**INTRODUCTION**

Multiple myeloma (MM) is a haematological disorder of clonal malignant plasma cells, accounts for 1-2% of all human cancers. Plasma cells are non-dividing cells of the B-cells, they secrete antibodies. Multiple myeloma cell is a malignant plasma cell. Multiple myeloma is characterised by proliferation of the tumour cells, mainly in the bone marrow, by production of large amounts of immunoglobulins and osteolytic lesions.

Interleukin-6 (IL-6) originally defined as B cell differentiation factor is a major proliferative factor for the malignant plasma cells (multiple myeloma cells).\(^1\) IL-6 exerts its biological function through binding to specific receptors on the membrane: (1) gp80 Interleukin-6 receptor alpha

**AIM:** To investigate the effect of exogenous IL-6 and IL-6 receptor antagonist (IL-6RA) on the proliferation and on the membrane expression of IL-6 receptor in human myeloma cell lines (a) having low spontaneous proliferation rate, (b) having high spontaneous proliferation rate.

**MATERIALS AND METHODS:** Three human multiple myeloma cell lines (RPMI-8226, OPM-2 and LP-1) were investigated in an in vitro model. Measured parameters: Viability, Membrane expression of IL-6 receptor, Cell proliferation.

**RESULTS:** IL-6 enhanced the proliferation in all myeloma cell lines with low spontaneous proliferation rate. In cell lines having high spontaneous proliferation IL-6 was ineffective. IL-6RA did not affect the spontaneous proliferation in the both groups. IL-6 up-regulated its membrane-bound receptor markedly in cells having low proliferation or high proliferation rate. IL-6RA reduced the membrane expression of IL-6 receptor down to an average of 55% (cells with low spontaneous proliferation ) resp. 77% (cells with high spontaneous proliferation) in the investigated cell lines.

**CONCLUSION:** The findings indicate that cells with low spontaneous proliferation have autocrine and paracrine regulation mechanisms, the cells with high spontaneous proliferation only an autocrine regulation mechanism. By the stromal cells produced IL-6 enhances the proliferation of myeloma cells with low spontaneous proliferation. This fact justifies the use of substances with cytostatic effect to inhibit the proliferation of myeloma cells.
Kovacs-Benke E. Proliferation, Multiple Myeloma, IL-6, IL-6 receptor antagonist

Parameters were measured after 24, 48 and 72 hours. To measure viability, membrane expression of IL-6 receptor the cells were cultured at a density of 0.5-0.7×10⁶ cells/mL. After 24 hours the cells were incubated with IL-6 (dose: 0.5 ng/10⁶ cells/100 μl). The parameter was measured after 24, 48 and 72 hours.

Treatment of cells with Interleukin-6 receptor antagonist (IL-6RA)
To measure viability and the membrane expression of IL-6 receptor the cells were cultured at a density of 0.5-0.7×10⁶ cells/mL. After 24 hours the cells were incubated with IL-6RA (dose: 0.2μg/10⁶ cells/mL) for 24, 48 and 72 hours.

To measure proliferation the cells were cultured at a density of 0.5-0.7×10⁶ cells/100 μl. After 24 hours the cells were incubated with IL-6RA (dose: 0.02μg/10⁶ cells/100 μl). The parameter was measured after 24, 48 and 72 hours.

Measurement of viability
The viabilities of the cultivated tumour cells were determined by using of 7-amoactinomycin D (7-AAD, No A1310, Life Technologies Europe, Switzerland), to exclude the non-viable cells in flow cytometric assays. The values are given in %.

Measurement of membrane expressions of IL-6 receptor (IL-6R)
For immunofluorescence staining 3×10⁵ cells/100 μl were incubated with 20 μl phycoerythrin (PE) conjugated monoclonal antibody (CD 126, Immunotech, France) for 30 min at 4°C. Then the cells were washed, sedimented and analysed in the FACSCalibur flow cytometer. For the expression of the membrane IL-6R (CD 126) the signal intensity (geometric mean of the fluorescence intensity x counts) was used as parameter.

The signal intensity of the treated samples was compared with that of untreated samples, which were taken as 100%.

Measurement of the proliferation
The proliferation was assessed using cell proliferation reagent WST-1 (Roche, Mannheim, Germany, No 1644 807). The colorimetric assay is based on the reduction of the tetrazolium salt WST-1 by viable cells. The reaction produces the soluble formazan salt. The quantity of the formazan dye is directly correlated to the number of the metabolically active cells. The proliferation rate was measured 1, 2 and 4 h after incubation with the reagents at time points 24, 48 and 72 h. The intra-sample variance of the untreated cells was <10% (3-8%).

Statistical analysis
For the evaluation of the parameters the Mann-Whitney U-test was used. The limit of significance was taken as p < 0.05.

