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Protein kinase ERK contributes to differential responsiveness of human myeloma cell lines to IFNα

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Abstract

Background: Despite IFNα has been used extensively in the treatment of multiple myeloma (MM), there are also several reports suggesting that IFNα may aggravate isease in some MM patients. That means the effect of IFNα on the growth of myeloma cells in vivo may be different. In this study, we selected two human myeloma cell lines that vary remarkably in response to IFNα and focused on elucidating the mechanism of differential IFNα responsiveness.

Results: Sko-007 is a myeloma cell line whose growth is arrested by IFNα; however, IFNα promoted the proliferation of the other myeloma cell line U266. We observed that the growth-stimulation effect of IFNα on U266 cells did not result from up-regulation of the IL-6 receptors on cell surface; while IFNα treatment on Sko-007 cells significantly reduced gp130 expression. Moreover, the transcription factors STAT3 and STAT1, which are involved in the JAK/STAT signal transduction pathway, can be activated in both IFNα-stimulated and -inhibited myeloma cell lines; while the activation of the protein kinase ERK, which is involved in the Ras/MAPK signal transduction pathway, can be down-regulated in IFNα-arrested Sko-007 cells and up-regulated in IFNα-stimulated U266 cells. In addition, both IFNα-induced growth-stimulation effect and the up-regulated activation of ERK in U266 cells were efficiently inhibited by PD98059, the specific inhibitor of MAPK/ERK kinase (MEK).

Conclusion: Myeloma cells responsiveness to IFNα is heterogeneous and the activation state of ERK in the Ras/MAPK signalling pathway mainly contributed to this difference.

Background

The typically growth-inhibitory action of Interferonα (IFNα) has made it a commonly used therapeutic agent in the treatment of a wide variety of human malignancies, including multiple myeloma (MM) [1]. However, although a considerable number of the clinical trials have addressed the effectiveness of IFNα on MM therapy [2], other reports also showed that in some MM patients, IFNα can promote the proliferation of myeloma cells in vivo and result in the development of aggressive plasma cell leukemia (PCL) [3]. Therefore, the action of IFNα on MM cell growth is a matter of debate, and the mechanism of this discrepancy is the topic of this study.

Interleukin-6 (IL-6) is the major growth and survival factor for myeloma cells. IFNα and IL-6 clearly use distinct

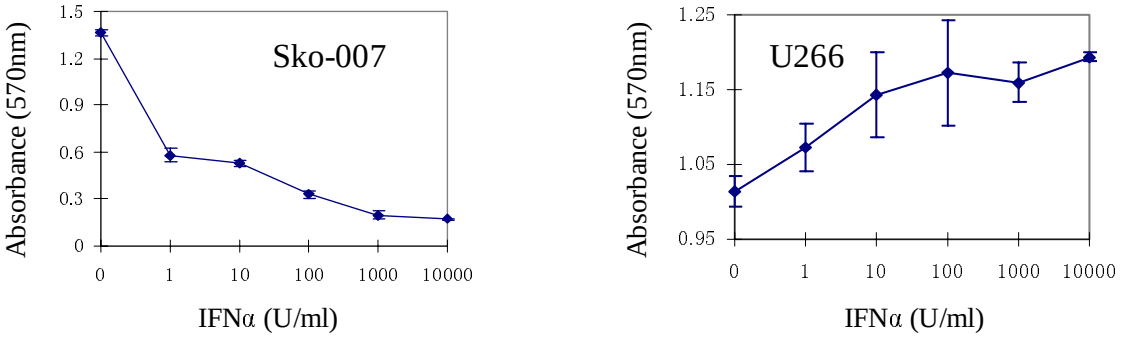

Figure 1
Effect of IFNα on the growth of Sko-0007 and U266 cells. 2×10^3 Sko-007 or U266 cells/well were cultured in 96-well tissue culture plates in the absence or presence of increasing concentrations of IFNα. After incubation for 48 h at 37°, MTT was mixed into each well and the incubation was prolonged for another 6 h, then the dark blue crystal were dissolved in 100 μl of 10% SDS in 0.01 N HCl. Absorbance was measured at 570 nm.

