Expression of Vascular Endothelial Growth Factor Receptors *VEGFR1* in Cultured Multiple Myeloma Cells: Correlation with Immunophenotype and Drug Resistance

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We studied the expression of genes encoding vascular endothelial growth factors *VEGF-A*, *VEGF-C*, *VEGF-D* and their receptors in cell cultures of human multiple myeloma IM9, RPMI 1640, RPMI 8226. The studied cells did not differ by the expression of growth factors. Expression of *VEGFR1* receptor was detected only in IM9 cells and *VEGFR2* and *VEGFR3* receptors were not expressed in multiple myeloma cells. A dependence between the aberrant CD45/CD56 phenotype of human multiple myeloma cells and *VEGFR1* expression in them was revealed. The only *VEGFR1*-positive IM9 cell culture was most resistant to Velcade (bortezomib).

Key Words: *multiple myeloma; gene expression; endothelial growth factors; cultures of human multiple myeloma cells*

Multiple myeloma (MM) is a malignant disease and a result of multistage process of transformation and clonal reproduction of plasma cells. The latter are the terminally differentiated hemopoietic B-cell lineage cells and are responsible for antibody synthesis and secretion in the body.

Molecular events underlying or typical of certain stages of MM development are not completely understood. It is known that MM cell population is characterized by phenotypic and morphological heterogeneity. There are data that expression of differentiation markers CD45, CD56, and CD19 by myeloma cells can correlate with relative severity of the disease [2]. The appearance of CD56 is more typical of myeloma cells, whereas normal polyclonal plasma cells, plasmacytes, in MGUS patients do not express this marker [8,10]. Expression of CD45 marker seems to be a positive prognostic factor. The lifespan of patients with CD45-positive plasma cells was longer than in CD45-negative patients; malignization of the disease was associated with a decrease in the expression of this marker.

Bone marrow plasma cells in MM patients express vascular endothelial growth factors VEGF and their receptors (VEGFR) [9]. Hence, signal systems activated as a result of co-expression of VEGF and the corresponding receptors can contribute to the regulation of plasma cell propagation. At the same time, changes in the expression of VEGF and their receptors during MM progression and the expression of these genes in different MM cell population were never studied.

Here we studied the expression of VEGF and VEGFR genes in cultures of human MM cells IM9, RPMI 1640, and RPMI 8226 differing by the expression of differentiation markers CD45, CD56, and CD19 and evaluated the sensitivity of these cultures to Velcade (bortezomib).

MATERIALS AND METHODS

Human MM cells IM9, RPMI 1640, and RPMI 8226 were obtained from All-Russian Collection of Cell

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Cultures (Institute of Cytology, Russian Academy of Sciences, St. Petersburg).

The cells were grown in RPMI-1640 medium (PanEko) containing 10% ECS (HyClone) and 50 μg/ml gentamicin and glutamine at 37°C and 5% CO_2.

Expression of differentiation markers on the surface of MM cells was studied using phycoerythrin (PE)-labeled antibodies: CD56-PE, CD45-PE, and CD19-PE, or FITC-labeled antibodies: CD138-FITC, CD38-FITC (BD). Analysis was performed on FACScan flow cytometer (Becton Dickinson). Plasma cell population gate was set on the basis of combination of forward (FSC) and side (SSC) light scatter and coexpression of CD38 and CD138. The data were processed statistically using WinMDI 2.8 software.

Cytotoxic activity of bortezomib was evaluated by the reaction of MTT reduction. The tumor cells (2×10^5/ml) were seeded to wells of 96-well plates (Costar; 150 μl per well) and incubated in RPMI-1640 with 10% ECS for 72 h at 37°C and 5% CO_2. Then, 20 μl 5% MTT (Sigma) was added to each well and after 3-h incubation at 37°C 60 μl DMSO (PanEko) was added and cell lysis (in %) was evaluated by optical density at 540 nm (Uniplan spectrophotometer).

RNA was isolated from cell cultures using TRI Reagent (Sigma) according to the standard protocol. cDNA synthesis and PCR with reverse transcription were carried out routinely [1]. Nucleotide sequences presented in Table 1 were used as specific primers.

RESULTS

Expression of some differentiation markers is an important characteristic of MM. We characterized three MM cell strains (IM9, RPMI 1640, and RPMI 8226) by the expression of differentiation markers CD38, CD138, CD45, CD56, and CD19 (Table 2).

