MM are most clearly presented in groups of patients with uncomplicated MM and in MM with kidney damage.

2. In the group of patients with MM complicated by plasmacytomas, there were fewer significant differences between PlC BM and CTC MM, from which we can conclude about the probable biological identity of these subclones.

3. The peculiarities of clonal bone marrow PlC in plasmacytomas are significantly less pronounced expression of adhesion molecules and more pronounced expression of antigens of the less "mature" stage of differentiation, which may be additional prognostic criteria for the development of local tumor growth.

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