3

Effects of Recombinant Human Tumor Necrosis Factor-α and Its Combination with Native Human Leukocyte Interferon-α on P3-X63-Ag8.653 Mouse Myeloma Cell Growth

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1. Introduction

Multiple myeloma (MM) is a malignant B-cell disease, characterized by uncontrolled proliferation of differentiated plasma cells in bone marrow (BM), osteolytic bone lesions, monoclonal protein peaks in serum or urine and suppression of normal antibody production. Patients with MM usually present with a number of clinical signs and symptoms, including fatigue, infection, severe bone pain, bone fractures, hypercalcaemia, and renal disease (Bommert et al., 2006; Raman et al., 2007; Redzepovic et al., 2008). Despite clinical responses produced by conventional chemotherapy, radiotherapy, and an increasing number of new compounds and improvements in supportive therapy, MM remains largely incurable (Katzel et al., 2007; Ozdemir et al., 2004; Redzepovic et al., 2008).

Tumor necrosis factor-α (TNF-α) is a known survival and proliferation factor for myeloma cell lines. It is produced by tumor and stromal cells in BM of patients with MM and induces tumor cell proliferation, migration, survival, drug resistance, and blood vessel proliferation (Harrison et al., 2006; Jourdan et al., 1999). Although TNF-α secreted by MM cells does not induce significant growth and drug resistance in tumor cells, it stimulates interleukin-6 (IL-6) secretion in bone marrow stromal cells more potently than vascular endothelial growth factor (VEGF) or transforming growth factor-β (TGF-β) (Yasui et al., 2005). Out of BM environment, circulating TNF-α levels are increased in MM patients with manifest bone disease, whose osteoblasts constitutively overexpress receptors for TNF-related apoptosis-inducing ligand, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) (Silvestris et al., 2004).

In our previous study, treatment with native human leukocyte interferon-α (nhIFN-α), recombinant human interferon-α2a (rhIFN-α2a) and recombinant human interferon-α2b (rhIFN-α2b) in doses of 500 IU/ml, 1000/ml and 2000 IU/ml resulted in differential effects on P3-X63-Ag8.653 mouse myeloma cells. A statistically significant dose-dependent decrease in

cell viability was observed in P3-X63-Ag8.653 mouse myeloma cells treated with nhIFN-α in comparison with matched negative controls. Conversely, a statistically significant increase in cell viability was observed in P3-X63-Ag8.653 mouse myeloma cells treated with rhIFN-α2a and rhIFN-α2b. This increase in cell viability occurred only in relation to their matched negative controls and was not dose-dependent (Plesničar et al., 2009). The differences in effects on P3-X63-Ag8.653 mouse myeloma cell viability between nhIFN-α and recombinant interferons probably occurred because nhIFN-α is composed of many subtypes of nhIFN-α and also contains trace amounts of IFN-γ, TNF-α, TNF-β, interleukin (IL)-1α, IL-1β, IL-2, IL-6, granulocyte-macrophage colony-stimulating factor and platelet-derived growth factor. Therefore, the decrease of cell viability in nhIFN-α treated P3-X63-Ag8.653 mouse myeloma cell cultures may have occurred in consequence of a synergistic effect of the various cytokines in nhIFN-α preparation. The quantities and the synergistic effect of the cytokines in nhIFN-α preparation are very small at lower concentrations and probably become active only at higher concentrations, thus accounting for the dose-dependent effects observed on cell growth (Plesničar et al., 2009; Šantak et al., 2007, Zidovec & Mažuran, 1999). In contrast to nhIFN-α, rhIFN-α2a and rhIFN-α2b are each preparations of only one subtype of IFN-α. The increase in cell viability in P3-X63-Ag8.653 mouse myeloma cell culture groups treated with rhIFN-α2a and rhIFN-α2b in our study was in accordance with a number of reports suggesting that IFN-α could induce uncontrolled cell proliferation in some patients with MM (Plesničar et al., 2009; Puthier et al., 2001; Sawamura et al., 1992). Interferon-α has been recognized as a survival factor in MM in some studies, the data supporting this claim are based on the results of studies using recombinant interferons-α (Cheriyath et al., 2007; Ferlin-Bezombes et al., 1998; Puthier et al., 2001).

The P3-X63-Ag8.653 mouse myeloma cell line is routinely cultured in several types of growth media. The cells in P3-X63-Ag8.653 mouse myeloma cell line propagate in suspension and do not secrete immunoglobulin. They can be used as fusion partners for producing hybridomas and show lymphocyte-like morphology (Kearney et al., 1979). Human myeloma blood cells were described as carrying surface membrane monoclonal or idiotypic immunoglobulin structures, and were morphologically classified as atypical small to medium-sized lymphocytes, lymphoblasts, lymphoplasmacytoid, plasmacytoid cells or myeloma cells (Mellstedt et al., 1984). With regard to morphology and despite the differences, it may be possible that P3-X63-Ag8.653 mouse myeloma cells, growing in suspension cell cultures, share at least some common properties with circulating clonogenic CD19 positive and CD138 negative cells, described as phenotypically resembling mature B cells (Cremer et al., 2001; Matsui et al., 2004).