receptor complexes; however, both cytokines have been shown to use two common signal transduction pathways to mediate their biological activities. One is the janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway which results to the activation of the protein tyrosine kinases JAKs and the transcription factors STAT3 and STAT1; the other is the Ras-dependent mitogen-activated protein kinase (Ras/MAPK) pathway which involves the subsequent activation of a series of serine/threonine kinases including extracucullar signal-regulated kianse (ERK) [4,5]. In the previous report, we have investigated in detail the mechanism of IL-6 signal transduction in different human myeloma cell lines [6]. In this study, we analyzed IFNα-induced activation of STAT3, STAT1 and ERK in Sko-007 and U266 human myeloma cell lines, which displayed overall different response to IFNα. And we demonstrated the evidence that the activation state of the Ras/MAPK in stead of the JAK/STAT signal transduction pathway accounted mainly for the differential effect of IFNα on myeloma cells.

Results

Myeloma cell lines are heterogeneous in response to IFNα

To determine the effect of IFNα on the growth of myeloma cells, MTT assay was firstly performed on four human myeloma cell lines-Sko-007, U266, KM-3 and XG-7 [7]. The growth of three myeloma cell lines (Sko-007, KM-3 and XG-7) were significantly inhibited in the presence of IFNα; whereas IFNα moderately promoted the proliferation of the forth myeloma cell line U266. Fig. 1 displayed a concentration-dependent growth-inhibition and -stimulation effect of IFNα on Sko-007 and U266 cells, respectively. This result supported the conclusion that there are two distinct patterns of IFNα response in human myeloma cells.

Effect of IFNα on the expression of two IL-6 receptor chains on myeloma cell surface

IL-6 is the major growth factor for myeloma cells. It has been reported that the effect of many other growth-stimulating or growth-inhibitory factors on myeloma cells is a secondary consequence that is mediated by the regulation on the expression or the activity of IL-6 or its receptors [4]. To determine whether a similar mechanism accounted for the different effect of IFNα on Sko-007 and U266 cells, IFNα-treated and untreated cells were incubated respectively with the specific antibodies to the two IL-6 receptor chains, gp80 (IL-6R) and gp130, then FACS analysis was performed. As shown in Table 1, the expression level (represented by both the average strength of fluorescence on cell surface and the percentage of the positive cells) of gp130 was down-regulated significantly in Sko-007 cells with the treatment of IFNα for 48 h, while there was no obvious changes on IL-6R level at the same conditions. This result indicated that growth-inhibition effect of IFNα on Sko-007 cells was partially mediated by down-regulated expression of gp130 on cell surface. In contrast, growth-stimulation of U266 cells in the presence of IFNα did not involve any induction of IL-6R and gp130 expression. Taken these results together, we concluded that IFNα-induced regulation of IL-6 receptors expression on cell surface is not a common mechanism to mediate the effect of IFNα on myeloma cells.