RESULTS
Viability of the myeloma cells
The viabilities of control cells without the treatment lay in the range of 62-69 % for RPMI-8226, 81-85 % for LP-1 and 65-73 % for OPM-2. The values present the range of all investigations. IL-6 or IL-6RA did not alter the viability of treated cells 24, 48 and 72 hours after incubation.

The effect of exogenous Interleukin-6 and the IL-6 receptor antagonist on the proliferation of human myeloma cells having low/high spontaneous proliferation rate.
Figure 1 shows the values of untreated and treated samples in each investigated cell line. The values present (1) the mean of the absorbance and (2) the range of these absorbance values expressed
in percentage. The percentage of the untreated samples was taken as 100%.

**RPMI-8226**

There were carried out 6 independent measurements. The mean of absorbance was 0.62 (range: 0.3-1.7) in myeloma cells with low spontaneous proliferation rate and 2.15 (range: 2-2.4) for the myeloma cells with high spontaneous proliferation rate.

With IL-6 the proliferation lay between 120-171% for cells having low spontaneous proliferation rate and 96-107% for the myeloma cells having high spontaneous proliferation rate.

With IL-RA the values of spontaneous proliferation lay in the same range of untreated samples both in cells with low (98-113%) and high proliferation rate (80-110%).

**OPM-2**

Five independent measurements were carried out. Myeloma cells (1) with low spontaneous proliferation: mean of the absorbance 0.97 (range 0.4-1.5); (2) with high spontaneous proliferation rate: mean of the absorbance 2.0 (range 1.7-2.2).

Interleukin-6 increased the proliferation in the cells with low spontaneous proliferation rate on average up to 122 % (range 110-150%). In cells with high spontaneous proliferation rate the values lay in the range of untreated samples: 93-108%

IL-6RA did not affect spontaneous proliferation of the cells both in cells having low proliferation and high proliferation rate (low proliferation rate: 93-114%) (high proliferation rate: 81-105%).

**LP-1**

Five independent measurements were carried out. The mean of absorbance was 1.5 (range: 1.2-1.7) for the myeloma cells having low spontaneous proliferation and 2.5 (range: 2.2-2.9) for the myeloma cells having high spontaneous proliferation rate.

With IL-6 the proliferation lay between 115-140% for cells having low spontaneous proliferation and 98-108% for the myeloma cells having high spontaneous proliferation rate.

With IL-6RA the spontaneous proliferation of the cells was slightly decreased below that of the untreated samples (low proliferation rate: 85-102%) (high proliferation rate: 85-97%).

The effect of exogenous Interleukin-6 and the IL-6 receptor antagonist on the membrane expression of IL-6 receptor human myeloma cells with low/high spontaneous proliferation rate.

Table 1 presents the signal intensities as parameter for the expression of surface IL-6 receptor in myeloma cell lines RPMI-8226 and OPM-2. The values of four independent measurements are also expressed in percentage of untreated samples.

**RPMI-8226**

Exogenous Interleukin-6 up-regulated significantly (p<0.01) its membrane receptor in cells having low and in cells high spontaneous proliferation rate with the same intensity (range: 132-180% resp. 135-185%).

![Figure 1](image-url) The effect of IL-6 and IL-6RA on the proliferation of human myeloma cells having low or high proliferation rate. The cells were incubated with IL-6 (dose: 0.5ng/105cells/100 μl) or with IL-6RA (dose:0.02ug/105cells/100 μl). The parameter was measured after 24, 48 and 72 hours. The results of 5-6 independent measurements present the mean values of absorbance and the range of these values expressed in percentage. The percentage of the untreated samples was taken as 100%.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Low spontaneous proliferation</th>
<th>High spontaneous proliferation</th>
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<tbody>
<tr>
<td></td>
<td>IL-6 mean (range in %)</td>
<td>IL-6RA mean (range in %)</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>255,076 (132-180%)</td>
<td>282,484 (120-162%)</td>
</tr>
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<td>OPM-2</td>
<td>167,943 (128-145%)</td>
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The cells were incubated for 24 hours and 48 hours with IL-6 (5ng/10^5 cells/ml) or with IL-6RA (0.2ug/10^5cells/ml). For the expression of the membrane IL-6R the signal intensity was taken as parameter. The percentage of untreated samples was taken as 100%.

### Table 2 Schematic representation of the effect of IL-6 and IL-6RA on the proliferation and the membrane expression of IL-6R in human myeloma cell lines (n=3).

<table>
<thead>
<tr>
<th>Low spontaneous proliferation</th>
<th>High spontaneous proliferation</th>
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</thead>
<tbody>
<tr>
<td>untreated</td>
<td>IL-6 RA</td>
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</table>

<table>
<thead>
<tr>
<th>Proliferation</th>
<th>Untreated IL-6</th>
<th>Untreated IL-6RA</th>
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<tr>
<td>Membrane expression</td>
<td>~</td>
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Symbols = ~ unchanged, ↑ increased, ↓ reduced.
IL-6 receptor antagonist inhibited the membrane receptor expression down to 46-57% in cells having low spontaneous proliferation rate and down to 50-77% in cells having high spontaneous proliferation rate.

