

Additive/Synergistic Effects of Interleukin-6 and Interleukin-10 on the Proliferation of Human Myeloma Cells

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ABSTRACT

Multiple myeloma (MM) is a haematological disorder of malignant plasma cells. Interleukin-6 (IL-6) is a major proliferative factor for the malignant plasma cells. Interleukin-10 (IL-10) is an Interleukin-6 related growth factor for multiple myeloma cells.

AIM: (1) to investigate the combination treatment with IL-6 and IL-10 on the proliferation in human myeloma cells, (2) to investigate the uptake of exogenous IL-6 by cultivated myeloma cells.

MATERIALS AND METHODS: Four human multiple myeloma cell lines were investigated in an in vitro model. Parameters: Viability, Cytokine production, Membrane expressions of IL-6 receptor and IL-10 receptor, Cell proliferation.

RESULTS: IL-6 increased the production of IL-10 (spontaneous: ND-79, induced up to 939 pg/ml). Both cytokines enhanced the proliferation in all cell lines. The proliferation rate for IL-6 lay between 120-138%, for IL-10 between 116-128%. The combination of IL-6 with IL-10 led to additive/synergistic effects in the proliferation of myeloma cells. IL-6 increased its receptor up to 192%. The up-regulation of the IL-10 receptor by IL-6 was less (110-129%). After 1 hour of incubation already 62-76 % of exogenous IL-6 was taken into the cells. At 24 hours the uptake of IL-6 was 84-90%.

CONCLUSION: The additive/synergistic effects of IL-6 and IL-10 have an important value in the treatment of multiple myeloma. The key position of the complex IL-6/IL-6R gives reasons for a specific-

targeted therapy with new perspectives for the future: When the cytokine1 induces the synthesis of cytokine2 leading to enhanced cell-proliferation then by the inhibition of cytokine1 the effect of cytokine2 also will be abolished.

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Key words: Interleukin-6; Interleukin-10; Multiple myeloma

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INTRODUCTION

Multiple myeloma (MM) is a haematological disorder of malignant plasma cells. It is characterised (A) by slow proliferation, mainly in the bone marrow, (B) by production of large amounts of immunoglobulins and (C) osteolytic lesions. Interleukin-6 (IL-6) originally defined as a B cell differentiation factor is produced by different cell types and certain tumour cells.

Interleukin-6 is a major proliferative factor for the malignant plasma cells. This cytokine is produced by the malignant plasma cells (endogenous production) and by bone marrow stromal cells (exogenous production).

In case of an endogenous production the cytokine affects the same type of cell (autocrine regulation mechanism). If the cytokine is released, then it is measurable in the supernatant. In case of an exogenous production the target cell is near to the cytokine produced cell (paracrine regulation mechanism).

The biological activity of IL-6 depends on binding to its specific receptors. These membrane receptors are composed of the glycoprotein gp80 Interleukin-6 receptor alpha (IL-6R, also called CD126) and the signal-transducing component gp130 (also called

CD 130). The complex IL-6 + IL-6R + gp130 initiate a signal transduction cascade through JAKs (Janus kinases) and STATs (Signal Transducer-Activator of Transcription) and activate the RAS/MAPKs (RAS/mitogen activated protein kinase) pathways. The JAK/STAT pathway is involved in the survival the RAS/MAPKs pathway is in the proliferation. The membrane receptors are released from the cells as soluble receptor proteins: sIL-6R as agonist and sgp130 as antagonist. The serum values of IL-6 in 35% or in 97% or in 42% of multiple myeloma patients are significantly higher than in healthy persons^[1-3]. The serum level of the sIL-6R is an important parameter in the evaluation and in the progression of multiple myeloma^[4,5].

Interleukin-10 (IL-10) is known as a human cytokine synthesis inhibitory factor (CSIF). It is produced by Thelper2 cells, monocytes/macrophages, B lymphocytes and some tumour cells. Interleukin-10 enhances the survival and proliferation of B cells. Three out of seven resp. four of ten human myeloma cell lines produce IL-10 spontaneously^[6,7].

Elevated IL-10 levels were detected in serum from about 50% of patients having multiple myeloma showing a relation to the clinical manifestation^[8]. IL-10 exerts its biologic effects on cells by specific cell surface receptors which are composed of two distinct subunits, activating the JAK/STAT signalling pathway.

