Erythrocyte deformability profile evaluated by laser diffractometry in patients with Multiple Myeloma: re-examination of our cases

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Highlights:

- Multiple myeloma is a complex pathology in the field of haematological neoplasms
- Multiple myeloma can often present changes in the hemorheological profile
- Several explanations of the behaviour of erythrocyte deformability in Multiple myeloma
- Hemorheological changes can interfere with the release of oxygen to peripheral tissues

Abstract

Background: Multiple myeloma is a complex pathology which represents about 10% of all haematological neoplasms. It can often present changes in the hemorheological profile and, in relation to this last topic, our aim was to evaluate the hemorheological profile in a group of patients with multiple myeloma, with reference to erythrocyte deformability. Methods: we have examined the profile of the erythrocyte deformability in multiple myeloma enrolling 29 patients; this profile, expressed as elongation index at several shear stress, has been obtained using the diffractometric method. Results: by comparing normal controls and MM patients, we observed a significant decrease in erythrocyte deformability, especially at low shear stresses; however, we did not observe any significant differences about this profile, subdividing the whole group of MM patients according to the degree of bone marrow plasma cell infiltration, to the red blood cell distribution width and to the serum values of LDH. Conclusions: we have taken in consideration all the hypotheses to explain the behaviour of the reduced erythrocyte deformability in multiple myeloma. Hemorheological changes, including erythrocyte deformability, can interfere with the physiological release of oxygen to various peripheral tissues, with possible important clinical implications.

Introduction

Plasma cell dyscrasias are heterogeneous clinical conditions that can be characterized by various hemorheological alterations. Up to now the information relating to this topic has not been numerous, and often they derive from studies with small groups of subjects.

In the last years, our attention in the hemorheological field has been addressed to the examination of the hemorheological profile in subjects with monoclonal gammopathy of undetermined significance (MGUS) [1, 2] and in patients with multiple myeloma (MM) [3-6], with reference to blood and plasma viscosity and erythrocyte deformability.

In the spectrum of plasmacellular dyscrasias, MGUS is defined by a serum monoclonal protein <3 gr/dl, <10% pathological plasma cells in bone marrow and the absence of end organ damage. MGUS may progress to MM, also through the stage known as smoldering multiple myeloma (SMM) [7].

MM is a heterogeneous neoplasm of plasma cells, and it represents the second most common hematological malignancy. It is characterized by a significant morbidity due to its and-organ damage, often referable to the deposition of abnormal immunoglobulin chains in different tissues.

The diagnosis of multiple myeloma requires 10% or more clonal plasma cells and/or ≥ 3 gr/dl of a serum monoclonal protein. Symptomatic myeloma is defined by the presence of end-organ destruction sings (CRAB: hypercalcemia, renal insufficiency, anaemia, bone lesions). In many MM patients there is a cluster of clinical, laboratory, radiological and pathological findings. A M-protein is observed in the serum or urine in about 97% of patients (IgG 50%, IgA 20%, light chain 20%, IgD, IgE, IgM and biclonal <10%); ~3% of cases are non-secretory. In 90% of MM patients there is a decrease in polyclonal Ig (<50% of normal). Other laboratory findings include hypercalcemia (20%), elevated creatinine (20-30%), hyperuricemia (>50%) and hypoalbuminemia (~15%).

Radiological studies show, at initial diagnosis, lytic lesions, osteoporosis, or fractures in 70% of cases.

The M-protein component in serum and urine is detected and evaluated by specific analysis (serum and urine electrophoresis and immunofixation). Furthermore, serum free light chain assay has a high sensitivity [8].

In MM, the high concentration of monoclonal plasma proteins alters plasma viscosity and then the whole blood viscosity; as for the blood viscosity, this is determined by haematocrit, plasma viscosity, red cell aggregation and erythrocyte deformability.