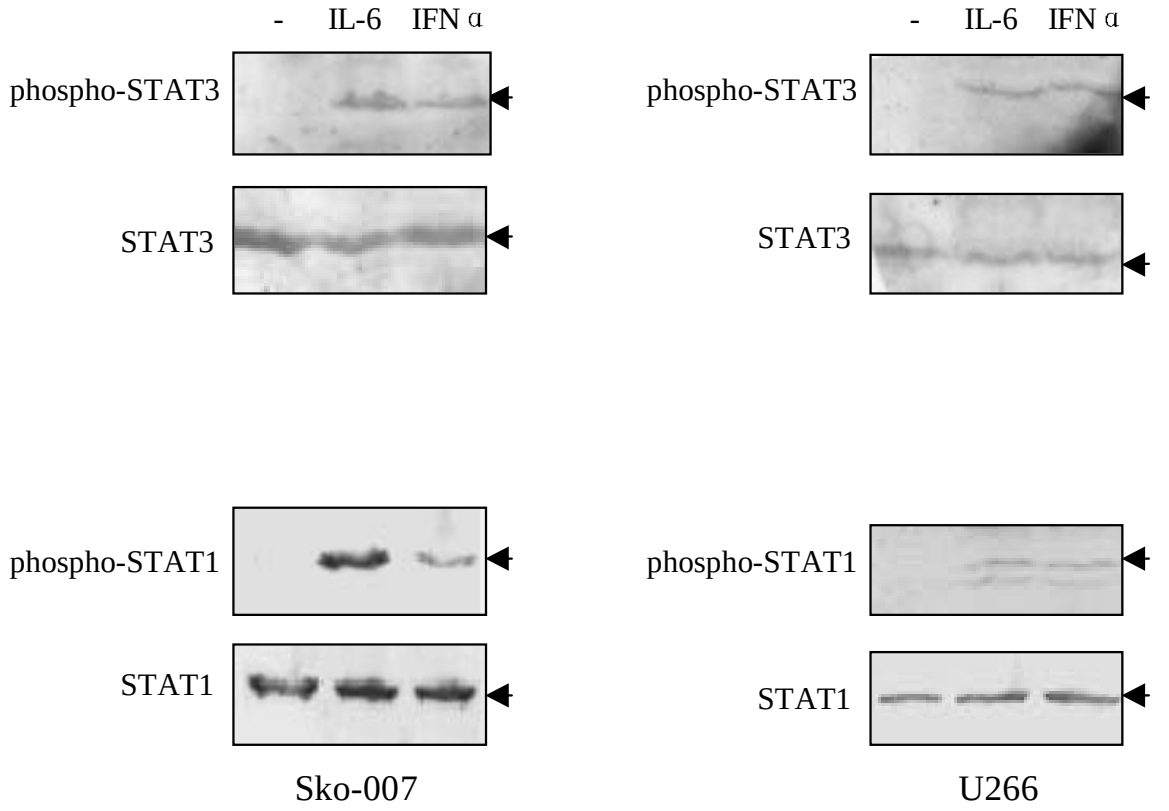

Figure 2
Activation states of STAT3 and STAT1 in Sko-007 and U266 cells in the absence or presence of IL-6 or IFNα. 2 × 10^6 Sko-007 or U266 cells were left untreated or treated with IL-6 (10 ng/ml) or IFNα (100 U/ml) for 10 min and lyzed in cell lysis buffer. Then 30 μg cytoplasmic proteins were separated by 10% polyacrylamide-SDS gel and transferred elechtrophoretically to Immobilon. After blocking with 5% dry milk, the membranes were blotted with anti-STAT3, STAT1, phospho-STAT3 or phospho-STAT1 antibodies and the relevant HRP-labeled second antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, USA).

Table 1: Expression of IL-6R and gp130 on the surface of Sko-007 and U266 cells in the absence or presence of IFNα.

	IFNα (-)		IFNα (+)	
	%	mean	%	mean
IL-6R Sko-007	13.00 ± 1.21	20.57 ± 2.74	10.11 ± 0.96	19.80 ± 0.47
Gp130	99.06 ± 1.01	60.68 ± 1.70	65.77 ± 3.19*	34.21 ± 1.31*
IL-6R U266	1.50 ± 0.33	50.44 ± 2.09	0.92 ± 0.36	50.09 ± 4.28
Gp130	8.66 ± 0.64	29.19 ± 2.13	9.33 ± 1.05	26.66 ± 0.63

2 × 10^5 or cells were treated with or without IFNα (100 U/ml) for 48 h and then subsequently incubated with anti-IL-6R or anti-gp130 antibodies for 30 min and with the relevant FITC-labeled second antibodies for 20 min. Cell samples were harvested and subjected to FACS analysis equipped with ModFitLT software. "mean" represents the average strength of fluorescence. "%" represents the percentage of the cells expressing target antigen (positive cells). The *p* values were determined by paired *t*-test. *significantly different ($p < 0.01$) from the corresponding values in cultures without IFNα.

