

Low/High Proliferation Rate of Human Myeloma Cells, Investigation of Interleukin-6 and Interleukin-6 Receptor Antagonist.

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ABSTRACT

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.

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)^[1-2].

It produces by the malignant plasma cells (endogenous production) and by bone marrow stromal cells (exogenous production). In case of an endogenous production the IL-6 affects the same type of cell (autocrine regulation mechanism or autocrine signalling). In case of an exogenous production the target cell is near to the IL-6 produced cell (paracrine regulation mechanism or paracrine signalling).

Multiple myeloma cells can be classified into three groups depending on exogenous IL-6: (a) both proliferation and survival of the cells are dependent on IL-6, (b) only proliferation of the myeloma cells is affected by IL-6, (c) the cells are dependent on IL-6 only for survival, but not for proliferation, (d) cell lines that are independent of IL-6 both for survival and proliferation^[3].

IL-6 exerts its biological function through binding to specific receptors on the membrane: (1) gp80 Interleukin-6 receptor alpha

(IL-6R, also called CD126) and (2) signal-transducing component gp130 (also called CD130). The complex IL-6 + IL-6R + gp130 activate the intracellular signalling cascades: JAKs (Janus kinases) and STATs (Signal Transducer-Activator of Transcription) and the RAS/MAPKs (RAS/mitogen activated protein kinase) pathways.

The bone marrow provides unique environment for the malignant plasma cell via autocrine and paracrine interactions between malignant plasma cells and other marrow cells. Whereas normal bone marrow contains less than 5% plasma cells, bone marrow of patients with multiple myeloma contains over 10% plasma cells^[4]. Qing Yi *et al*^[5] reported that myeloma plasma cells constitute 10% to 90% of the total bone marrow cell count in patients with multiple myeloma.

High levels of peripheral blood circulating plasma cells are a specific risk factor for progression of multiple myeloma^[6]. Circulating myeloma cells in blood have a slower proliferation rate compared to those in the marrow. Higher proliferation rate means a poorer prognosis^[7].

Previously we analysed the cell cycle phases in human myeloma cell line RPMI-8226. The numbers of cells increased in G0/G1 phases and decrease in phases S and G2/M. With IL-6 the number of cells in phase G0/G1 was lower and markedly increased in the S phase^[8].

In the present study we investigated in an in vitro model the effect of exogenous IL-6 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. Additionally we asked how does IL-6 receptor antagonist (IL-6RA) affect the proliferation and the expression of the membrane IL-6 receptor in these cell lines. We used myeloma cell lines RPMI-8226, OPM-2 and LP-1. All three cell lines sourced from blood.

MATERIALS AND METHODS

Test substances

Recombinant human interleukin-6 (rh IL-6) were obtained from R & D Systems (No 206-IL, United Kingdom) and reconstituted in phosphate-buffered saline with 0.18% bovine serum albumin. Human interleukin-6 receptor antagonist (IL-6RA) was obtained from MedSystems (No BMS 135, Diagnostic GmbH, Austria) and reconstituted in phosphate-buffered saline.

Cells and culture condition

Human myeloma cell lines: RPMI-8226, LP-1, OPM-2 were obtained from DSMZ (Braunschweig, Germany). The cell lines derived from blood. The cells were cultivated in RPMI 1640 supplemented with 10-15% foetal calf serum, 2 mM L-glutamine and 1 % gentamicin in a humidified atmosphere with 5% CO₂ at 37°C. The doubling times of tumour cell lines were 60-70 hours for RPMI-8226, 70-80 hours for LP-1, 50-60 hours for OPM-2. For the measurement of the parameters the cell cultures were used within 4-6 weeks after thawing.

The proliferation and the viability were checked each day after splitting of the cells to distinguish cell cultures with low or high proliferation rate.

Treatment of cells with Interleukin-6 (IL-6)

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: 5 ng/10⁶ cells/mL). The parameters were measured after 24, 48 and 72 hours.

To measure proliferation the cells were cultured at a density of 0.5-0.7×10⁵ cells/100 µl. After 24 hours the cells were incubated with IL-6 (dose: 0.5 ng/10⁵ 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: 0.2ug/10⁶ cells/mL) for 24, 48 and 72 hours.

