Research Article

TNFα Mediated IL-6 Secretion Is Regulated by JAK/STAT Pathway but Not by MEK Phosphorylation and AKT Phosphorylation in U266 Multiple Myeloma Cells

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IL-6 and TNFα were significantly increased in the bone marrow aspirate samples of patients with active multiple myeloma (MM) compared to those of normal controls. Furthermore, MM patients with advanced aggressive disease had significantly higher levels of IL-6 and TNFα than those with MM in plateau phase. TNFα increased interleukin-6 (IL-6) production from MM cells. However, the detailed mechanisms involved in signaling pathways by which TNFα promotes IL-6 secretion from MM cells are largely unknown. In our study, we found that TNFα treatments induce MEK and AKT phosphorylation. TNFα-stimulated IL-6 production was abolished by inhibition of JAK2 and IKKβ or by small interfering RNA (siRNA) targeting TNF receptors (TNFR) but not by MEK, p38, and PI3K inhibitors. Also, TNFα increased phosphorylation of STAT3 (ser727) including c-Myc and cyclin D1. Three different types of JAK inhibitors decreased the activation of the previously mentioned pathways. In conclusion, blockage of JAK/STAT-mediated NF-κB activation was highly effective in controlling the growth of MM cells and, consequently, an inhibitor of TNFα-mediated IL-6 secretion would be a potential new therapeutic agent for patients with multiple myeloma.

1. Introduction

Despite intensive investigations during the past decade, multiple myeloma (MM) remains an incurable disease. One challenge lies in that the bone marrow (BM) microenvironment and the mutual interaction between multiple myeloma cells and bone marrow stromal cells contribute to modulation of biological behaviors of tumor cells in each patient. Therefore, novel therapeutic approaches targeting both tumor cells and stromal cells are being eagerly sought [1–3].

The bone marrow microenvironment is composed of many cellular and cellular components such as endothelial cells, stromal cells, osteoclasts, osteoblast, immune cells, fat cells, and extracellular matrices. These cells interact by direct adhesion or by secretion of numerous cytokines to support tumor progression and confer drug resistance [4–7]. A number of factors present in BM microenvironment have been dysregulated in patients with MM [8–10]. Cytokines including IL-6, VEGF, IGF-1, TNFα, and HGF produced from bone marrow stromal cells (BMSCs) and tumor cells directly and/or indirectly influence MM cells in autocrine and paracrine manners. Also, overexpressed cytokines in BM microenvironment eventually contribute to the acquisition of resistance against anticancer drugs [4]. Therefore, biology of interaction between MM cells and BMSCs needs to be verified to improve treatment outcome of MM.

TNFα is one of the factors elevated in multiple myeloma patients. As a 7cytokine, TNFα is associated with various
physiological and pathological processes such as cell growth, apoptosis, and proliferation. Moreover, TNFα is known for promoting osteoclastogenesis and inhibiting osteoblastogenesis. It is also responsible for regulating homeostasis in other diseases such as type I diabetes [11] and inflammatory arthritis [12]. The major mechanism by which TNFα mediates progression of multiple myeloma cells is via regulation of nuclear factor kappa B (NF-κB) transcription factor. This molecular signaling regulation is also related to IL-6 secretion by stromal cells and osteoblasts which is a potent growth factor for MM cells [13, 14]. Thus, it is of utmost importance by understanding pathways of TNFα in relation to IL-6 regulation to develop effective therapeutic strategy against MM.

We found that the levels of TNFα and IL-6 were elevated in bone marrow aspirates of multiple myeloma patients. We also analyzed the patterns of correlation between TNFα and IL-6 and the mechanisms of TNFα-induced IL-6 secretion from multiple myeloma cells. Furthermore, we investigated the biological consequences of IL-6 suppression by inhibition of TNFα-mediated intracellular signaling. Importantly, JAK/STAT pathway was directly involved in the signal pathways of TNFα in relation to IL-6 and inhibitor of NF-κB, TPCK, was most effective compared to other types of inhibitors.