The aim of the present study was to compare the effects of different doses of rTNF-α on the *in-vitro* growth of P3-X63-Ag8.653 mouse myeloma cells. Additionally, in one cell culture study group the aim was also to compare the effect of a combination of rTNF-α and nhIFN-α with the effects of corresponding doses of single cytokines on the *in-vitro* growth of P3-X63-Ag8.653 mouse myeloma cells.

2. Materials and methods

2.1 P3-X63-Ag8.653 mouse myeloma cell preparation

The P3-X63-Ag8.653 mouse myeloma cells were retrieved from the frozen storage at -80 °C and cultured in 25 cm² cell culture flasks (Cole Parmer, Vernon Hills, IL, USA) in Dulbecco's

modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) and gentamycin (Krka, tovarna zdravil, d. d., Novo Mesto, Slovenia). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 hours.

In preparation for this study, P3-X63-Ag8.653 mouse myeloma cell growth curves on logarithmic scale plots were established when the most convenient seeding density to be used was determined. Various time zero values ranged from 5 X 10^3 to 6 X 10^4 cells/ml and S-shaped growth curves were observed after cell concentrations measured in 24 hour intervals over the 96 hours were plotted on Keuffel & Esser 464970 Semi-Logarithmic Grids general purpose drawing paper. Time zero density of 10^4 P3-X63-Ag8.653 mouse myeloma cells/ml was found to be the most appropriate for the study. With the use of Keuffel & Esser 464970 graph paper it was also possible to observe that P3-X63-Ag8.653 mouse myeloma cells started to enter the log phase in approximately 24 hours (one day) and the plateau phase in approximately 72 hours (three days).

2.2 Recombinant human tumor necrosis factor-α, native human interferon-α and cell culture study groups

Actively growing P3-X63.Ag8.653 mouse myeloma cells were seeded into 35 mm Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated in each study group with three different concentrations of rTNF-α (Prospecbio, East Brunswick, NJ, USA). In the first study group the cells were incubated with 2, 10 and 20 IU/ml of rTNF-α, in the second with 30, 40 and 50 IU/ml of rTNF-α, in the third with 100, 200 and 300 IU/ml of rTNF-α, and in the fourth study group with 400, 800 and 1200 IU/ml of rTNF-α. After the experiments with rTNF-α, in one study group the cells were incubated with a combination of 10 IU/ml of rTNF-α and 2000 IU/ML of nhIFN-α (Institute of Immunology Inc., Zagreb, Croatia). The combination was compared to the corresponding doses of single cytokines. Matched negative controls that consisted of P3-X63-Ag8.653 mouse myeloma cells cultured in the absence of cytokines were established for each of the different cytokine study groups. All experiments were replicated five times and 20 Petri dishes were used for each cytokine cell culture study group and their negative controls. Cell viability was assessed by Trypan blue exclusion in 24 hour intervals (days 1-4).

2.3 Statistical analysis

In proliferating cell lines, it is difficult to distinguish between early cell loss and prolonged lag phase in which cells are still adapting to their new environment (Wilson, 1994). The effects of different concentrations of rTNF-α and its combination with nhIFN-α were thus estimated with the use of whole growth curves to reduce the possibility of misinterpretation. Statistical evaluation was performed using SPSS® software package, version 12.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Analysis of variance (ANOVA) was used to assess the differences between and within different treatment groups and their negative control groups. *P*-values of < 0.05 were considered to be statistically significant.

3. Results

Treatment of P3-X63-Ag8.653 mouse myeloma cells with rTNF-α showed a statistically significant reduction in cell viability in comparison with negative control cells. The

reduction in cell viability occurred in dose-dependent manner, with higher doses having a greater effect (Table 1, Figures 1-4). Treatment of P3-X63-Ag8.653 mouse myeloma cells with 400, 800 and 1200 IU/ml of rTNF-α showed a complete cessation of cell growth (Table 1), with the cells being unable to enter the log phase of the S-shaped growth curve (Figure 4). Treatment of P3-X63-Ag8.653 mouse myeloma cells with a combination of rTNF-α and nhIFN-α showed a statistically significant reduction in cell viability in comparison with negative control cells and cells treated exclusively with either rTNF-α or nhIFN-α. The addition of a small dose of rTNF-α (10 IU/ml) to the treatment of P3-X63-Ag8.653 mouse myeloma cells with a relatively high dose of nhIFN-α (2000 IU/ml) resulted in a further, although small, reduction in cell viability (Table 1, Figure 5).

