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골수미세환경과 관련된 다발골수종 진행기전

Mechanisms of myeloma progression related to the bone marrow microenvironment

2014년 8월

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지도교수 윤 성 수

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Mechanisms of myeloma progression related to the bone marrow microenvironment

by

LEE, Chansu

(Directed by Yoon, Sung-Soo, M.D., Ph.D.)

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Approved by thesis Committee:

Professor Dong Goon Lee **Chairperson**

Professor Sungsu Yoon **Vice Chairperson**

Professor _____

Professor Seokah Seo

Professor Jin seok Kim

Abstract

The dynamic interplay between multiple myeloma and bone marrow stromal cells directly affect disease progression. Various cytokines and chemokines are produced by bone marrow stromal cells, and they lead to alter the biologic behaviors of MM cells becoming a malignant type of MM cells. To understand the role of cytokines, first, I examine the expression levels of various cytokines in the serum of MM patients' bone marrow at diagnosis as well as at relapse. The results indicated that the expression levels of IL-6, sIL-6R, HGF, TNFα, TGFβ, DKK1, Flt3L and OPN, such as well-known factors in MM, were detectable. According to the correlation analysis among the level of these cytokines, some cytokines might affect the expressions of other cytokines in an autocrine and/or paracrine manner. In this study, I address the biological roles and correlation of cytokines, such as TNFα, HGF, OPN, and Flt3L, releasing from BMSCs in the progression of MM.

Previous reports stated that TNFα increased interleukin-6 (IL-6) production from MM cells. However, the detailed mechanisms involved in the signaling pathways by which TNFα promotes IL-6 secretion from MM cells are largely unknown. I found that TNFα treatments induce MEK, STAT3 and AKT phosphorylation in MM cells. IL-6 production stimulated by TNFα was suppressed by the inhibition of JAK2, IKKβ and small interfering RNA (siRNA) for TNF receptors (TNFR), but not by MEK, p38, and PI3K inhibitors. This implies that TNFα induced the

regulation of IL-6 secretion via JAK/STAT mediated NF-κB activation in MM.

The levels of HGF expression were correlated with those of OPN in the BM plasma of an MM patient. The induction of OPN was regulated by HGF released from BMSCs. HGF activated both the MAPK and PI3K/AKT pathways. HGF also stimulated the increase of the RUNX2 mRNA level that contributed to the OPN expression in multiple myeloma cells. However, the expression of OPN mediated by HGF could only be suppressed by the MEK inhibitor (PD98059) and not by the PI3K inhibitor (LY294002). In addition, I observed that MMP-9 mRNA expression was increased in OPN treated BMSCs. However, its expression was suppressed by the LY294002. These results indicate that HGF induced OPN expression via the activation of the MAPK pathway and activated MMP-9 expression in BMSCs via OPN-mediated PI3/AKT pathway. It means High level of HGF in BM microenvironment could contribute to modulate the biologic behaviors of BMSCs related to MM progression.

CD44 is widely distributed in diverse biologic processes as lymphocyte homing and leukocyte activation. In particular, some reports stated that HGF-induced signaling depend on the presence of some CD44 isoforms and activation of receptor-tyrosine kinase through CD44; moreover, its CD44 variants supports cell survival and progression in MM. I found that CD44s were expressed in MM cells. As

the results conveyed, CD44s were detected in the MM cell line and CD138 positive cells were obtained from the MM patient's bone marrow. Further, the down-regulation of CD44s expression by CD44 shRNA controlled the HGF-mediated MAPK signaling pathway as well as the HGF-mediated induction of DKK1. It demonstrated that CD44s also can contribute to the development of bone erosion mediated by HGF stimulation.

Flt3 ligand (Flt3L) is known to be involved in hematopoietic cell differentiation and early B-cell differentiation. However, the role of Flt3L still remains unknown in MM. I found that the level of Flt3L in MM patients' BM plasma was related to poor prognostic parameters. Moreover, up regulation of Flt3 phosphorylation mediated by Flt3L was related with relapse after the autologous bone marrow transplantation in MM. In addition, Flt3 activation induced DKK1 secretion mediated via JAK/STAT and AKT signaling pathways. These results implied that a high level of FLT3L is significantly related with MM progression.