As it is known, the human erythrocyte is a non-nucleated, biconcave discoid shaped cell which has a diameter of 8 micron. During its 120 days lifespan in the circulation, it undergoes continuous passive shape changes: in arteries, it responds to shear stress becoming an ellipsoid; in the microcirculation, it must go through capillaries which have a transversal diameter that is a third of its own; in the spleen it changes shape, passing through the small endothelial slits that separate the cords from sinuses. The red blood cell has exclusive mechanical properties that render it greatly elastic, able to respond quickly to applied stresses and to undergo wide and reversible linear deformation conserving constant the area of its membrane surface: in fact, an increase of 4% is enough to cause the cellular lysis. This behaviour depends on its peculiar structural organization: its elasticity, that is the predisposition to maintain its own shape, depends on the protein composition while its viscosity is determined by the properties of the lipid structure. Erythrocyte membrane proteins are distinguished in integral and in peripheral proteins. The most represented integral proteins are the Band 3, the glycophorins and the glycosylated phosphatidylinositol-anchored proteins. The peripheral membrane proteins may be subdivided into anchoring and cytoskeletal proteins. Anchoring proteins are ankyrin, protein 4.1, protein 4.2, adducin, dermatin, p55, tropomodulin and tropomyosin while the cytoskeletal proteins are alpha and beta-spectrin and actin. The spectrin plays a dominant role in determining the stability of the red cell membrane, but also its flexibility. Erythrocyte membrane lipids are represented by cholesterol (28%), phospholipids

(62%), and glycolipids (5-10%). A property of the erythrocyte membrane is the asymmetric distribution of phospholipids: phosphatidylcholine (30%) and sphingomyelin (25%) are neutral molecules localized in the external leaflet while phosphatidylethanolamine (27%) and phosphatidylserine (14%) –negatively charged- are localized in the internal leaflet. The asymmetry is physiologically conserved through the action of some enzymes such as flippases (the phospholipid flippase ATP11C particularly) and floppases that localized within the membrane translocate lipids against gradient with an energy-dependent mechanism. The scramblases (the phospholipid scramblase 1 specifically), instead are erythrocyte membrane enzymes that move phospholipids in both directions dissipating the transmembrane gradient via an ATP-independent mechanism. As the mature erythrocyte cannot synthesize lipids by itself, so it repairs and renews its membrane using plasma fatty acids via the Lands pathway [9].

Whole blood is an example of a non-Newtonian fluid, because of its nature, it is a suspension of viscoelastic cellular components in plasma, therefore the blood viscosity in the circulation varies in relation with the shear rate; clinical and experimental results have clearly demonstrated that red cell deformability and plasma viscosity are very significant at high shear flow, while red cell aggregation occurs at low shear flow.

In the last years, some authors have described an altered lipid profile in the erythrocyte membrane [10] and in plasma of MM patients [11]. Instead, others have suggested that the increasing fatty acid synthase (FAS), observed in these patients, should be the focus for pharmacological treatment [12]. Moreover, it has been observed how the proliferation of human myeloma cells may be contained with the inhibition of the fatty acid synthesis [13]. Using the atomic force microscopy (AFM), that examines the morphological and ultrastructural properties of membrane erythrocyte at nanometer scales, other authors have observed significant differences in MM patients in comparison with controls [14].

The purpose of this research was to focus on the behaviour of the erythrocyte deformability profile in this malignant disease of plasma cells respect to a group of normal control, and to evaluate the

possible explanations for this hemorheological profile.

Materials and Methods

Population

We enrolled 29 patients (11 women and 18 men; mean age 67.9 ± 10.6 years) with a diagnosis of MM. The group included 9 IgA, 16 IgG, 1 IgM and 3 non-secretory MM. Sixteen patients were recently diagnosed and at the initial stage of therapy, 8 were on consolidation/conservation therapy, whereas 5 patients had achieved a complete remission. The principal laboratory findings in this group of patients are reported in Table1. The control group included 31 subjects (18 women and 13 men; age range 23-65 years) free of diseases based on clinical history, physical examination, electrocardiography, routine hematological and urine analysis.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital of Palermo (report N° 01/2022).

Methods

Venous blood samples were collected in the morning by venous puncture from the antecubital vein of fasting subjects and immediately transferred to anticoagulated glass tubes for the evaluation of the erythrocyte deformability: to evaluate this hemorheological parameter, a 30 µl of anticoagulated blood were mixed with 2 ml of dextran solution at a viscosity of 24 mPa. The measurement was obtained by using the diffractometer Rheodyn SSD of Myrenne, which measures the diffraction pattern of a laser beam passing through erythrocytes suspended in a viscous medium and deformed by a force with defined shear stress. The shear stresses employed were 6, 12, 30 and 60 Pa. The erythrocyte deformation was expressed as elongation index (EI) = (l − w/l + w) x100, where l = length and w = width of the erythrocytes.