2. Materials and Methods

2.1. Cell Culture and Multiple Myeloma Patient Samples. The human multiple myeloma, U266 cells, was generously provided by Dr. Dongsoo Lee (Seoul National University, College of Medicine, Seoul, Republic of Korea). IM9 was obtained from the Korean Cell Line Bank (Chongro-gu, Seoul, Republic of Korea). These cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with sodium pyruvate, essential vitamins, L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) (Gibco, Grand Island, NY, USA), and 10% heat-inactivated fetal bovine serum, except for U266 cell line (cultured in 15% FBS containing medium). The cells were incubated in a highly humidified atmosphere of 5% CO2 and 95% air at 37°C.

All experiments were conducted using cells in logarithmic growth phase. Clinical and laboratory parameters of 45 patients with multiple myeloma were shown in Table I. 11 patients had normal radiographic findings of the skeleton and overall; 4 patients died.

2.2. Antibodies and Reagents. Total and phospho form of mTOR, c-Raf, STAT3 (ser727), phospho-MAPK sampler kit, phospho-AKT (ser473) sampler kit, phospho-MEK1/2 (cell signaling technology, Beverly, MA, USA), c-Myc, cyclin D1, phospho-JNK, JNK, and GAPDH (Santa Cruz, CA, USA) were used as primary antibodies. In addition, horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch laboratories Inc., PA, USA) was used. Bay 11-7082(E)-3-[(4-merhylphenyl)sulfonyl]-2-propeninitrile, TPCCK (Nα-Tosyl-Phe Chloromethyl Ketone), PDTC (10 Pyrrolidinecarbodithioic Acid, Ammonium Salt), used as NF-κB inhibitors, PI3K inhibitor (LY294002), JNK inhibitor II, MEKI/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580), JAK inhibitor I/II, and JAK/STAT inhibitor (AG490) were purchased from Calbiochem corp. (San Diego, CA, USA). These inhibitors were dissolved in DMSO as stock solutions, stored at −20°C, and subsequently diluted with serum-free RPMI1640 medium prior to use. Concentrations of various pharmacologic inhibitors were adapted from IC50 values from the manufacturer’s manual. Since the treatment duration was just 1h instead of several days for usual cytotoxic assay, cells were not affected for their viability. Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN, USA), rehydrated in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and stored as a stock solution at −20°C.

2.3. Western Blot Analysis. Cells stimulated with specific factors and treated for indicated periods were collected and washed using cold phosphate buffered saline (PBS). Cell pellets were lysed in Kinexus protein lysis buffer containing 20 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate (pH 7.2), 20 mM sodium pyrophosphate, 1 mM sodium, orthovanadate, 1% Triton X-100, 1 mM PMSF, aprotinin, leupeptin and pepstatin 1 µg/mL), stored in −20°C. Prepared protein samples were separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membrane was blocked with Tris-buffered saline solution containing 5% Tween-20 (TBS/T) and 5% nonfat dry milk for 1h in room temperature. Blots on membranes were probed with specific primary antibodies for overnight and diluted secondary antibody (rabbit for 1: 20,000, mouse for 1: 10,000) was used for detecting primary

| Parameters |
|----------------|
| Male/female (number) | 22/23 (45) |
| TNF alpha level (pg/mL) | 40* (3.17–308.7)† |
| IL-6 level (pg/mL) | 109.7 (10.73–2695.7) |
| Age (yr) | 63.3 (33–91) |
| OS (month) | 22 (0.2–71.7) |
| PLT (10^3/µL) | 231.1 (115–518) |
| Hb (g/dL) | 11.16 (7.7–32.1) |
| WBC (×10^9/L) | 6780.79 (1190–31530) |
| Serum κ/λ | 21.72 (0.01–414.39) |
| Serum Cr | 3.25 (0.5–15.2) |
| Serum Ca | 3.93 (72–13.3) |
| LDH (IU/L) | 200 (107–392) |
| β2-MG (serum) | 9.48 (1.42–72.4) |
| Serum albumin | 3.32 (1.8–4.8) |
| Bone lesion (%) | 44% (20/45) |