To improve the clinical outcome of MM patients, many studies have been attempting to develop novel targeted drugs. Using the newly developed anti-MM drug, CKD-581, a novel HDAC inhibitor, its effect was examined in various MM cell lines. CKD-581 showed a potentially anti-myeloma effect in MM cell lines. In a combined treatment with bortezomib, CKD-581 synergistically increased bortezomib-mediated apoptosis in MM cells, and these effects were similar to the effect of

co-cultured MM cells with BMSCs. This result suggested that CKD581 could overcome the interferences of BMSCs in the treatment of the anti-MM drug. In an ex vivo study using patients' derived buffy coat, CD138 positive cells were decreased significantly compare to other cells when cells were treated with CKD-581. In the vivo model, mice treated with CKD-581 alone showed significantly longer survival duration compared to non-treated control mice. Mice treated with combined CKD-581 and Bortezomib also showed survival benefit; further, these mice showed a slightly longer survival compared to CKD-581 treated mice. These data indicated that CKD-581 had anti-tumor effects in MM and moreover, it has the potential to show a synergistic effect with Bortezomib.

Keywords

Multiple myeloma, Microenvironment, BMSCs, Cytokine, HDAC inhibitor

Student number : 2010-31164

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Introduction

Multiple myeloma (MM) is a tumor characterized by the clonal proliferation of abnormal and malignant plasma cells [1,2]. MM is the third most common hematological malignancy after non-Hodgkin's lymphoma and acute myeloid leukemia in Korea [3]. Furthermore, the incidence of multiple myeloma in Korea has been increasing consistent over the past 30 years. Despite the fact that many researchers have been focusing on intensive investigations to overcome the incurable disease, clues for complete eradication of MM still remains unknown [3]. To overcome the blockages of anti-MM chemotherapy, it is necessary for us to investigate the dynamic interplay between MM cells and their microenvironment. So far, we know that the biologic behaviors of MM cells play a critical role in their chemotherapeutic response and progression.

Generally, B-cells are terminally differentiated to plasma cells by the interaction with an antigen or antigen presented T cells, which produce antigen specific antibodies [4]. Even though dyregulation of the production of antibody could be a major causing factor in MM, various factors, which are growth factors-mediated signaling and NF-kB-mediated cell signaling, are involved in the incidence and the progression of MM. In particular, multistep genetic alternations of MM cells under their microenvironments lead to the transformation of these

cells into a malignant neoplasm [5]. Various factors released from bone marrow stromal cells could directly affect the growth of MM cells. In general, most differentiated plasma cells settle in the bone marrow, where they can receive survival signals from stromal cells and remain for several months to years [4]. In a similar way, the niches of MM patient's bone marrow contribute to the accumulation of poor differentiated MM cells, which produced IgM within the bone marrow results in MM [6]. Increasing accumulations of MM cells lead them to be a malignant phenotype of MM cells. After dyregulating both the differentiations of cell lineages and the apoptosis of MM cells in the bone marrow, the main clinical manifestations of MM are the development of devastating osteolytic bone lesions, bone pain, hypercalcaemia, renal insufficiency, suppressed haematopoietic function, reduced polyclonal immunoglobulin production, and increased BM angiogenesis [7].

In the progression of MM, MM usually evolves from an asymptomatic premalignant stage of clonal plasma cell proliferation termed "monoclonal gammopathy of undetermined significance" (MGUS) [6]. MGUS develops "smoldering multiple myeloma" (SMM). Finally, it develops in a more advanced premalignant stage, a progress to myeloma [6,8]. Not only many oncogenic events, but also alternations of bone marrow niches directly affect the biologic transition from

normal plasma cells to MGUS to SMM to advanced malignant MM [9]. In the past few years, early, partially overlapping genetic events include, at a minimum, primary IgH translocations, hyperdiploidy, and del13, which lead directly or indirectly to the dysregulation of a CCND gene [4,10]. Secondary translocations, involving MYC (8q24), MAFB (20q12), and IRF4 (6p25), can occur at any stage of plasma cell dyscrasia [4]. Furthermore, activating mutations of NRAS, KRAS, FGFR3, MYC dysregulation, deletion in p18, or loss of expression or mutation in TP53 are found only in multiple myeloma patients compared with MGUS patients [11]. The inactivation of cyclin-dependent kinase inhibitors, CDKN2A and CDKN2C, are also found in MM. Other genetic abnormalities also involve epigenetic dyregulation, such as an alteration in microRNA expression and gene methylation modifications [5].