Cell study group	**Cytokine type**	**Cytokine concentration (IU/ml)**	**No. of cells (10^4/ml) Mean +/- SE over days 0-4**	**Statistical significance[1]**
rTNF-α (1)		Negative control	20.400 +/- 0.83306	P = 0.001
	rTNF-α	2	20.187 +/- 1.65707	
	rTNF-α	10	16.675 +/- 1.99607	
	rTNF-α	20	13.425 +/- 0.54608	
rTNF-α (2)		Negative control	23.3250 +/- 1.96338	P = 0.000
	rTNF-α	30	12.8750 +/- 0.55234	
	rTNF-α	40	10.4250 +/- 0.71709	
	rTNF-α	50	9.3750 +/- 0.20444	
rTNF-α (3)		Negative control	22.9000 +/- 0.79096	P = 0.000
	rTNF-α	100	8.5125 +/- 0.32918	
	rTNF-α	200	5.9625 +/- 0.34403	
	rTNF-α	300	1.6750 +/- 0.07500	
rTNF-α (4)		Negative control	22.2000 +/- 1.16011	P = 0.000
	rTNF-α	400	1.0375 +/- 0.05078	
	rTNF-α	800	0.9250 +/- 0.06673	
	rTNF-α	1200	0.6750 +/- 0.03644	
rTNF-α and **nhIFN-α**		Negative control	25.9625 +/- 0.62581	P = 0.000
	rTNF-α	10	19.6375 +/- 1.07591	
	nhIFN-α	2000	5.8000 +/- 0.10346	
	rTNF-α and **nhIFN-α**	10 and 2000	4.8500 +/- 0.35609	

[1]Comparison between active treatment overall and the corresponding negative control in each cell study group.

Table 1. Effect of recombinant human tumor necrosis factor-α (rTNF-α) at different concentrations, native human interferon-α (nhIFN-α) and the combination of rTNF-α and nhIFN-α on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth.

As expected, when the cell numbers for each cell study group of P3-X63-Ag8.653 mouse myeloma cells treated with the different concentrations of rTNF-α and with the combination of rTNF-α and nhIFN-α or their negative controls were plotted on a logarithmic scale for the

whole 96 hours (four days) period over which cell viability was measured, the growth curves were S-shaped. P3-X63-Ag8.653 mouse myeloma cells started to enter the log phase at approximately 24 hours (one day) and reached the plateau phase at approximately 72 hours (three days) from incubation with the different concentrations of rTNF-α and with the combination of rTNF-α and nhIFN-α. The intermediate portions (log phase) of the S-shaped growth curves, approximately between 24 and 72 hours, were linear. The slopes of the growth curves in the treated cell culture study groups and their negative controls were not identical (Figures 1-5).