*Median; † the parenthesis means the range. OS: overall survival. Hb: Hemoglobin. WBC: white blood cells, β2-MG, beta-2 microglobulin.
antibody. It was enhanced by chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.4. ELISA. IL-2, IL-4, IL-6, IL-10, IL-17, TNFα, TGFβ₁, and IFNγ released from multiple myeloma cells were measured using ELISA kit (R&D systems, Minneapolis, MN, USA). Cells were pretreated with specific reagents for indicated period and then cell-free supernatants were harvested and stored in −70°C. Bone marrow aspirates obtained from 45 patients with multiple myeloma were measured for cytokines concentration in accordance with the manufacturer’s instructions. The optical density of the samples was determined using a microplate reader set at 450 nm.

2.5. Transfection of TNFR siRNA. Small interference RNA (siRNA) for siGENOME Human TNFR siRNA (M-005197-00) and siGENOME Nontargeting siRNA Pool (D-001206-13) were purchased from Dharmacon (Lafayette, CO.). Transient transfection of U266 was performed using the Human Cell Line Nucleofector Kit C (VACA-1004; Amaxa Biosystems, Gaithersburg, MD), according to the manufacturer’s protocols. Briefly, siRNA (3 μg) was added to 1 × 10⁶ U266 cells suspended in 100 μL of Nucleofector TM solution. The mixture was then transferred into the electroporation cuvette and subjected to electroporation using the X-005 program, according to the manufacturer’s instructions. Immediately after electroporation, the cells were suspended in 500 μL of cell culture medium and transferred into culture dishes or plates. The transfected cells were then grown until the knocked-down effect of TNFR siRNA was evident by RT-PCR.

2.6. Statistical Analysis. The statistical significance of differences observed in experimental arm versus control was analyzed by Student’s t-test, using the statistical software GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at a level of P < 0.05.

3. Results

3.1. The Cytokine Patterns in the Bone Marrow Environment of Multiple Myeloma Patients. To identify which among various cytokines are retained with high concentration in the serum of multiple myeloma patients, 8 cytokine (IL-2, IL-4, IL-6, IL-10, IL-17, TNFα, TGFβ₁, and IFNγ) levels were measured using 45 bone marrow aspirate samples of multiple myeloma patients (Table 1). Levels of TNFα, IL-6, and TGFβ₁ were elevated, but cytokines such as IL-2, IL-4, and IL-10 were too low to be detected by ELISA. The level of IL-6 showed positive correlation with the level of TNFα (Figure 1), and these cytokines showed further correlation with poor prognostic factors such as high level of serum κ/λ light chain ratio and β₂MG, as well as short overall survival (data not shown).

3.2. Effect of TNFα on IL-6 Release from Multiple Myeloma. It has been previously reported that TNFα plays a key role in facilitation of IL-6 secretion [15]. Figure 1 showed that there was correlation between IL-6 and TNFα level in bone marrow aspirate samples of patients. We examined whether TNFα could be the stimulator that releases IL-6 from multiple myeloma cells. In U266 cells, IL-6 secretion was markedly increased in response to TNFα, whereas other MM cell lines
Figure 2: Regulation of IL-6 release by TNFα in vitro. After serum starvation, U266 and IM9 multiple myeloma cells were treated with or without 1 ng/mL TNFα for indicated times. Cell supernatants from each experimental sample were harvested for ELISA and IL-6 concentrations were determined at different time points with TNFα treatment. Relative fold changes compared to TNFα nontreated samples as a control were shown in graph. Bars represent the mean ± SEM from three independent experiments.

did not show drastic IL-6 secretion. As shown in Figure 2, IL-6 concentration was 1.5 folds higher in TNFα-treated U266 cells compared with untreated cells. However, induction of IL-6 by TNFα treatment was not detected in IM9 cells.