Chronic inflammation could be one of the major causing factors for developing and progressing MM. Various cytokines and chemokines are produced by bone marrow stromal cells, and they lead to alter the biologic behaviors of MM cells from becoming malignant type of MM cells. In bone marrow niches of MM patients, several sets of cytokines, such as IL-6, sIL-6R, TNFα, HGF, TGF-β, DKK1, and OPN, were highly expressed when compared to the case controls. Indeed, the dynamic interplay between those cytokines and their receptor in MM

cells triggers the activation of phosphatidylinositol 3-kinase (PI3-K)/AKT and mitogen-activated protein kinase (MAPK) pathway, which lead to proliferation, chemotaxis, and protection against apoptosis, and thereby suggesting a major role for NF-kB activation in the pathophysiology of MM.

Typically, the development of lytic bone lesions occurs only in the area of the bone adjacent to myeloma cells. It suggests that lytic lesions result from the local overproduction of osteoclast stimulatory factors, which are secreted by MM cells, bone marrow stromal cells (BMSCs), or both. In addition, various cytokines and chemokines released from BMSCs contribute to the survival of MM cells against apoptotic signals. A recent study suggested that several cytokines from the bone marrow microenvironment have been implicated in contributing to the development of MM. In particular, Interleukin 6 (IL-6) has been frequently suggested to be crucial for the pathogenesis of MM. Moreover, it has been shown to act as an autocrine, paracrine growth, and survival factor. Subsequently, the constitutive activation of the nuclear factor κB (NFκB) pathway is mediated by mutations in MM during progression. In addition to these oncogenic events, the tumor cells are strongly dependent on the bone marrow microenvironment [6]. Bone marrow (BM) microenvironment and mutual interaction between multiple myeloma cells and bone marrow stromal cells contribute to modulate the biological behaviors of MM cells in each patient [12,13].

The interaction between MM cells and the BMSCs is a matter of central importance with regard to the pathophysiology of MM [14].

The bone marrow microenvironment is composed of many cellular and cellular components, such as endothelial cells, stromal cells, osteoclasts, osteoblast, immune cells, fat cells as well as extracellular matrices. And this environment can provide a highly vascular environment rich in nutrients and growth factors to the contained cells [15,16]. In the bone marrow environment, cancer cells are located in specific niches containing bone marrow stromal cells [17]. Consequently, the bone marrow microenvironment has been shown to contribute to the survival of the malignant cells as well as mediate tumor progression. Because bone marrow stromal cells interact by direct adhesion or by the secretion of numerous cytokines to support multiple myeloma survival, progression and drug resistance [13,16], multiple myeloma cells and the bone marrow microenvironment should be linked by a composite network of interactions mediated by soluble factors and adhesion molecules [18]. The levels of expression and activity of adhesion molecules are controlled by cytoplasmic operating mechanisms, as well as by extracellular factors, including enzymes, growth factors and microenvironmental conditions [18,19].

Genetic abnormalities alter the expression of adhesion molecules and

growth factors on myeloma cells [5,20]. For example; the 1q21 amplification can causes an increase in the IL-6 receptor and CCR1, CD40L, FGFR3, HGF, ICAM1, IGF1, MIP1 α, and MUC1 [4,21]. Also, MM cells adhere to the extracellular matrix proteins and BMSCs via a series of adhesion molecules, including the β1-integrin family with several very late antigens (VLA; VLA-4, VLA-5, and VLA-6), as well as the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [13,22]. These Integrins and syndecan-1/CD138, as a neoplastic plasma cell marker, are the principal multiple myeloma receptor systems of extracellular matrix components, as well as of surface molecules of stromal cells [10,18,23]. The SDF-1/CXCR4 axis is a key factor in the homing of multiple myeloma cells to the bone marrow [18,24]. Several signaling responses are activated by adhesive interactions of multiple myeloma cells, and their outcomes affect the survival, proliferation and migration of these cells. Thus, this interaction mediated by abnormal expression affects the production of growth factors, which stimulate plasma cells survival, angiogenesis, bone resorption, and drug resistance [18,25].