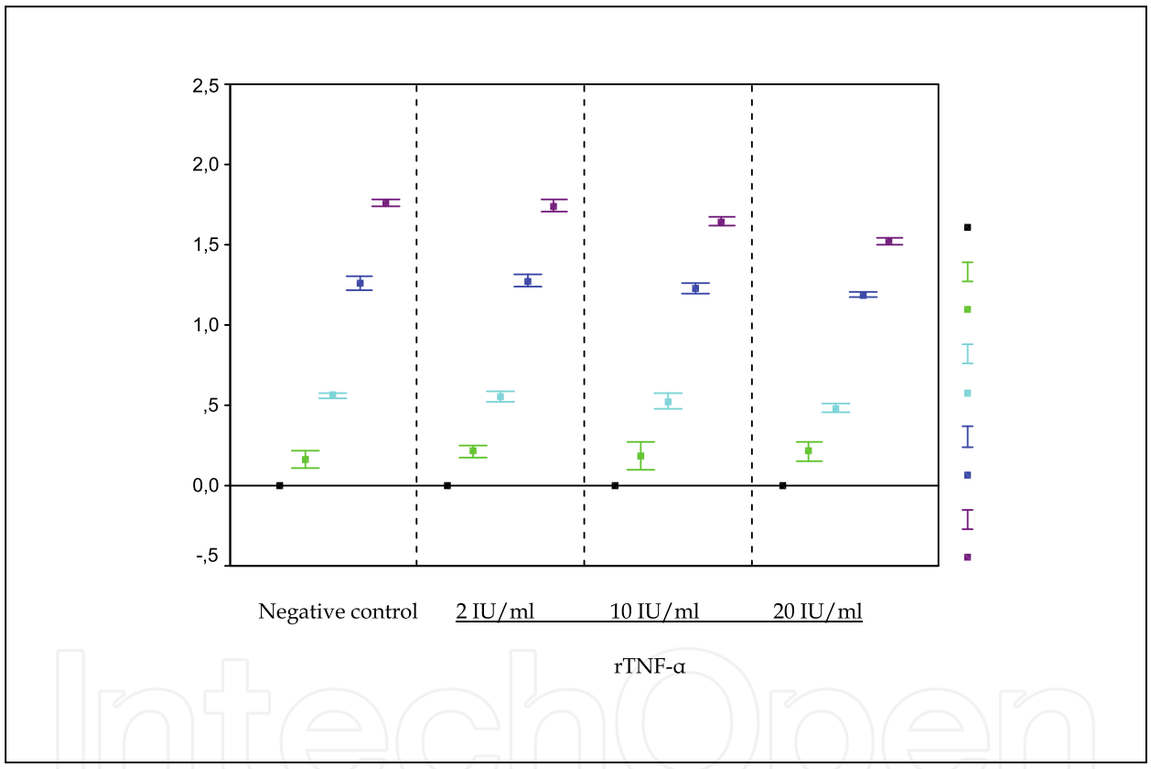

Fig. 1. The effect of 2, 10 and 20 IU/ml of human recombinant TNF-α (rTNF-α) on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth plotted on a logarithmic scale (logarithmic number of P3-X63-Ag8.653 cells/ml), showing a dose-dependent reduction in cell viability over four days of treatment. The reduction in cell growth observed with rTNF-α was statistically significant in comparison with negative control (P = 0.001). Legend: black, day 0; green, day 1; light blue, day 2; dark blue, day 3; violet, day 4.

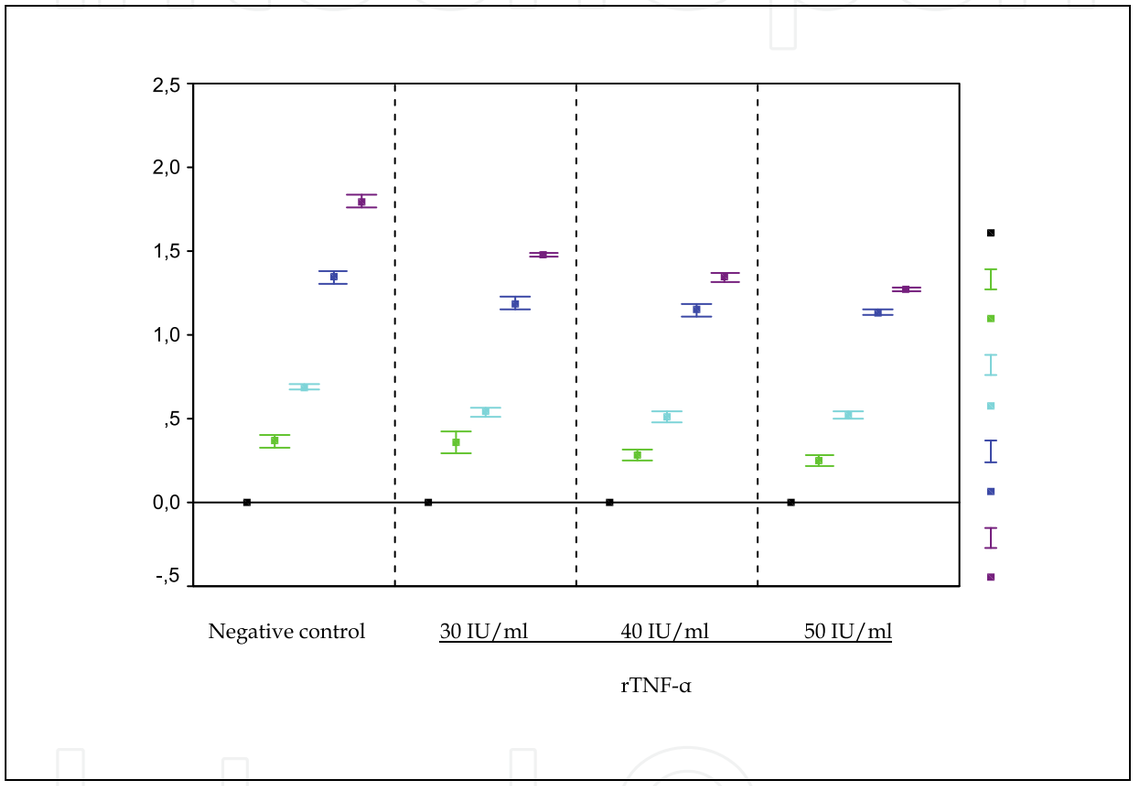

Fig. 2. The effect of 30, 40 and 50 IU/ml of human recombinant tumor necrosis factor-α (rTNF-α) on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth plotted on a logarithmic scale (logarithmic number of P3-X63-Ag8.653 cells/ml), showing a dose-dependent reduction in cell viability over four days of treatment. The reduction in cell growth observed with rTNF-α was statistically significant in comparison with negative control *(P* = 0.000). Legend: black, day 0; green, day 1; light blue, day 2; dark blue, day 3; violet, day 4.