3.3. Activation of Various Signaling Pathways by TNFα and Suppression of IL-6 Release with Inhibitors. To evaluate the signaling mechanism of TNFα on IL-6 secretion, we first examined molecules activated by TNFα by western blot analysis in U266 and IM9 cells. As a result of TNFα stimulation after serum starvation, various signaling molecules were regulated by TNFα including Raf/MEK/Erk, JNK, and PI3K/AKT pathways in both cell lines (Figure 3(a)). Since IM9 showed little difference in the level of IL-6 secretion despite TNFα stimulation, we opted to use U266 cell line for further study since the aim of our study was to delineate the role of TNFα on the secretion of IL-6. Cells were preincubated with PD98059, LY294002, SB203580, and JNK inhibitor II for suppression of the phosphorylation of p44/42MAPK, PI3K/AKT, p38 MAPK, and JNK, respectively, and then stimulated with TNFα for IL-6 release from cells. The inhibitors were not potent in blocking secretion of IL-6 from U266 multiple myeloma cells (Figure 3(b)).

3.4. Regulation of IL-6 Secretion Induced by TNFα via JAK/STAT Pathway. To further investigate whether TNFα induces the activation of JAK/STAT pathway which is associated with NF-κB activation, TNFα mediated STAT3 phosphorylation and the induction of c-myc and cyclin D1, downstream genes of JAK/STAT activation, were examined in U266 cells. As shown in Figure 4(a), TNFα induced phosphorylated STAT3 as well as increased the expression levels of cMyc and cyclin D1. So, we used JAK/STAT inhibitors such as AG490, JAK inhibitor I, and JAK inhibitor II to examine whether IL-6 secretion by TNFα is regulated by JAK/STAT pathway. As shown in Figure 4(b), all inhibitors that target JAK/STAT signaling resulted in the suppression of IL-6 secretion by TNFα. Similar suppression was also achieved by targeting TNFα receptor using siRNA.

3.5. Suppression of IL-6 Secretion by TPCK. Numerous studies revealed that TNFα induces IL-6 secretion from multiple myeloma cells and bone marrow stromal cells through NF-κB pathway [16]. Moreover, most multiple myeloma cells constitutively activate nuclear NF-κB signaling. So, we examined whether inhibition of NF-κB pathway affects secretion of IL-6 by TNFα. Figure 5(a) describes that only the cells treated with TPCK, which inhibits the DNA binding of transiently expressed p65/RelA by suppression of IKK-beta, effectively decreased the level of IL-6 secretion. To examine whether NF-κB inhibitors directly affect the signaling pathways, such as MEK/ERK, PDK1/AKT, and JAK/STAT, signaling molecules, such as p-ERK, p-PDK1, p-AKT, and p-STAT3, were examined after treating with three different inhibitors. Figure 5(b) describes that none of the NF-κB inhibitors suppresses TNFα-induced phosphorylation of signaling molecules. Since TPCK did not directly inhibit the TNFα-induced p-STAT3, we can assume that TPCK action is not mediated by STAT3 pathway but by downstream of JAK/STAT pathway (i.e., NF-κB pathway).

4. Discussion

Chemokines and cytokines existed in BM Microenvironment directly and indirectly influence MM cells. The well-known factors including IL-6, TNFα, TGFβ1, and IFNγ, which are produced from BMSCs and MM cells, directly affect the survival and growth of MM cells. Several of proinflammatory factors such as IL-2, IL-4, IL-10, and IL-17 are also reported for their association with survival and growth of MM cells [9, 17, 18].
In this study, we investigated the biological mechanism of action of the TNFα in human myeloma cells. TNFα, as a proinflammatory cytokine, is associated with various processes for multiple myeloma progression such as cell growth, death, and differentiation [19, 20]. In the aspirates of patients with multiple myeloma, elevated TNFα level has been observed in numerous studies and this is correlated with poor prognosis [8, 10]. There are also multiple lines of evidence suggesting that TNFα is predictive of progression-free survival after thalidomide therapy in MM patients [21, 22]. Consistent with previous reports, we found that not only TNFα but also IL-6 level was highly present in aspirates of patients with multiple myeloma, while no difference was seen with T-cell related or produced cytokines such as IL-2, IL-4, IL-10, IL-17, and IFN γ (data not shown). We also verified the correlation between TNFα and IL-6.