CD138 is a specific marker of plasma cells and its expression suggests that cells of all three strains are plasmacytes. This is also confirmed by the absence of CD19, a specific B-cell marker expressed in normal plasmacytes at all stages of differentiation of these cells except malignant plasmacytes.

Expression of CD45 and CD56 markers was different in these cell strains: IM9 was the only cell strain expressing CD45, while CD56 was detected only in RPMI 8226 cells.

Thus, taking into account the known data on the interaction between the expression of differentiation markers CD45 and CD56 and malignization of plasma cells, the detected differences in the expression of differentiation markers probably suggest that the three studied cell strains are at different stages of malignant transformation and that IM9 cells are least transformed.

Using a semiquantitative PCR, we studied the expression of *VEGF* and *VEGFR* genes in these cells (Fig. 1). *VEGF-A* and *VEGF-D* genes were similarly expressed in all three strains; no expression of *VEGF-C* genes was detected. Similar data were reported previously [9]: no significant quantitative changes in *VEGF-A* expression were observed in plasma cells of patients with active and nonactive MM and MGUS patients, *i.e.* the level of *VEGF-A* expression did not depend on plasmacyte malignancy degree.

VEGFR1 mRNA was present only in IM9 cells, whereas expression of *VEGFR3* and *VEGFR2* genes was not detected (data on *VEGFR2* expression are

TABLE 1. Primers Used in the Study

Primer	Product length, b.p.
VEGF-A forward: 5'-AGT GGT GAA GTT CAT GGA TGT C-3'	
VEGF-A reverse: 5'-TGG TCT ATC TTT CTT TGG TCT G-3'	295
VEGF-C forward: 5'-CAG TTA CGG TCT GTG TCC AGT GTA G-3'	
VEGF-C reverse: 5'-GGA CAC ACA TGG AGG TTT AAA GAA G-3'	300
VEGF-D forward: 5'-TCC AGA TCC CTG AAG AAG ATC GCT G-3'	
VEGF-D reverse: 5'-ATG CTT TGC ACA TGC TGT TTT GC-3'	387
VEGFR1 forward: 5'-CAG CTC CAA ATA TCT AGC TGT ACC-3'	
VEGFR1 reverse: 5'-GAG GAC AAG AGT ATG GCC TCT AAG-3'	436
VEGFR2 forward: 5'-ATG GCC TCT TCT GTA AGA CAC TCA C-3'	
VEGFR2 reverse: 5'-AAG AAG GGT ATT CCC AGT TGA AGT C-3'	556
VEGFR3 forward: 5'-CTT GTC GGT ACC GGC GTC ATC-3'	
VEGFR3 reverse: 5'-GAG GAT CTT GAG CTC CGA CAT CAG- 3'	366

TABLE 2. Expression of Differentiation Markers in Cultures of Human MM Cells IM9, RPMI 1640, and RPMI 8226

Cell strain	CD138	CD38	CD45	CD56	CD19
IM9	+	—	+	—	—
RPMI 1640	+	+	—	±	—
RPMI 8226	+	+	—	±	—

not presented). Hence, co-expression of growth factor *VEGF-A* and its receptor *VEGFR1* was observed only in IM9 cells (Fig. 2), and therefore, the VEGF-A/VEGFR1-dependent system can be active in these cells.

Of the studied cell strains, IM9 is the only strain expressing CD45. The processes of cell signaling and proliferation are different in MM cells expressing and not expressing CD45. For instance, IL-6, the main regulator of myeloma cell proliferation, stimulated the growth of both $CD45^+$ and $CD45^-$ MM cells colonies. However, the growth of $CD45^-$ cells was also stimulated by other growth factors: IGF-1, FGF, HGF, and HB-EGF [3,5]. The authors hypothesized that poorer survival of $CD45^-$MM patients can be explained by the fact that their cells respond to not only IL-6, but also other growth factors.

The data obtained in our study on different status of the VEGF-dependent systems also attest to differences in cell signaling between $CD45^+$ and $CD45^-$ plasma cells.

The sensitivity of these cells to Velcade, the drug that is often used in the therapy of MM, was evaluated in MTT test (Fig. 3).