In a previous publication it was reported, that Interleukin-6 leads to a marked production of Interleukin-10 in several human multiple myeloma cells and Interleukin-10 is an Interleukin-6 related growth factor for these tumour cells^[7]. It was a significant correlation between the proliferative effects of IL-6 and IL-10^[7].

The aim of this study was (1) to investigate the combination treatment with IL-6 and IL-10 on the proliferation in human myeloma cells; (2) to investigate the uptake of exogenous IL-6 by cultivated myeloma cells.

MATERIALS AND METHODS

Test substances

Recombinant human interleukin-6 (rh IL-6) and recombinant human interleukin-10 (rh IL-10) were obtained from R & D Systems (No 206-IL and No. 1064-IL, United Kingdom) and reconstituted in phosphate-buffered saline with 0.18% bovine serum albumin.

Cells and culture condition

Human myeloma cell lines: RPMI-8226, LP-1, OPM-2, COLO-677 were obtained from DSMZ (Braunschweig, Germany). Three cell lines derived from blood, COLO-677 from lymph node. The cytogenetic analysis of COLO-677 showed cross-contamination with cell line RPMI-8226: COLO-677 is derivative of RPMI-8226. All cell lines produced immunoglobulins of type IgG. The cells were cultivated in RPMI 1640 supplemented with 10-15% foetal calf serum, 2mM L-glutamine and 1 % gentamicin in a humidified atmosphere with 5% CO₂ at 37°C. The doubling times of tumour cell lines were between 35 and 80 hours. For the measurement of the parameters the cell cultures were used within 4-6 weeks after thawing.

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

To measure viability, IL-10 production, membrane expression of IL-6 receptor and membrane expression of IL-10 receptor the cells were cultured at a density of $0.5-0.7 \times 10^6$ cells/ml, except for COLO-677 (0.2×10^6 cells/mL). After 24 hours the cells were incubated with IL-6 (dose: 5 ng/ 10^6 cells/mL). The parameters were measured after 24, 48 and 72 hours.

To measure proliferation the cells were cultured at a density of $0.5-0.7 \times 10^5$ cells/100 μ l, except for COLO-677 (0.2×10^5 cells/100 μ l). After 24 hours the cells were incubated with IL-6 (dose: 0.5 ng/ 10^5 cells/100 μ l). The parameter was measured after 24, 48 and 72 hours.

Treatment of cells with Interleukin-10 (IL-10)

To measure viability the cells were cultured at a density of $0.5-0.7 \times 10^6$ cells/mL, except for COLO-677 (0.2×10^6 cells/mL). After 24 hours the cells were incubated with IL-10 (dose: 10 ng/ 10^6 cells/mL). The parameter was measured after 24, 48 and 72 hours.

To measure proliferation the cells were cultured at a density of $0.5-0.7 \times 10^5$ cells/100 μ l, except for COLO-677 (0.2×10^5 cells/100 μ l). After 24 hours the cells were incubated with IL-10 (dose: 2ng/ 10^5 cells/100 μ l). The parameter was measured after 24, 48 and 72 hours.

Combination treatment of cells with Interleukin-6 and Interleukin 10

To measure proliferation the cells were cultured. After 24 hours the cell were incubated with IL-6 (dose: 0.5 ng/ 10^5 cells/100 μ l). Four hours later IL-10 (dose: 2 ng/ 10^5 cells/100 μ l) was added to the cell cultures. 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 cytokine production and the amount of cytokine in the supernatant

The IL-10 production and the amount of IL-6 in the supernatant of the cultured cells were determined by chemiluminescent enzyme-labeled immunometric assay (Immulite 1000, Böhmann Laboratories AG, Switzerland). The lowest detectable level was 5pg/ml or 2pg/mL.

Measurement of membrane expressions of IL-6 receptor (IL-6R) and IL-10 receptor (IL-10R)

For immunofluorescence staining 3×10^5 cells/100 μ l were incubated with 20 μ l phycoerythrin (PE) conjugated monoclonal antibody (CD 126, Immunotech, France) or with 20 μ l phycoerythrin (PE) conjugated monoclonal antibody (CD 210, Pharmingen/BD Biosciences, Germany) for 30 min at 40C. Then the cells were washed, sedimented and analysed in the FACSCalibur flow cytometer. For the expression of the membrane IL-6R (CD 126) or IL-10R (CD210) 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 upper limit of the absorbance was 2.0–2.1. The intra-sample variance of the untreated cells was <10% (3–8%).