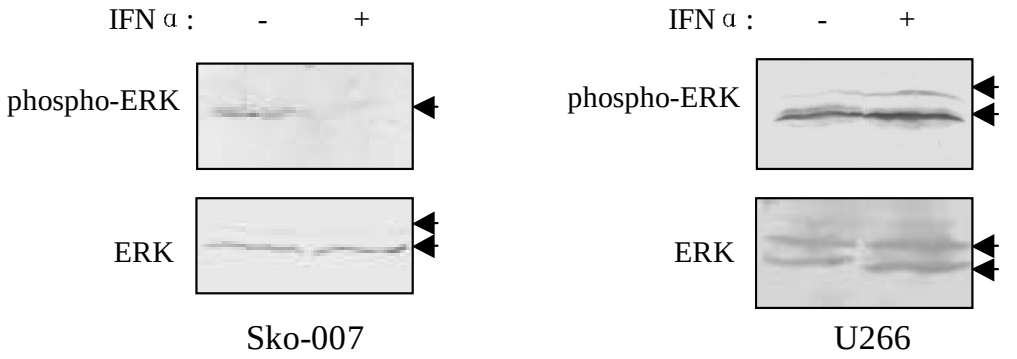

Figure 3
Activation state of ERK in Sko-007 and U266 cells in the absence or presence of IFNα. 2×10^6 Sko-007 or U266 cells were left untreated or treated with IFNα (100 U/ml) for 48 h and lyzed in cell lysis buffer. Then cytoplasmic proteins were extracted and the expressions of phospho-ERK and ERK were determined by immunoblot assay as similar as in Fig. 2 except using anti-ERK or phospho-ERK antibodies.

Activation of the transcription factors STAT3 and STAT1 in myeloma cells stimulated by IFNα

JAK/STAT pathway is one of two common signal transduction pathways used by both IL-6 and IFNα to mediate their activities [4,5]. To address whether there exists any difference in the activation of the JAK/STAT pathway in Sko-007 and U266 cells stimulated by IFNα, immunoblot assay was performed using anti-phospho-STAT3 and phospho-STAT1 antibodies to detect the activation of the transcription factors STAT3 and STAT1 in the JAK/STAT pathway. As shown in Fig. 2, there was no activation of STAT3 and STAT1 in unstimulated Sko-007 and U266 cells, while IL-6 and IFNα can activate both two transcription factors in the both two cell lines. Anti-STAT3 and STAT1 antibodies blotting showed equal amount of STAT3 and STAT1 proteins in untreated and IL-6 or IFNα-treated cells. These results indicated that the activation of the JAK/STAT pathway did not account for the different effect of IFNα on Sko-007 and U266 cells.

Activation state of the protein kinase ERK in myeloma cells stimulated by IFNα

Ras/MAPK pathway is the other common signal transduction cascade used by both IL-6 and IFNα [4,5]. In the previous report, we have proved that the survival of Sko-007 and U266 cells were mediated by the constitutive activation of this pathway in the presence of the autocrine IL-6 [7]. To analyze whether the activation of this pathway was different in above two cell lines in the presence of IFNα, a similar immunoblot assay was performed using anti-phospho-ERK antibody to detect the activation state of the protein kinase ERK, the most important kinase in the Ras/MAPK pathway. As shown in Fig. 3, constitutive activation of ERK can be observed in both Sko-007 and U266 cells in the absence of IFNα; and IFNα simulation down-regulated ERK activation in Sko-007 cells and up-regulated ERK activation in U266 cells. This result strongly suggested that different regulation of the protein kinase ERK in the Ras/MAPK signal transduction pathway might play a key role in responding to IFNα in Sko-007 and U266 cells.

PD98059 inhibited IFNα-induced growth-stimulation effect and up-regulation of ERK activation in U266 cells

To further confirm the result from Fig. 3, we next analyzed whether the regulation on ERK activation can result to the corresponding changes on the activity of IFNα on myeloma cells. As shown in Fig. 4, when IFNα-induced increase of ERK activation in U266 cells was efficiently inhibited by treatment of PD98059, the specific inhibitor of the upstream kinase of ERK, MAPK/ERK kinase (MEK), in the Ras/MAPK pathway, IFNα-mediated growth-stimulation effect was also decreased at the same conditions. This finding confirmed the result that protein kinase ERK in the Ras/MAPK pathway mainly contributed to the differential responsiveness of myeloma cells to IFNα.