Statistical Analysis

Data were expressed as means +/- S.D. The Student's t test for unpaired data was used to compare normal controls and MM patients. Afterwards, we compared the values of the elongation index evaluated for each shear stress in the several subgroups of MM patients; these subgroups have been established subdividing the entire group of MM patients according to the degree of bone marrow plasma cell infiltration, to the red blood cell distribution width and to the values of LDH, respectively.

Results

A significant decrease in erythrocyte deformability profile was observed in MM patients, compared to normal control (Table 2). This reduction is more evident at low shear stress and less at high shear stresses. In fact, at the shear stress of 60 Pa the percentage of reduction between normal controls and MM patients was almost by7%, at the shear stress of 30 Pa this percentage was nearly by 13% $\pm$ 2% , while at the shear stress of 12 Pa was almost of 24 $\pm$ 3%% and at the shear stress of 6 Pa resulted by 35 $\pm$ 4%. This marked percentage difference in EI between the group of normal subjects and that of MM patients was not detected systematically when the shear rate changed.

In attempt to clarify the anomalies of erythrocyte deformability observed in MM patients, we stratified the group of patients under examination in relation to some important clinical and laboratory parameters, such as bone marrow plasma cells infiltration rate, the hemometric parameter RDW and the value of LDH. No statistical difference regarding the elongation index at all shear stresses was found dividing the MM patients according respectively to the degree of bone marrow plasma cell infiltration (Table 3), to the red cell distribution width (Table 4) and to the serum levels of LDH (Table 5).

Discussion

MM is a rare disease, but the second most common hematologic malignancy. It is found in the spectrum of plasma cell dyscrasias, which often begins with monoclonal gammopathy of unknown significance (MGUS), to overt plasma cell leukemia and extramedullary myeloma, and it is associated with significant morbidity due to its end-organ destruction.

MM, as emerges from the various works aimed at investigating the hemorheological profile, may present various alterations relating to blood and plasma viscosity; however, data relating to alterations of the erythrocyte deformability begin to emerge.

In our analysis, we observed in MM patients an evident decrease of the erythrocyte deformability; preliminary, we evaluated the role of some factors, such as the bone marrow plasma cell infiltration, the red blood cell distribution width (RDW) and the serum levels of LDH: however, in this small cohort of MM patients, the trend in erythrocyte deformability was not influenced by these parameters, considered biomarkers and/or prognostic indicators of this hematological disease [15-24].

The reduction of erythrocyte deformability was more evident at low shear stresses; considering the methodological approach employed for the evaluation of this hemorheological parameter and the previous reports suggesting that the behavior of this parameter is referable especially to the biochemical composition of erythrocyte membrane found in MM patients, our results support the finding reported by other authors [25]. In fact, with this approach, at low and at very low shear stress, the variation of red cell deformability appears related to the membrane viscoelasticity. As it is known, physiologically, the decrease of red cell deformability may depend on a reduced surface/volume ratio, an increased cytosolic viscosity, or an alteration of the membrane dynamic properties; the latter refers to the qualitative and/or quantitative alteration of membrane lipids and proteins.

In MM patients, an alteration of the red cell membrane fatty acid profile has been found, and specifically, an increase in saturated fatty acids and in total polyunsaturated fatty acids (n-6), and a decrease in monounsaturated fatty acids, in total polyunsaturated fatty acids (n-3), in total trans fatty

acids, and in the ratio n-3/n-6 (10). The plasma fatty acid profile in MM patients shows an increase in saturated fatty acids, in monounsaturated fatty acids and in total polyunsaturated fatty acid (n-6) and a decrease in polyunsaturated fatty acids (n-3), in total trans fatty acids, and in the ratio n-3/n-6. These alterations are related to the functional alteration of the desaturases and the elongases, which have a specific role in the maintenance of the lipid network in biological membranes [26-29].