Statistical analysis

Four to six independent measurements were carried out. 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 49-71 %. The values present the range of all investigations. IL-6 and IL-10 did not alter the viability of treated cells 24, 48 and 72 hours after incubation.

The cell proliferation induced by IL-6 or IL-10 or IL-6+IL-10

The cells were treated for 24, 48 and 72 h. The proliferation was measured at each time point and the mean values were calculated.

Figure 1 shows the proliferation effects of these cytokines. The absorbance values are expressed as percentage of untreated samples (100%) and present the mean values with SD of 4-6 independent measurements. We have taken the lower limit as 110%.

IL-6 and IL-10 increased the proliferation in all investigated myeloma cell lines. The mean values lay for IL-6 between 120-138 %, for IL-10 between 116-128 % in the four cell lines. With IL-6 the values were significantly higher ($p < 0.05$) than those with IL-10 in cell lines RPMI-8226, LP-1.

The combination of IL-6 with IL-10 led to a higher proliferation rate than with separate treatment of IL-6 or IL-10 in all investigated cell lines. There were a significance (a) between the proliferative effects of IL-6 and IL-6 + IL-10 = $p < 0.01$; (b) between the proliferative effects of IL-10 and IL-6 + IL-10 = $p < 0.002$ in cell lines RPMI-8226, LP-1 and COLO-677.

We calculated the sum of the separate effects of IL-6 and IL-10 on cell proliferation.

In the cell lines RPMI-8226, LP-1 and COLO-677 the combined effects were on average higher than those of the sum of each separate effect. In two cell lines (RPMI-8226 and LP-1) these differences were significant ($p < 0.05$). In the cell line OPM-2 the proliferative effect of combined treatment with IL-6 + IL-10 corresponded to the sum of the separate effects of these two cytokines.

Production of IL-10 in myeloma cells

The IL-10 production of myeloma cells was measured without addition of IL-6 (spontaneous cytokine production) then 24 and 48 hours after treatment with IL-6 (induced cytokine production). Table 1 presents the IL-10 production in pg/ml. The values are the range of 4 independent measurements. Spontaneous IL-10 production was found in cell lines: RPMI-8226 > LP-1 > COLO-677. With IL-6 the IL-10 production was increased markedly in RPMI-8226. The induced IL-10 production was less in the cell lines LP-1 > OPM-2 = COLO-677.

Membrane expressions of IL-6 receptor (IL-6R) and IL-10 receptor (IL-10R) after IL-6 treatment

Table 1 presents the signal intensities as parameter for the expression of surface IL-6R and IL-10R in the four myeloma cell lines. This parameter was measured after 48 hours of incubation with exogenous IL-6. The values are the range of 4 independent measurements and expressed in percentage of the untreated samples as taken 100%.

IL-6 led to a marked increase of its receptor after 48 of incubation in all cell lines: OPM-2 = RPMI-8226 > LP-1 > COLO-677.

With exogenous IL-6 the membrane expression of IL-10R also was

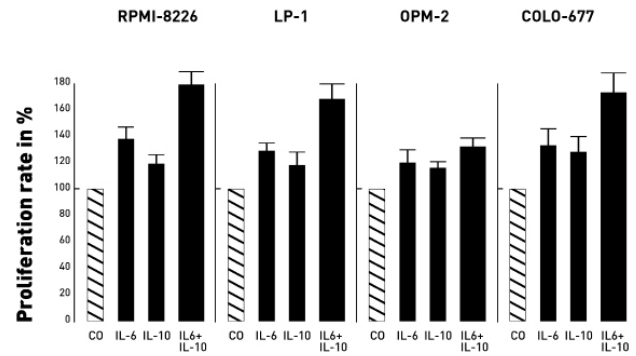


Figure 1 The effects of IL-6 or IL-10 or IL-6 + IL-10 on the proliferation of human myeloma cells. The cells were incubated with IL-6 (dose: $0.5 \text{ ng}/10^5 \text{ cells}/100 \text{ }\mu\text{l}$) or with IL-10 (dose: $2 \text{ ng}/10^5 \text{ cells}/100 \text{ }\mu\text{l}$). For the combination treatment the cells were incubated with IL-6 (dose: $0.5 \text{ ng}/10^5 \text{ cells}/100 \text{ }\mu\text{l}$) and four hours later with IL-10 (dose: $2 \text{ ng}/10^5 \text{ cells}/100 \text{ }\mu\text{l}$). The parameter was measured after 24, 48 and 72 hours. The results of 4-6 independent measurements are expressed as percentage of untreated samples (100%) and present the mean values with SD.