Discussion

Up till now, the clinical value of IFNα on multiple myeloma therapy remains debatable due to the conflicting in vivo results reported by different clinical groups [2,3]. Therefore in this study, we analyzed the responsiveness of

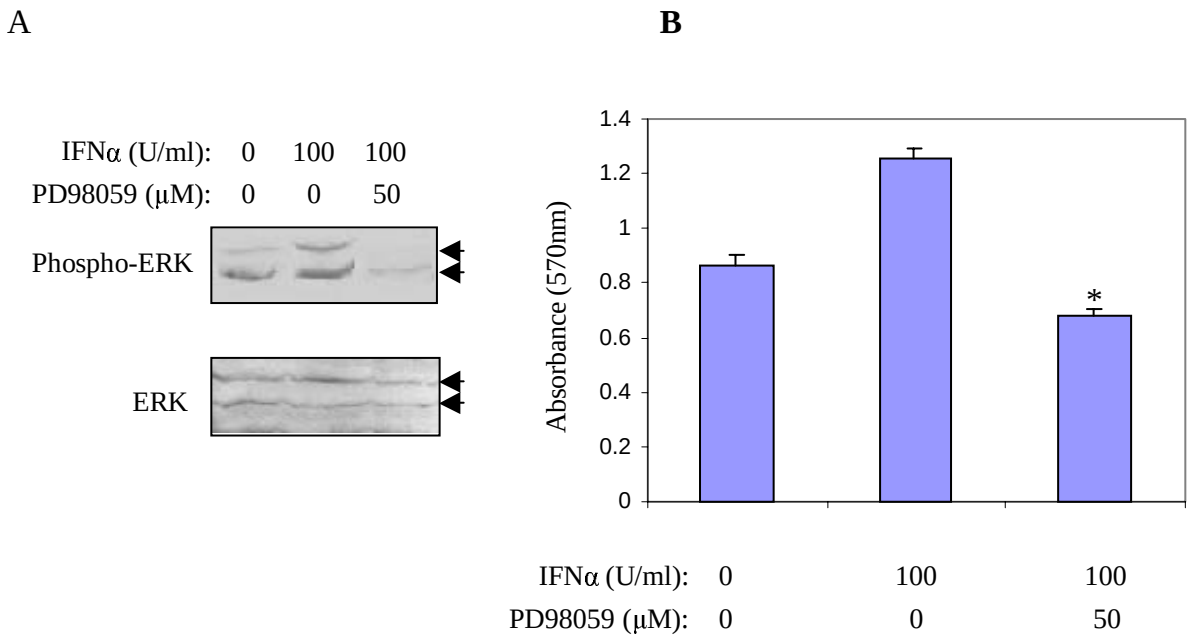

Figure 4
PD98059 inhibited IFNα-induced growth-stimulation effect and increase of ERK activation in U266 cells. (A) U266 cells were left untreated or treated with IFNα (100 U/ml) or pre-treated with PD98059 (50 μM) before IFNα was added. Then cytoplasmic proteins were extracted and the expressions of phospho-ERK and ERK were determined by immunoblot assay as same as in Fig. 3. (B) U266 cells (2×10^6 /well) were cultured in 96-well tissue culture plates in the absence or presence of IFNα (100 U/ml) alone or IFNα together with PD98059 (50 μM). MTT assay was preformed as described in Fig. 1. The *p* values were determined by paired *t*-test. *significantly different ($p < 0.01$) from the corresponding values in cultures with IFNα alone.

a panel of human myeloma cell lines to IFNα in vitro and the mechanism of myeloma cells heterogeneous response to IFNα.