CD44, a distributed cell surface glycoprotein, is one of the major hyaluronan receptors and adhesion molecules expressed on MM cells. CD44 is a widely distributed protein implicated in such diverse biologic processes as lymphocyte homing and leukocyte activation [26,27]. The CD44 transcript is composed of at least 20 exons and 10 variant exons

(v1–10), which can be alternatively spliced in various combinations, thereby generating numerous splice variants (CD44v) from a single gene [26]. HGF-induced Met activation and signaling depends absolutely on the presence of some CD44 isoforms. CD44 and its variants are also involved in multiple myeloma progression and bone disease by the interaction between myeloma cells and BMSC via IL-6 regulation [28].

The BM cells have interaction by the secretion of numerous cytokines to support tumor development and progression [6,29]. A number of factors present in the BM microenvironment have been dysregulated in patients with MM [30,31,32]. Cytokines, including IL-6, VEGF, IGF-1, TNFα and HGF produced from BMSCs, and tumor cells directly/indirectly influence MM cells via autocrine and paracrine manners. Cytokines trigger signaling pathways that promote cell proliferation and prevent apoptosis [12]. IL6 and IL6 receptor complexes (IL6R and gp130) are the best characterized myeloma growth factors. This IL6 complex enhances the proliferation and survival of myeloma cells that mediate the activation of both the STAT and MAPK pathways [33,34]. TNFα mediates the progression of MM cells is via regulation of the NF-κB transcription factor. This molecular signaling regulation is also related to IL-6 secretion by stromal cells and osteoblasts [35]. TNFα is also known for promoting

osteoclastogenesis and inhibiting osteoblastogenesis [36,37]. HGF is a mitogenic factor involved in blood vessel formation. HGF involves in the development of osteolytic bone disease, and HGF-mediated c-Met activation regulates several physiological processes, including cell proliferation, invasion, and angiogenesis in tumors [38]. Previousreports have stated that HGF inhibited bone osteoblastogenesis in multiple myeloma and high serum concentration of HGF, which is associated with poor prognosis in patients with myeloma [35].

The concerted action of cytokines secreted locally in the bone marrow controls the maintenance, expansion, and differentiation of hematopoietic stem cells (HSCs) [39]. Given that the progression of MM is regulated by growth factors released from bone marrow stromal cells, aberrant cytokine signaling contributes to the oncogenic transformation during multi-lineage hematopoietic cell differentiation. The Flt3-ligand (Flt3L) is capable of inducing multi-lineage hematopoietic cell differentiation [40]. In particular, Flt3L-stimulated signaling is one of the major factors for early B-cell differentiation [41]. B-cell progenitors up-regulate Flt3, Flt3L receptor and efficiently reconstitute the lympho-myeloid, but not the erythro-megakaryocytic lineages [42]. Also, Jeny Maree Lean et al. (2001) reported that Flt3L participated in the maintenance of osteoclastic precursors on bone

surfaces and induced osteolysis in the animal model [43]. Even though abnormal bone resorption is the most frequent manifestation in MM patients, the exact role of Flt3-ligand still remains unknown in MM.

The development of bone lesions is a pathologic feature that evolves on the progression of MM [44]. A number of factors secreted by the interaction between MM cells and BMSCs are involved with promoting osteoclastogenesis and decreasing osteogenesis. The increased osteoclast activity is due to an imbalance in the ratio between RANK and OPG, as a result of enhanced production of RANK ligand (RANKL) and reduced production of OPG [45]. Osteoblast activity is also suppressed by the production of dickkopf homolog 1 (DKK1) by plasma cells [46]. Dkk1, such as extracellular antagonist of the Wnt signaling pathway, has been previously identified as a potential mediator of osteoblast dysfunction in myeloma bone disease [47] [48].

Recently, to overcome drug resistance against classical therapies, many studies have been performed to develop a novel drug that targeted small molecules. Many molecular-targeted compounds, such as bortezomib (Proteasome inhibitor), Thalidomide, and lenalidomide (immunomodulatory drugs), along with several targeted inhibitors have been developed for MM treatment [49]. Recent studies have reported that combined treatment strategies using conventional regimens and

new molecular-targeted compounds are sufficiently effective on MM patients. However, most patients with MM eventually become resistant or relapse against chemotherapies and die of disease progression within 10 years [50]. In many cases, MM patients experience relapses during the course of therapeutic treatment. It is generally believed that the BM microenvironment has been shown to protect myeloma cells from common cytostatic or cytotoxic drugs. Therefore, novel therapeutic approaches must target both tumor cells and stromal cells [51].