TNFα has a central role in bone pathophysiology along with the receptor activator of NF-κB ligand (RANKL) as a skeletal catabolic agent. It is known to be responsible for promoting osteoclastogenesis and inhibiting osteoblast function. Bone destruction is generated in multiple myeloma patients which is unique from other hematologic malignancies; this phenomenon might be related to increase TNFα levels. So we analyzed the correlation patterns between TNFα levels

![Graph showing regulation of IL-6 secretion](image)

**Figure 3:** Effect of TNFα on activation of MAPK, JNK, and PI3K/AKT signaling pathways and effect of indicated inhibitors on IL-6 secretion. (a) U266 and IM9, multiple myeloma cell lines were starved for 8 h and then treated with or without 1 ng/mL TNFα for 10 min. Each cell line was harvested and prepared for immunoblotting. Upregulation of MAPK, JNK, and PI3K/AKT by TNFα was examined with indicated antibodies. GAPDH was used to ensure equal loading. (b) U266 cells were pretreated with various signal inhibitors for 1 h, respectively. These cells were then stimulated with TNFα for 10 min, and cell supernatants were harvested for ELISA to determine IL-6 concentration induced by TNFα. Bars represent the mean ± SEM from three independent experiments. c: control; 1: TNFα only; 2: TNFα plus 10 μM PD98059; 3: TNFα plus 20 μM LY294002; 4: TNFα plus 10 μM SB203580; 5: TNFα plus 40 nM JNK inhibitor II.
and bone lesion development. Patients having higher levels of TNFα in bone marrow aspirate developed bone lesions more easily by almost 1.5 folds than those having lower levels of TNFα (data not shown), consistent with previous report [23]. In addition to TNFα, IL-6, produced from multiple myeloma cells as well as bone marrow stromal cells, is also a major growth factor for tumor cells regulating various biological signaling. One of the mechanisms regulating osteoclastogenesis is through NF-κB-dependent IL-6 secretion. TNFα potently stimulates IL-6 secretion by stromal cells and osteoblasts, and accumulated IL-6 stimulates growth of multiple myeloma cells. Furthermore, it mediates the effects of other inflammatory cytokines on osteoclast formation, such as IL-1 and TNFα again [16]. Recently, it was shown that it can induce apoptosis of mature osteoblast as well as inhibit the proliferation of mesenchymal stem cells [19]. We found that some of myeloma cells including U266 cells showed stronger response to exogenous IL-6 (data not shown), and these cells also secreted more IL-6 in response to TNFα in vitro. So, we hypothesized that TNFα is one of the major factors that regulate IL-6 secretion from multiple myeloma cells. First, we examined the signaling pathways that may be the candidate bridge between TNFα and IL-6. We found that TNFα could regulate cell proliferation, survival, and antiapoptosis by inducing various signaling pathways such as PI3K/AKT, JNK, MAPK, and JAK/STAT pathways. Thus, we blocked above TNFα-induced molecules to investigate the major signaling pathway involved in IL-6 secretion. There was no significant change in IL-6 secretion from multiple myeloma cells when PI3K/AKT, JNK, or MAPK pathways were inhibited. However, JAK/STAT inhibitors led to considerable decrease in IL-6 level. It indicated that IL-6 secretion by TNFα was mainly dependent on JAK/STAT pathway. Moreover, we demonstrated that TNFα up-regulates cyclin D1 as well as c-Myc. These are well known for cell proliferation related to cell cycle progression and antiapoptosis.