**OPM-2**

Exogenous Interleukin-6 led to an increased membrane expression of its receptor ($p < 0.01$). The values were in the similar range in cells with low spontaneous proliferation rate: 120-162% and in cells with high spontaneous proliferation rate 128-140%.

IL-6 receptor antagonist inhibited the membrane receptor expression down to 50-69% in cells having low spontaneous proliferation rate and down to 67-89% in cells having high spontaneous proliferation rate.

**DISCUSSION**

Cytokines can affect the cells via autocrine and/or paracrine regulation mechanisms. Cytokine binds to a specific receptor and causes change(s) in function or in development of the target cell. In both cases, i.e. autocrine and paracrine regulation mechanisms the expression of the membrane receptor is altered.

Interleukin-6 is an important cytokine in the proliferation of different MM cells via autocrine signalling (endogenous IL-6 production) and/or paracrine signalling (exogenous IL-6 production).

It was reported that IL-6 affects the growth of myeloma cells rather by paracrine than autocrine regulation mechanisms[9]. Bone marrow stromal cells represent a major source for the production of IL-6[10]. The serum values of IL-6 are on average in 50 % of multiple myeloma patients significantly higher than in healthy persons[11].

In the present study we investigated in an in vitro model the effect of exogenous IL-6 and IL-6 receptor antagonist on the proliferation and on the membrane expression of IL-6 receptor in three human myeloma cell lines: RPMI-8226, OPM2 and LP-1 having low or high spontaneous proliferation rate.

Multiple myeloma is characterised by slow proliferation of the tumour cells in the bone marrow. This means that myeloma cells have a low spontaneous proliferation rate. Our experimental studies presented that some cell passage of different cell lines also have a high proliferation rate[12].

The absorbance values in the cell lines RPMI-8226, OPM2, LP-1 having low or high spontaneous proliferation rate.

It is suggested that the high proliferation due to the increased endogenous IL-6 production (autocrine signalling). As the result of this increased production:

1. The membrane expression of IL-6 receptor will be up-regulated.

2. The complex IL-6/IL-6R initiates the signal transduction cascade through JAKs/STATs activating the RAS/MAPKs pathways leading to enhanced spontaneous proliferation of the myeloma cell.

We plan a study to measure the intracellular IL-6 production and the cell cycle phases in human myeloma cell lines with low and high spontaneous proliferation rate to confirm this hypothesis.

Table 2 presents schematically the results of this study and gives an overview of the findings: In cells with low spontaneous proliferation rate: (A) Interleukin-6 enhanced the cell-proliferation and up-regulated its membrane receptor markedly, (B) IL-6 receptor antagonist reduced the membrane receptor down to an average of 55%, the proliferation was not affected.

In cells with high spontaneous proliferation rate: (A) IL-6 did not enhance the proliferation, but up-regulated significantly its membrane receptor, (B) IL-6 receptor antagonist down-regulated the expression of the membrane receptor down to an average of 77%, the proliferation was not affected. These facts indicate that cells with low spontaneous proliferation have autocrine and paracrine regulation mechanisms, the cells with high spontaneous proliferation only an autocrine regulation mechanism.

In an earlier study we found that a substance using in the therapy of malignancy was more effective in the inhibition of cell-growth when the cells have a high spontaneous proliferation rate than those with low rate[12]. In a further investigation we confirmed this finding (unpublished results).

It is known that there are two important pathways against tumours: (1) to inhibit the tumour cell proliferation: cytostatic effect and/or (2) to induce the death of tumour cells: cytocidal effect (apoptosis or necrosis). The apoptosis is a physiological process in the life of healthy cells, whereas necrosis is a pathological process for tumour cells. It is clear that substance(s) to inhibit of cell proliferation are different from that of substance(s) with cytocidal effect.

**CONCLUSION**

The findings of this study motivate to further experimental studies and give important information in the therapy of myeloma. In the future therapy modalities should be specific-focused in interest of the patients. Serum values of IL-6 are elevated on average in 50 % of multiple myeloma patients, indicating disease activity. In these patients by the stromal cells produced IL-6 enhances the proliferation of myeloma cells. This fact justifies the use of substance(s) with cytocidal effect to inhibit the proliferation of myeloma cells.

**ACKNOWLEDGMENTS**

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**CONFLICT OF INTEREST**

The author declare no conflicts of interest.

**REFERENCES**


2. Urbanska-Rys H, Wierzbowksa A, Stepian H, Robak T. Relationship between circulating interleukin-10 (IL-10) with interleukin-6 (IL-6) type cytokines interleukin-11 (IL-11) oncostatin M (OSM) and soluble interleukin-6 receptor (sIL-6R) in patients with multiple myeloma. Eur Cytokine Netw 2000; 11: 445-51


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