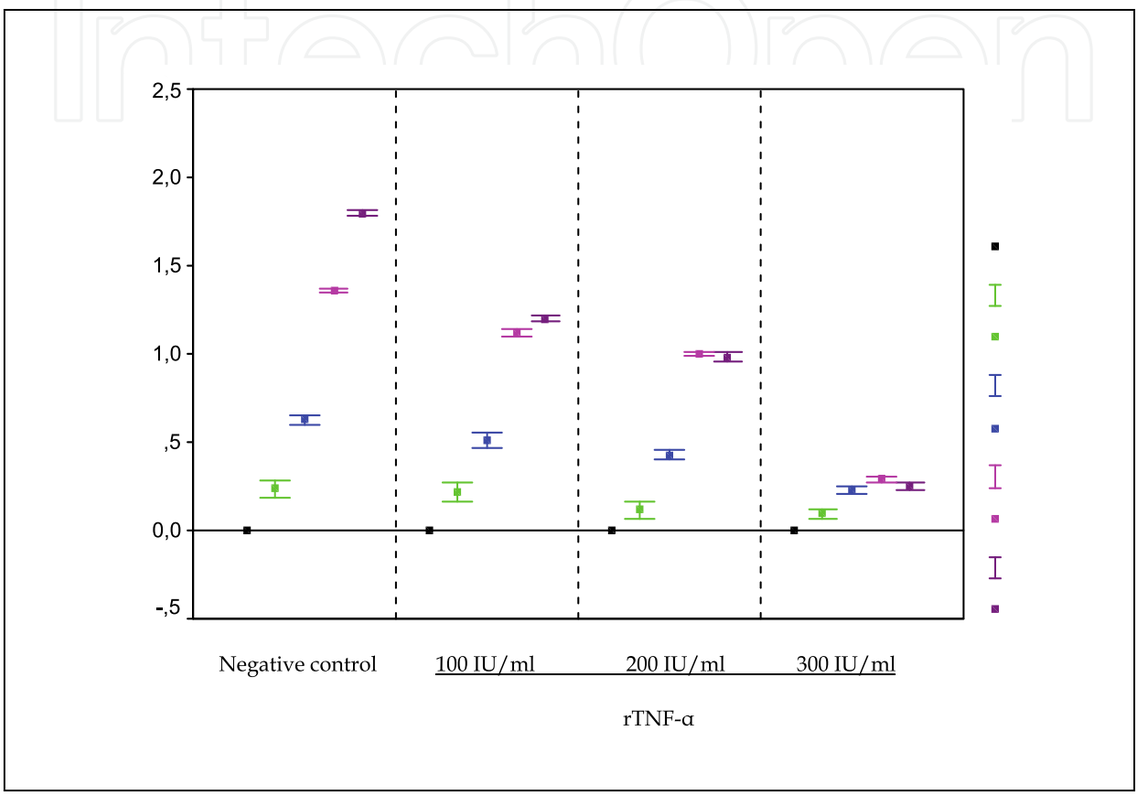

Fig. 3. The effect of 100, 200 and 300 IU/ml of human recombinant TNF-α (rTNF-α) on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth plotted on a logarithmic scale (logarithmic number of P3-X63-Ag8.653 cells/ml), showing a dose-dependent reduction in cell viability over four days of treatment. The reduction in cell growth observed with rTNF-α was statistically significant in comparison with negative control *(P* = 0.000). Legend: black, day 0; green, day 1; blue, day 2; light violet, day 3; dark violet, day 4.