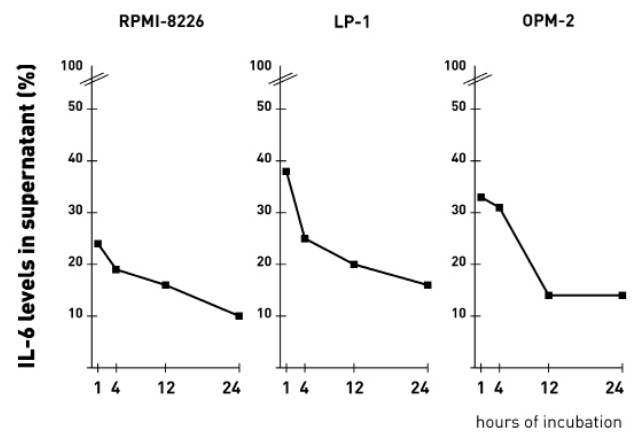


Figure 2 Time course (1, 4, 12 and 24 hours) of the uptake of exogenous IL-6 by human myeloma cells. The cells were treated with dose of $5 \text{ ng}/10^6 \text{ cells}/\text{mL}$. The results present the mean values of three independent measurements.

Table 1 The effect of exogenous IL-6 on the IL-10 production, on the membrane expressions of IL-6 receptor and IL-10 receptor.

Cell line	IL-10 production (pg/mL)		Membrane receptor (%)	
	Spontaneous	Induced	IL-6 receptor	IL-10 receptor
RPMI-8226	42-79	386-939	150-171	115-128
LP-1	9-48	41-208	138-150	111-120
OPM-2	ND	15-53	137-192	110-129
COLO-677	ND-20	11-49	120-132	117-125

To measure the IL-10 production the cells were incubated for 24 and 48 hours with IL-6 ($5 \text{ ng}/10^6 \text{ cells}/\text{mL}$). To measure the membrane expression of the receptors the cells were incubated for 48 hours with IL-6 ($5 \text{ ng}/10^6 \text{ cells}/\text{mL}$).

up-regulated in the four cell lines. The values were in the same range. The up-regulation of the IL-10 receptor was in all cases clearly less than that of the IL-6 receptors in all cases.

Uptake of exogenous IL-6 by myeloma cells

The uptake of IL-6 was investigated in 3 myeloma cell lines RPMI-8226, LP-1, OPM-2 after 1, 4, 12 and 24 hours of incubation with exogenous IL-6. The cells were treated with dose of $5 \text{ ng}/10^6 \text{ cells}/\text{mL}$. Figure 2 presents the mean values of three independent measurements in % at different time points. The amounts of IL-6 in

the supernatant lay in range between 38-24 % already after 1 hour of incubation. The values were reduced down to 16-10% during the next 23 hours.

DISCUSSION

It is known that some cytokines can induce the synthesis of others. In an earlier study it was reported that IL-6 as an important factor in the survival and in the proliferation of myeloma cells leads to a markedly increased IL-10 production in several human multiple myeloma cell lines^[7]. The results have shown that IL-10 is an IL-6 related factor in the proliferation of myeloma cells: There was a significant correlation between the proliferative effects of IL-6 and IL-10^[7].

In this study we investigated whether a synergistic effect exists between IL-6 and IL-10 regarding proliferation in myeloma cells.

There are three types of interactions between medications: Additive-effect, synergistic-effect and antagonistic-effect; (1) Additive: The effect of two chemicals/agents is equal to the sum of the effect of the two chemical/agents taken separately; (2) Synergistic: The effect of two chemical/agents taken together is greater than the sum of their separate effect.

In the present investigation we confirmed earlier findings: IL-6 increased the IL-10 production in all investigated ($n=4$) human myeloma cell lines. The IL-10 production was marked in RPMI-8226. The induced IL-10 production was considerably less in the cell lines LP-1>OPM-2=COLO-677.