Besides to the alterations regarding the fatty acid profile observed in red cell membrane and in plasma of MM patients, interesting data underline that the patients with MM have a significant higher percentage of phosphatidylserine (PS) in red blood cell in comparison with healthy subjects (RBC 1.1+/- 0.58 vs 0.54+/-0.29% ; p<0.05) [30]. Moreover, the exposition of PS at the outer leaflet is present in all myeloma cell lines but also in primary myeloma samples [31]. It is not possible to exclude that the exposition of this phospholipid at the outer leaflet of erythrocyte membrane may be dependent on the functional alteration of ATP11C [32, 33] in the red blood cells of patents with MM. Therefore, further data are required on role played by this phospholipid in MM, because PS is an amino-phospholipid normally confined to the cytoplasmatic side of the erythrocyte membrane, but it is present, in high percentage, on the outer leaflet of the membrane, interfering with the deformability of red blood cells and determining the loss of membrane phospholipid asymmetry [34]. This physiological asymmetry has been suggested to control membrane curvature and other conditions of erythrocyte membrane structure; taking into the account, indeed, the compositional changes between the inner and outer leaflets of the lipid bilayer, it has been proposed that the outer leaflet shows a neutral curvature, whereas the inner leaflet may prefer negative curvature [35]. This asymmetric phospholipid distribution has been linked to the erythrocyte function [36]. Moreover, this peculiar erythrocyte lipid pattern permits the interaction of membrane lipid network with the integral proteins such as Band 3 and glycophorins.

By the way of the PS, in oncologic research, up to now in *in vitro* studies it has been demonstrated that some antitumor peptides, tested for the melanoma cells, need phosphatidylserine and cholesterol to fulfill their activity [37]. Moreover, it has been proved that in animal models the

stable fluorogenic peptide (termed Apo-15) appears to have phosphatidylserine as molecular target [38]. Other authors [39] have ascertained that the lipid composition of the cellular membrane of tumor cells has a specific role in the action of chemotherapeutic drugs.

Previously, the increase in fatty acid synthase (FAS) observed in human myeloma cell lines had suggested that fat acid synthase should be a target of the pharmacological treatment in MM [12]. In fact, these authors demonstrated an increased FAS expression in bone marrow samples obtained from MM patients but also in two human MM cell lines (U266 and RPMI8226). Thereafter, they treated in vitro U266 cells with cerulenin, a native inhibitor of FAS and this treatment reduced the metabolic activity/cell proliferation of the cell lines and activated the apoptosis. Further considerations [13] have been made considering the inhibition of fatty acid metabolism obtained with some drugs such as etomoxir (an inhibitor of the carnitine palmitoyl-transferase 1a) or orlistat (a pancreatic lipase inhibitor) in human myeloma cells. These molecules inhibited significantly the beta-oxidation and "de novo" fatty acid synthesis, without changes in the glucose metabolism; each molecule reduced by 40-70% the myeloma cell proliferation while the use of both molecules produced an additive inhibitory effect. All data regarding these metabolic alterations described in multiple myeloma and in particular the fatty acid synthesis have been reported by different authors [40].

By employing the atomic force microscopy (AFM), that is able to examine the changes in the morphological and biomechanical properties of the erythrocyte at a nanometer scale, Zhang et al [41] have noted that in MM patients the red cell surface architecture is extremely deformed and the centre of its surface is swollen so that the erythrocytes do not exhibit their normal biconcave profile, while the topographic images indicate that the red cell is flat and large in diameter and its surface shows large holes and several protrusions. The authors have, therefore, assessed that the surface topographic image, the height profile, and the surface ultrastructure clearly discriminate the erythrocyte of healthy subjects from those of MM patients. Liu et al [14] have, moreover, found in MM erythrocytes a marked irregularity of the outline of the histograms of the particle size extracted

from the surface ultrastructure. The altered membrane lipid profile, as well as the marked ultrastructural alterations observed using the AFM, may explain the decrease of erythrocyte deformability observed in MM.