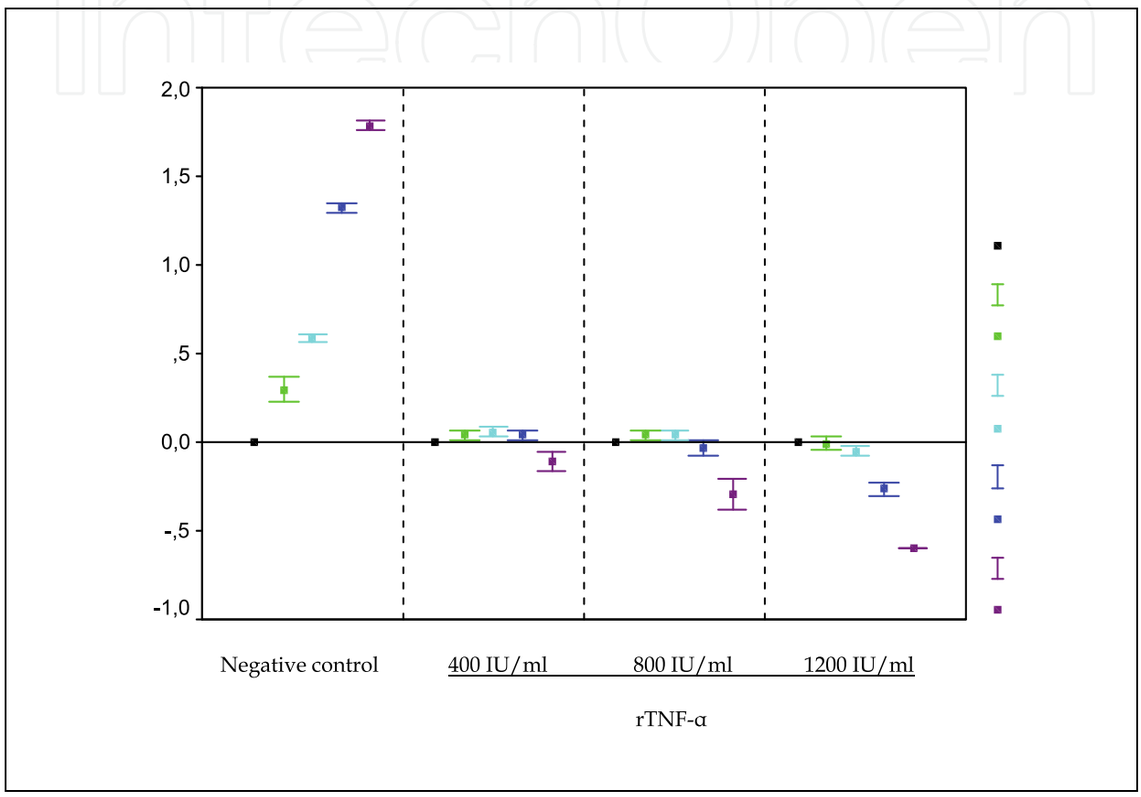

Fig. 4. The effect of 400, 800 and 1200 IU/ml of human recombinant TNF-α (rTNF-α) on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth plotted on a logarithmic scale (logarithmic number of P3-X63-Ag8.653 cells/ml), showing a dose-dependent reduction in cell viability over four days of treatment. The reduction in cell growth observed with rTNF-α was statistically significant in comparison with negative control ($P = 0.000$). Legend: black, day 0; green, day 1; light blue, day 2; dark blue, day 3; violet, day 4.