The results in this study showed that IFNα exerted different effects on the growth of different myeloma cell lines. The growth-inhibition effect of IFNα was significant while the growth-stimulation effect was moderate (Fig. 1). This result is in accordance with the fact that IFNα usually exerts a strongly inhibitory or a slowly stimulatory effect on the proliferation of myeloma cells in different patients with MM [2,3].

To elucidate the mechanism responsible for the different activity of IFNα on myeloma cells, we firstly examined the regulation of IL-6R and gp130 expression on cell surface in the presence of IFNα, which may be one of the reasons for the heterogeneous IFNα responsiveness according to the previous reports [8]. Interestingly, we only detected down-regulated expression of gp130 in IFNα-arrested Sko-007 cells, while not any changes on two IL-6 receptor chains level in IFNα-stimulated U266 cells. Therefore, the regulation of IL-6 receptors expression may not mainly account for the different effect of IFNα on myeloma cells.

In the further study, we focused on investigating the activation states of IFNα-induced signal transduction pathways in myeloma cellls. We observed that both STAT3 and STAT1 could be activated in the both two myeloma cell lines irrespective of growth outcome upon IFNα stimulation; while the activation state of ERK correlated tightly with the growth effect of IFNα (Fig. 2, 3 and 4). That means, the Ras/MAPK in stead of the JAK/STAT signal transduction pathway mainly contributed to the different response of myeloma cells to IFNα. Identification of the target gene(s) regulated by protein kinase ERK in the Ras/MAPK pathway in IFNα-arrested and stimulated myeloma cells will be helpful to further elucidate the mechanism of IFNα on myeloma cells.

Conclusions

Our data in this study clearly demonstrated that IFNα can exert growth-stimulatory or -inhibitory effect on different

myeloma cells in vitro and protein kinase ERK played a key role in myeloma cells in response to IFNα.

Materials and methods

Reagents

Human recombinant IFNα was provided by Institute of Biotechnology (Beijing, China). Anti-human IL-6R and gp130 antibodies were prepared in our lab. Anti-ERK and phospho-ERK antibodies was purchased from Santa Cruz Biotechnology (USA). Anti-STAT3, STAT1, phospho-STAT3 and phospho-STAT1 antibodies were purchased from NEB Biotechnology (USA).

Human myeloma cell lines

Human myeloma cell line U266 was kindly provided by Prof. Xueguang Zhang (Suzhou University, China). Sko-007 myeloma cell line was obtained from Medical School of Stanford University (USA). The cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 80 U/ml streptomycin.

Effect of IFNα on the growth of myeloma cells

The proliferation of myeloma cells in the presence of IFNα was measured by MTT assay as described previously [7].

Expression of two IL-6 receptor chains (IL-6R and gp130) on myeloma cell surface

2×10^5 myeloma cells were treated with or without IFNα (100 U/ml) for 48 h and then incubated with anti-IL-6R or anti-gp130 antibodies for 30 min and with the relevant FITC-labeled second antibodies for 20 min. Cell samples were harvested and subjected to FACS analysis.

Activation of the protein kinase ERK and the transcription factors STAT3 and STAT1 in myeloma cells induced by IFNα

2×10^6 myeloma cells were treated with IFNα (100 U/ml) for 10 min (to detect the activation of STAT3 and STAT1) or 48 h (to detect the activation of ERK) and then lyzed in 100 μL lysis buffer (10 mM Tris-Cl pH 7.6, 150 mM NaCl, 0.5% NP40, 5 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 1 mM NaF, 2 μg/mL aprotinin). After 30 min on ice, cell lysates were cleared by configuration at $12000 \times g$ for 30 min at 4°. The concentrations of the cytoplasmic proteins were measured by Bradford assay [9]. Then 30 μg cytoplasmic proteins were separated by 10% polyacrylamide-SDS gel and transferred elechtrophoretically to Immobilon (Millipore). After blocking with 5% dry milk, the membranes were blotted with anti-ERK, STAT3, STAT1 or anti-phospho-ERK, phospho-STAT3, phospho-STAT1 antibodies and the relevant HRP-labeled second antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham, USA).

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