Increasing cytokines released from BMSCs help MM cells to adapt themselves to new bone marrow niches; further, they trigger MM cells into malignant phenotype. First, I examine the expression levels of various cytokines in the serum of MM patients' bone marrow at diagnosis and at relapse. Moreover, the correlation among cytokines examines to understand the progression of MM and MM pathogenesis. In this study, I address the role of cytokines, such as TNFα, HGF, OPN, and Flt3L, released from BMSCs in the progression of MM. In addition, I examine the role of BMSCs in the response of chemotherapeutic agents, such as bortezomib and novel HDAC inhibitor in the in vitro and in vivo model.

Objective

Based on the background described above, following objectives were pursued.

1. The expression level of several cytokines associated with MM progression was investigated in MM plasma. And correlations among cytokines retained in the bone marrow microenvironment were sought.

2. Mechanisms for expression regulation of correlated cytokines in MM were assessed and roles of cytokines related to MM progression were evaluated.

3. The novel pan-HDAC inhibitor, CKD-581 was examined as an anti-myeloma drug in MM cells co-cultured with bone marrow stromal cells and orthotopic MM mouse model.

These aims will be addressed in the following chapters.

Materials and Methods

Cell lines

The human multiple myeloma cell lines, U266 and RPMI8226, were generously provided by Dr. Dong soon Lee (Seoul National University, College of Medicine, Seoul, Korea) and IM9 was obtained from the Korea Cell Line Bank (Chongno-gu, Seoul, Korea). MOLP8, KMS-12-BM, KMS-12-PE, EJM, and LP-1 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). U266, RPMI8226, MOLP8, KMS-12-BM, and KMS-12-PE were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with sodium pyruvate, 10% heat-inactivated fetal bovine serum, essential vitamins, L-glutamine, penicillin (100 U/mL), and streptomycin (100 g/mL) (GIBCO, Grand Island, NY, USA). EJM, and LP-1 were maintained in IMDM, Iscove's Modified Dulbecco's Medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with sodium pyruvate, sodium bicarbonate, 20% heat-inactivated fetal bovine serum, essential vitamins, L-glutamine, penicillin (100 U/mL), and streptomycin (100 g/mL). All Cells were maintained in a highly humidified atmosphere of 5% CO2 and 95% air at 37°C. All experiments were performed using cells in logarithmic growth phase.

Clinical samples

All patients, diagnosed with multiple myeloma, referred to centers in Korea—Seoul National University Hospital, who received induction chemotherapy. Patients agreed to donate bone marrow samples for molecular testing on research base with informed consent and analyzed. Buffy coat layer was separated from each bone marrow samples by ficoll density gradient centrifugation

BMSC culture and co-culture

Bone marrow sample 200μl was seeded in 5ml Dulbecco's modified Eagle's medium (DMEM/F12) with 10% fetal bovine serum (FBS) in T-25 flasks. And after 2 days, the non-adherent cells were removed. The cultures were then continued for 3 weeks. Media were replaced with fresh media every 4 days. At subconfluence, cells were detached with trypsin and plated at a density of 10^5 cells/T-25 flask and were used at passages 2 to 3. Cultured primary BMSCs $1x10^4$ cells were seeded in appropriate culture ware (6 well). After 24 h, $1X10^6$ MM cells were added. After another 24 h, indicated dose of agent was treated.

Reagents

CKD-581 was provided by Chong Kun Dang Pharmaceutical Corp, Seoul, Korea. It was dissolved in saline solution and stored at 4°C

freezer as a stock solution protected from light. The specific proteasome inhibitor, bortezomib (VelcadeTM; formerly known as bortezomib), was generously provided by Janssen Korea, Ltd. (Seoul, Korea). It was dissolved in DMSO and stored at -20°C as a stock solution. All reagents were diluted in culture medium immediately before use.

Recombinant human FLT3L, HGF, TNFα and OPN were 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. PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor), JAK inhibitor I / II, Bay 11-7082((E)3-[4-Merhylphenyl)sulfonyl)]-2-propenenitrile), TPCK (Nα-Tosyl-Phe Chloromethyl Ketone), PDTC (10 Pyrrolidinecarbodithioic Acid, Ammonium Salt), used as NF-κB inhibitors, PI3K inhibitor (LY294002), JNK inhibitor II, p38 MAPK inhibitor (SB203580), and AG490 (JAK2 inhibitor) were purchased from Calbiochem Corp. (San Diego, CA, USA). These agents were dissolved in DMSO as stock solutions, stored at -80°C, and subsequently diluted with serum-free RPMI-1640 prior to use. Concentrations of various pharmacologic inhibitors were adapted from IC50 values from the manufacturer's manual.