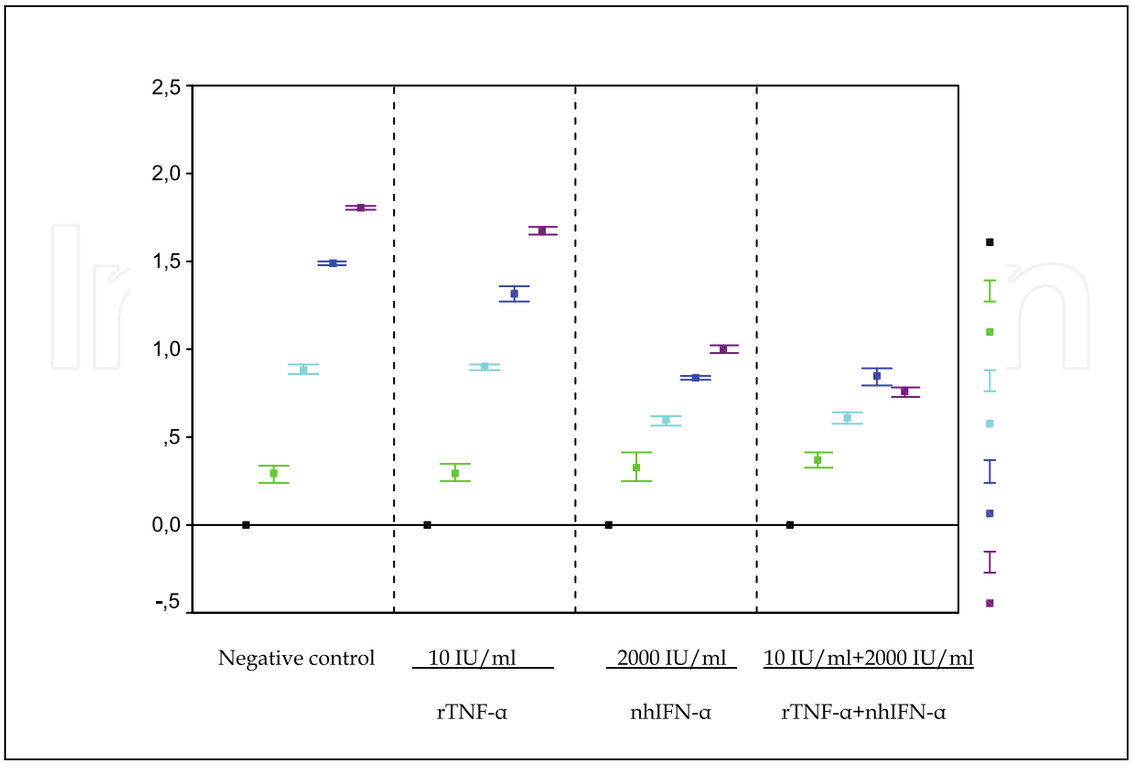

Fig. 5. The effect of 10 IU/ml of human recombinant TNF-α (rTNF-α), 2000 IU/ML of native human leukocyte interferon-α (nhIFN-α), and of a combination of 10 IU/ml of rTNF-α and 2000 IU/ML of nhIFN-α on *in-vitro* P3-X63-Ag8.653 mouse myeloma cell growth plotted on a logarithmic scale (logarithmic number of P3-X63-Ag8.653 cells/ml), showing a reduction in cell viability over four days of treatment. The reduction in cell growth observed after treatment with rTNF-α, nhIFN-α, and with the combination of rTNF-α and nhIFN-α, was statistically significant in comparison with negative control (P = 0.000). Legend: black, day 0; green, day 1; light blue, day 2; dark blue, day 3; violet, day 4.

4. Discussion

Treatment with rTNF-α at different doses had a negative effect on *in vitro* P3-X63-Ag8.653 mouse myeloma cell growth. A statistically significant dose-dependent reduction in cell viability was observed in P3-X63-Ag8.653 mouse myeloma cells treated with rTNF-α in comparison with negative controls. Additionally, a slightly enhanced reduction in P3-X63-Ag8.653 mouse myeloma cell viability was observed in cells treated with the combination of rTNF-α and nhIFN-α, in comparison with negative controls and cells treated exclusively with either rTNF-α or nhIFN-α.

The results of this study are surprising, as the treatment of P3-X63-Ag8.653 mouse myeloma with rTNF-α showed statistically significant reduction in cell viability compared with untreated control cells, with higher doses having greater effect. These results are in contradiction with numerous reports describing TNF-α as a survival and proliferation factor in MM (Harrison et al., 2006; Hideshima et al., 2004; Jourdan et al., 1999; Yasui et al., 2006;

Westendorf et al., 1996). However, TNF-α has previously also been described as an apoptotic factor in MM. The TNF-dependent trimerization of TNF receptors may lead to the recruitment of TRADD (TNF-R1 associated death domain protein), FADD (Fas-associated death domain protein) or RIP (receptor interacting protein) adapter proteins, resulting in activation and acceleration of caspase cascade (Baker & Reddy, 1996; Dai et al., 2003; Jourdan et al., 1999). This mechanism may further lead to apoptosis in MM cells (Jourdan et al., 1999).

Treatment of P3-X63-Ag8.653 mouse myeloma cells with the combination of rTNF-α and nHIFN-α resulted in an enhancement of the reduction in cell viability in comparison with negative control cells and cells treated exclusively with either rTNF-α or nhIFN-α. The nHIFN-α used in this study contains traces of a number of other cytokines produced by human peripheral blood leukocytes infected by Sendai virus (Šantak et al., 2007, Zidovec & Mažuran, 1999). The differences between the slopes of the S-shaped growth curves in the rTNF-α treated P3-X63-Ag8.653 mouse myeloma cell cultures and their controls, and equally prominent differences between the slopes of the growth curves of cells treated with the combination of rTNF-α and nHIFN-α and corresponding doses of single cytokines and their controls, may indicate that the active mechanisms associated with rTNF-α and nHIFN-α, and involved in reduction of cell viability, share some similarities and may possibly benefit from the synergy between rTNF-α, various subtypes of IFN-α and the small amounts of a number of other cytokines in the nHIFN-α preparation (Desmyter et al., 1968; Plesničar et al., 2009). In this context, it would be interesting to identify whether TNF-α and IFN-α share any signaling pathways leading to the reduction in MM cell viability and MM cell death.