NF-κB is known to be correlated with drug-resistant activity [24] and to be a major signaling pathway associated with multiple myeloma pathogenesis. Previous reports showed that not only multiple myeloma cells but also BMSCs adherent to multiple myeloma cells also release IL-6 via NF-κB pathway to support MM cell growth [25]. Therefore, NF-κB activation in BMSCs enhances positive loop with adherent multiple myeloma cells by secretion of growth factors such as IL-6, TNFα, and HGF. Therefore, there are numerous studies targeting NF-κB pathway using inhibitors to suppress tumor progression [26–28]. Among the three compounds that inhibit NF-κB pathway, our data showed that only TPCK suppressed IL-6 secretion remarkably from multiple myeloma cells as well as BMSCs. Previous reports showed that TPCK, known as chymotrypsin-like proteases, could be used as an NF-κB pathway inhibitor. It targets serine and cysteine activation loop of IKK beta, resulting in blocking of NF-κB binding to DNA in HeLa cells [29]. In addition, Wang et al. reported that TPCK abolishes constitutive RelA activity and uPA overexpression in pancreatic tumor cell lines with dexamethasone [30]. Although previous report demonstrated that TPCK inhibits TRAIL-mediated caspase activity and PDK/AKT signaling in human prostatic carcinoma cell lines [31], our data indicated that induced AKT signaling by TNFα was not affected by TPCK in myeloma cells (Figure 5). These observations do not exclude the possibility that TPCK inhibits other kinases but support the potential of TPCK as an NF-κB inhibitor, especially regulating IL-6 secretion in multiple myeloma cells.

**Figure 4:** IL-6 secretion by TNFα regulated by JAK/STAT pathway. U266 cells were serum starved for 8 h and treated with indicated JAK/STAT inhibitors for 1 h or transfected with TNFR siRNA. The cells were then stimulated with TNFα to induce IL-6 secretion and harvested for immunoblotting with indicated antibodies (a). Cell supernatants were collected to determine IL-6 levels using ELISA kit (b). TNF receptor targeted siRNA was used as a positive control. Data shown are the means ± SEM of three independent experiments. c: control; 1: TNFα only; 2: TNFα plus 20 μM AG490; 3: TNFα plus 10 μM JAK inhibitor I; 4: TNFα plus 10 μM JAK inhibitor II; 5: TNFα plus TNFR siRNA.

**Figure 5:** Regulation of IL-6 secretion from multiple myeloma cells treated with indicated JAK/STAT inhibitors. U266 cells were serum starved for 8 h and treated with indicated inhibitors for 1 h or transfected with TNFR siRNA. The cells were then stimulated with TNFα to induce IL-6 secretion and harvested for immunoblotting with indicated antibodies (a). Cell supernatants were collected to determine IL-6 levels using ELISA kit (b). TNF receptor targeted siRNA was used as a positive control. Data shown are the means ± SEM of three independent experiments. c: control; 1: TNFα only; 2: TNFα plus 20 μM AG490; 3: TNFα plus 10 μM JAK inhibitor I; 4: TNFα plus 10 μM JAK inhibitor II; 5: TNFα plus TNFR siRNA.
Figure 5: Effect of NF-κB inhibitors on TNFα induced IL-6 levels and signaling induction of its upstream pathways. U266 cells were serum starved for 8 hr and pretreated with NF-κB inhibitors (10 μM Bay11-7082, 10 μM PDTC, 20 μM TPCK) for 1 hr. These cells were then stimulated with 1 ng/mL TNFα for 10 min to stimulate IL-6 secretion. Cell supernatants were subjected to determine IL-6 level using ELISA (a), and proteins were prepared for detection of signaling activation using indicated antibodies (b). Data shown are the means ± SEM of three independent experiments.

Taken together, we analyzed the correlation of high levels of TNFα with several prognostic factors in multiple myeloma patients and the signaling regulation in vitro. In addition, the IL-6 secretion is effectively suppressed by specific inhibitors. TNFα and IL-6, as pivotal factors for myeloma, can be ideal targets for therapeutic purpose, either directly or by inhibiting interaction with BMSCs.

Conflicts of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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