IM9 cells were most resistant to bortezomib. RPMI 1640 and RPMI 8226 cells also differed by their sensitivity to bortezomib: RPMI 8226 cells with aberrant phenotype $CD56^+/CD45^-$ were most sensitive to this drug.

Plasma cell-specific differentiation marker CD138 was least expressed in IM9 cells. Similar data were reported previously [6]: $CD138^-$ precursors were more resistant to some antitumor drugs (including bortezomib) than mature $CD138^+$ plasma cells.

Thus, IM9 cells strain, the only strain where *VEGFR1* mRNA is expressed and, hence, VEGF-A/VEGFR1 signaling could be active, appeared to be most resistant to bortezomib. Expression of differentiation markers ($CD45^+/CD56^-$) in these cells suggests that these cells could be at an earlier stage of malig-

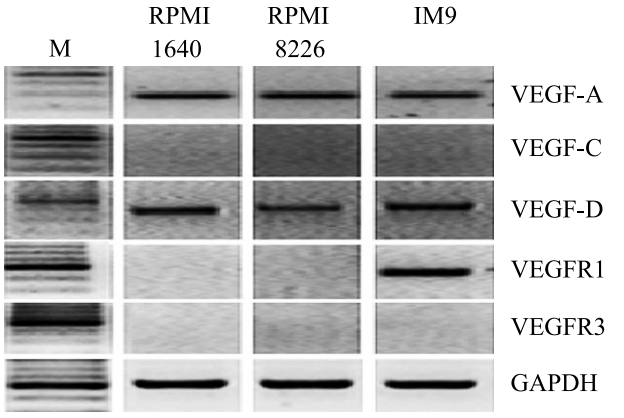

Fig. 1. Expression of mRNA of *VEGF* growth factor family and their receptors in cultured human MM cells. M: marker.

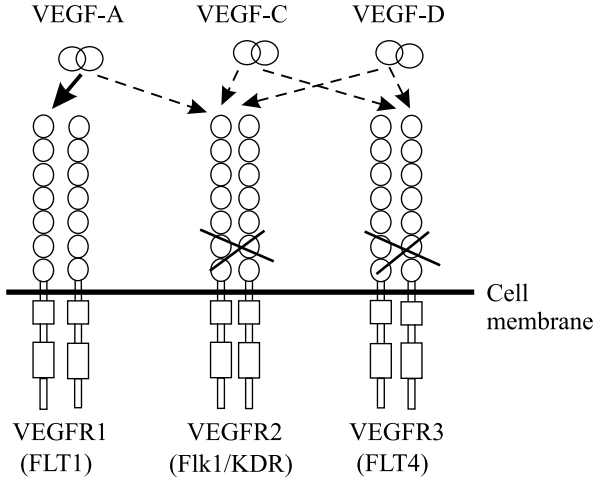

Fig. 2. Interaction of *VEGF* growth factors with their receptors.

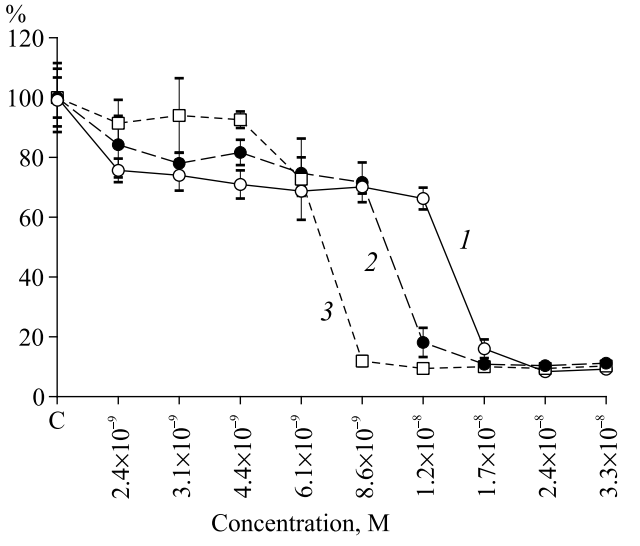

Fig. 3. Sensitivity of human MM cell cultures to proteasome inhibitor bortezomib. MTT-test. *1*) IM9; *2*) RPMI 1640; *3*) RPMI 8226. C: control.

nant transformation than RPMI 1640 and RPMI 8226 cells. It can be hypothesized that VEGFR1 expression is more typical of less transformed (according to some data) plasma cells.

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