Contrary to expectations, in this study treatment of P3-X63-Ag8.653 mouse myeloma cells with rTNF-α showed no increase, but a significant dose-dependent reduction in their cell viability. The P3-X63-Ag8.653 mouse myeloma cells propagate in suspension and show lymphocyte-like morphology (Kearney et al., 1979), and with this in mind, these cells may perhaps be useful in assessment of the effects rTNF-α may have on the growth of clonogenic B-cells in blood of patients with MM. Clonogenic B-cells represent the proliferating compartment in MM and possibly also a biologically distinct, drug-resistant MM progenitor population responsible for cell growth in tumor relapse after the treatment (Matsui et al., 2004; Matsui et al., 2008). In comparison to terminally differentiated plasma cells in MM, clonogenic B-cells appear to be relatively resistant to a number of anti-cancer agents, including dexamethasone, bortezomib, lenalidomide, and 4-hydroxycyclophosphamide (Agarwal & Matsui, 2010; Matsui et al., 2008). Possible similarities between P3-X63-Ag8.653 mouse myeloma cells and clonogenic B-cells in patients with MM, and because clonogenic B-cells are insensitive to standard cytotoxic chemotherapy and dexamethasone (Matsui et al., 2008), render the results observed in this study quite intriguing.

It is known that the activity of TNF-α as a survival and proliferation factor for MM is a part of a complex network of interactions between MM plasma cells, stromal cells and other cells in BM (Jourdan et al., 1999; Matsui et al., 2008). In this *in vitro* study, P3-X63-Ag8.653 mouse myeloma cells were grown in suspension culture, probably resembling the circumstances in which clonogenic B-cells in patients with MM grow without influences of BM microenvironment (Matsui et al., 2008). In a number of studies, serum levels of TNF-α were shown to be increased in patients with active MM and manifest bone disease, and to be

associated with poor prognosis (Alexandrakis et al., 2004; Fillela et al., 1996; Jourdan et al., 1999). Hypothetically, it may be possible to speculate that the increased serum levels of TNF-α in patients with active MM represent a part of a complex negative control loop mechanism that regulates and negatively affects the quantity of circulating clonogenic B-cells in such patients.

A heterologous system was used to evaluate the effects of rTNF-α and its combination with nHIFN-α on MM cells *in-vitro*. Recombinant human tumor necrosis factor-α and nHIFN-α used in this study were active in P3-X63-Ag8.653 mouse myeloma cells, again confirming the observations that cytokines synthesized in cells of one species may have a considerable effect in cells of another closely related species (Desmyter et al., 1968; Greenberg & Mosny, 1977; Ozdemir et al., 2004). Moreover, MM cells are difficult to grow in vitro (Barker et al., 1993). An important advantage of the P3-X63-Ag8.653 mouse myeloma cell line may also lie in its easy reproducibility, unlimited supply, infinite storability and recoverability, and consequently in important cost savings (Drexler & Matsuo, 2000).

The results of this study point to the importance of the study of differential effects TNF-α may exert on malignant cells in MM during specific phases of their development and differentiation. It is possible that TNF-α may have a role in future carefully planned personalized therapy approaches based on genetic features, age, and other risk factors in patients with MM (Durie, 2008; Ludwig et al., 2008). Such therapy could perhaps include patients' own TNF-α, IFN-α, other substances and their combinations, provided that effective procedures for the establishment and maintenance of *ex vivo* cell cultures of patients' own cytokine-producing cells become available.

5. Conclusion

The results of this study point to the importance of assessing the role of TNF-α in study and therapy of MM. Additional studies with other cytokines and human MM cells are required to obtain further information.

6. References

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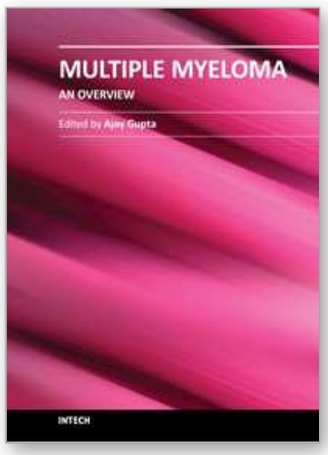

Multiple Myeloma - An Overview

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Multiple myeloma is a malignant disorder characterized by the proliferation of plasma cells. Much insight has been gained into the molecular pathways that lead to myeloma and indeed much more remains to be done. The understanding of these pathways is closely linked to their therapeutic implications and is stressed upon in the initial chapters. Recently, the introduction of newer agents such as bortezomib, lenalidomide, thalidomide, liposomal doxorubicin, etc. has led to a flurry of trials aimed at testing various combinations in order to improve survival. Higher response rates observed with these agents have led to their integration into induction therapies. The role of various new therapies vis a vis transplantation has also been examined. Recent advances in the management of plasmacytomas , renal dysfunction, dentistry as well as mobilization of stem cells in the context of myeloma have also found exclusive mention. Since brevity is the soul of wit our attempt has been to present before the reader a comprehensive yet brief text on this important subject.

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