An alternative hypothesis might be the presence of a paroxysmal nocturnal hemoglobinuria-like (PNH-like) defect in the erythrocyte membrane of MM patients [42-44]; the percentage of this laboratory finding, up to now, is more variable. In fact, while in the paper of Varma [45] this percentage was by 6.89%, in those of Meletis [43] and of Chatziantoniou [42] were respectively by 9.6% and 32.3% of the examined MM patients. Moreover, Terpos [44] studied 43 MM patients and detected this alteration in 56% of his group. It is well known that this defect is characterized by an altered synthesis of glycosylphosphatidylinositol, which is essential for the binding of some surface proteins, such as CD55 (decay accelerating factor or DAF) and CD59 (membrane inhibitor of reactive lysis or MIRL), to protect the red cells from intravascular lysis. Up to now, PNH-like clones have been also observed in different hematological diseases [46-48], but also in other clinical conditions [49, 50] and in patients with rheumatic diseases [51, such as rheumatoid arthritis, systemic lupus erythematosus, Sjogren syndrome, systemic sclerosis, vasculitis, dermatomyositis, ankylosing spondylitis, and mixed connective tissue diseases. In diabetes mellitus [52] it has been observed instead a decrease of the expression of CD55 and CD59 in endothelial cells incubated with high glucose concentration, normalized after co-incubation with verapamil.

Another aspect to underline is the role played by the modified erythrocyte deformability in the pathogenesis of anemia, detected in two thirds of patients with multiple myeloma. This clinical manifestation is the consequence of an increase in IL-6 production, in macrophage inflammatory protein-1α secretion by myeloma cells, in the apoptosis of erythrocyte precursors (immature erythroblasts). Moreover, low erythropoietin level has been reported, due partly to the kidney impairment, [53, 54] but also to the increase of serum level of hepcidin observed in MM patients [55, 56]. Furthermore, it is reasonable to suppose that the reduction of the red cell deformability observed in this hematological condition may facilitate their removal from blood circulation.

Conclusions

In conclusion, multiple myeloma is a hematological neoplasm that impairs the microcirculation because of the increased plasma viscosity, associated with a reduction in erythrocyte deformability; the behavior of these two hemorheological determinants, acting together and negatively in the microcirculation, affects the transport and the delivery of oxygen to the tissue [57-60], with significant clinical implications. Future studies are required to obtain a complete knowledge of the different hemorheological parameters in MM patients, aimed to assure them an optimal treatment strategy with significant impact on the quality of life.

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Tables:

Table 1: Erythrocyte deformability at different shear stresses in control subjects and in MM patients

	Control subjects			MM patients		
EI 60 Pa	45.19	±	2.52	42.34	±	4.65**
EI 30 Pa	41.67	±	2.20	36.43	±	5.07***
EI 12 Pa	33.29	±	2.18	25.54	±	5.31***
EI 6 Pa	24.94	±	2.32	16.25	±	4.87***

** $p < 0.01$, *** $p < 0.001$ vs control subjects

EI = Elongation Index

Table 2: Erythrocyte deformability at different shear stresses in MM patients subdivided according to the BMPC percentage

	BMPC % < median value			BMPC % ≥ median value		
EI 60 Pa	41.46	±	3.15	43.05	±	5.59
EI 30 Pa	35.22	±	3.61	37.41	±	5.94
EI 12 Pa	24.00	±	3.93	26.80	±	6.04
EI 6 Pa	14.74	±	3.68	17.48	±	5.47

BMPC = Bone Marrow Plasma-Cell

EI = Elongation Index

Table 3: Erythrocyte deformability at different shear stresses in MM patients subdivided according to the RDW percentage

	RDW % < median value			RDW % ≥ median value		
EI 60 Pa	42.11	±	3.50	42.55	±	5.64
EI 30 Pa	36.17	±	4.03	36.67	±	6.02
EI 12 Pa	25.29	±	4.28	25.78	±	6.27
EI 6 Pa	15.91	±	4.04	16.57	±	5.66

RDW = Red cell distribution width

EI = Elongation Index

Table 4: Erythrocyte deformability at different shear stresses in MM patients subdivided according to the plasma LDH level

	LDH < median value	LDH ≥ median value
EI 60 Pa	42.78 ± 4.93	41.93 ± 4.51
EI 30 Pa	36.84 ± 5.39	36.05 ± 4.92
EI 12 Pa	25.54 ± 5.47	25.55 ± 5.35
EI 6 Pa	15.88 ± 4.94	16.59 ± 4.95

LDH = Lactate dehydrogenase

EI = Elongation Index