To measure proliferation the cells were cultured at a density of 0.5-0.7×10⁵ cells/100 µl. After 24 hours the cells were incubated with IL-6RA (dose: 0.02ug/10⁵ 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-aminoactinomycin 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 lines. 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-six 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 the 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%).

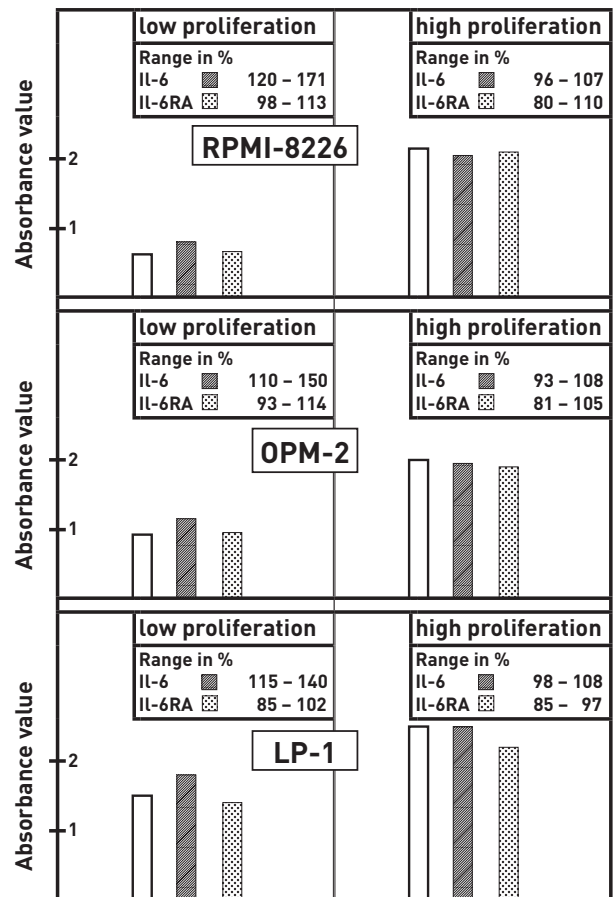


Figure 1 The effect of IL-6 and IL-6RA on the proliferation of human myeloma cells having low or high proliferation rate. The cells were incubated with IL-6 (dose: 0.5ng/105cells/100 µl) or with IL-6RA (dose:0.02ug/105cells/100 µl). The parameter was measured after 24, 48 and 72 hours. The results of 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 1 The effect of IL-6 and IL-6RA on the expression of the surface IL-6R in human myeloma cell lines.

Cell line	Low spontaneous proliferation			High spontaneous proliferation		
	untreated samples mean	IL-6 mean (range in%)	IL-6RA mean (range in%)	untreated samples mean	IL-6 mean (range in%)	IL-6RA mean (range in%)
RPMI-8226	255,076	393,382 (132-180%)	126,330 (46-57%)	587,876	952,321(135-85%)	363,949 (50-77%)
OPM-2	167,943	282,484 (120-162%)	114,021 (50-69%)	613,917	823,539(128-40%)	452,527 (67-89%)

The cells were incubated for 24 hours and 48 hours with IL-6 (5ng/10⁶ cells/ml) or with IL-6RA (0,2µg/10⁶ cells/ml). For the expression of the membrane IL-6R the signal intensity was taken as parameter. The percentage of ungreated 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).

	Low spontaneous proliferation			High spontaneous proliferation		
	untreated	IL-6	IL-6RA	untreated	IL-6	IL-6RA
Proliferation	~	↑	~	~	~	~
Membrane expression	~	↑	↓	~	↑	↓

Symbols = ~ unaltered, ↑ 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 spontaneous proliferation rate lay on average between 0.62-1.5. In cases of high proliferation rate the difference between the absorbance values were less: 2-2.5.

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: cytotoxic 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 cytotoxic effect.

CONCLUSION

The findings of this study motivate to further experimental studies and give important information in the therapy of myeloma. In the future the 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 cytostatic effect to inhibit the proliferation of myeloma cells.

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CONFLICT OF INTEREST

The author declare no conflicts of interest.

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