Reasons: (1) The response of the myeloma cells to IL-6 regarding IL-10 production can be different; (2) For the correct evaluation of the amount of the endogenous produced IL-10 it is necessary to measure the quantity of IL-10 both intracellular and in the supernatant.

Both cytokines enhanced the proliferation of human myeloma cells. The combination of IL-6 with IL-10 led to a significantly higher proliferation rate than with separate treatment of these cytokines.

We calculated the sum of the separate effects of IL-6 and IL-10 on cell proliferation.

In three cell lines (RPMI-8226, LP-1, COLO-677) the combined effects were on average higher than those of the sum of each separate effect. In the cell lines RPMI-8226 and LP-1 these differences were significant ($p<0.05$). In the cell line OPM-2 the final effect was equal to the sum of the effect of IL-6 and IL-10. The results indicate that IL-6 and IL-10 have additive and synergistic effects in the proliferation of human myeloma cell lines.

The JAK/STAT pathway is an intracellular signalling pathway that mediates the effects of a large number of growth factors and cytokines as IL-6 and IL-10. These bind to receptors to initiate the signalling.

I. The complex IL-6/IL-6R/gp130 activates the JAK/STAT and RAS/MAPK pathways^[9].

In the present study all myeloma cell lines proliferated spontaneously with a doubling time between 35-80 hours. (1) In the supernatant of the cultivated cells IL-6 was not detectable. (2) The surface IL-6 receptor was expressed. These findings indicate in these spontaneously proliferating cells an endogenous IL-6 production with an autocrine regulation mechanism.

Exogenous IL-6 up-regulated its (3) membrane receptors and (4) led to increases of proliferation in the investigated cell lines. These findings indicate that the cell lines RPMI-8226, LP-1, OPM-2 and COLO-677 also have an additional paracrine regulation mechanism.

II. IL-10 activates the JAK-STAT signalling pathway by its specific cell surface receptor^[10]. (1) Myeloma cells produce IL-10,

measurable in the supernatant of the cultivated cells. These findings indicate an endogenous IL-10 production with an autocrine regulation mechanism; (2) Exogenous IL-10 leads to an enhanced proliferation in myeloma cells, supporting an additional paracrine regulation mechanism.

In a previous study the time course of the membrane expression of IL-6 receptor and the IL-10 production in multiple myeloma cell line RPMI-8226 was investigated^[7]. The kinetic curve has been shown that the IL-10 production increased parallel and proportionally to the membrane expression of IL-6 receptor at each time point. This investigation was repeated in this study with the myeloma cell line LP-1. The findings were the same as that of RPMI-8226 (results are not shown).

Results presented that after 1 hour of incubation already 62-76 % of the exogenous IL-6 was taken into the cells. After 24 hours of incubation the uptake of IL-6 was practically complete (84-90%).

Effectors mechanism of IL-6 on the IL-10 production in human myeloma cells

(A) Myeloma cells produce IL-6 endogenously. The IL-6 up-regulates its membrane receptor (IL-6R). The complex IL-6/IL-6R leads to endogenous IL-10 production inducing the up-regulation of IL-10 receptor. IL-10 binds to its receptor and the complex initiates the JAK/STAT pathway.

(B) Bone marrow stem cells produce IL-6 exogenously. The IL-6 up-regulates its membrane receptor (IL-6R). The complex IL-6/IL-6R leads to endogenous IL-10 production. The IL-10 receptor will be up-regulated. IL-10 binds to its receptor and the complex initiates the JAK/STAT pathway.

We plan a study to measure the time course of (1) the spontaneous intracellular IL-10 production and (2) the intracellular IL-10 production induced by IL-6 and (3) the membrane expression of IL-10 receptor after incubation with IL-10. The results could confirm the working hypothesis.

CONCLUSION

In the present investigation we confirmed earlier findings: IL-6 increased the IL-10 production, both cytokines enhanced the proliferation of human myeloma cells.

The combination of IL-6 with IL-10 led to additive resp. to synergistic effects in the proliferation of myeloma cells. The findings have important value in the therapy of multiple myeloma.

The complex IL-6/IL-6R has a key position in the increased production of IL-10. This justifies a specific-targeted therapy: When the cytokine1 induces the synthesis of cytokine2 leading to enhanced cell-proliferation then by the inhibition of cytokine1 the effect of cytokine2 also will be abolished.

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CONFLICT OF INTERESTS

The author has no conflicts of interest to declare.

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