Cell proliferation assay

Cell proliferation assay was performed using Cell Counting Kit-8

(Dojindo Laboratories, Kumamoto, Japan). 100 μL of cell suspension was seeded in 96-well culture plates at 1X104 cells per well. Then, reagents were added into each well in the plate and cells were incubated for the indicated time period. Then, 10 μL of CCK-8 solution was added to each well for another 4 h. The absorbance of each well was measured in a microplate reader (Becton Dickinson Labware, Le Pont de Claix, France) at 450 nm. Means and standard deviations were generated from three independent experiments. Absorbance values were normalized to the values obtained from control group to determine the value for % of survival. Values are the mean ± S.D.

Reverse transcriptase and Real time polymerase chain reaction

Total RNA was extracted from appropriate cells using TRIsol reagent(Gibco BRL, Invitrogen, Carlsbad, CA, USA) and measured its concentration by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc Wilmington, DE 19810 USA). 1μg RNA per sample was reverse transcribed with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using random hexamers. The cDNA were synthesized by incubation at 45°C for 1 h. Then the reaction was inactivated by heating at 95°C for 5 min. Then the reaction was inactivated by heating at 95°C for 5 min.

Relative mRNA expression levels were measured by quantitative PCR using target specific primer and Premix buffer contained

SYBR/ROX dye (AccuPower® greenStar qPCR premix) purchased from Bioneer(Daejeon, Korea). Temperature conditions were as follows: qPCR process was done in 20 μl volume for 95°C for 5 min, 40 cycles at 95°C for 1 min, 60°C for 30 s. Plate document/experiment parameter for qPCR was followed by manufacturer's instructions and analyzed by Applied Biosystems 7300 Real-Time PCR System. Three independent qPCR reactions were performed on the cDNA derived total RNA isolates from the three experimental replicates and target mRNA levels were normalized relative to β-actin expression levels.

Western blot

Cells were treated with indicated reagents for the indicated time period, washed once with ice-cold phosphate buffered saline (PBS), and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, Na-deoxycholate 0.25%, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF and 1g/mL of Protein inhibitor cocktail purchased from Hoffmann-La Roche Ltd, Swiss).

The protein concentration of lysate was measured, 30 g of cytoplasmic protein extracts were boiled for 5 min and proteins were resolved in 10% SDS–polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h at room temperature

and incubated with appropriate primary antibody for 2h. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., PA, USA) and an enhanced chemiluminescence reagent, WEST-ZOL plus (iNtRon biotechnology, Seoul, Korea).

Primary antibodies for following proteins were used in this study; p-MAPK42/44, p-raf, c-raf, HDAC1, 3, 7, phospho-mTOR, phospho-c-Raf, phospho-STAT3 (ser727), phospho-MAPK sampler kit, phospho- AKT (ser473) sampler kit, phospho-MEK1/2 (cell signaling technology, Beverly, MA, USA) and c-Myc, cyclin D1, phospho-JNK, JNK, Cytochrome C, BAX, PARP, GAPDH and β-actin (Santa Cruz, CA, USA) were used as primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch laboratories Inc., PA, USA) were used.

Expression assay by HT-12 Whole-Genome Expression BeadChip

In two human MM cell lines, Gene expression assays were run in triplicate. RNA isolated from the cell line at the indicated time points was used for gene expression analysis using the HT-12 Whole-Genome Expression BeadChip (Illumina, San Diego, CA, USA), which generates expression profiles for more than 46,000 human transcripts. Biotin-labeled cRNA was produced using a linear amplification kit

(Ambion, Austin, TX, USA) using 300 ng of quality-checked total RNA as input. Chip hybridizations, washing, Cy3-streptavidin staining and scanning were performed on a BeadArray Reader (Illumina) platform using reagents and following protocols supplied by the manufacturer. The detection score was used to determine expression.

ELISA

The Enzyme-linked immunosorbent assays (ELISA assay) were performed with several cytokines such as FLT3L, IL-6, TNFα, TGF beta_1, HGF, DKK1, IL-2, IL-4, IL-10 and OPN. The expression levels of cytokines maintained in plasma of bone marrow were measured and the level of cytokines released bone marrow stromal cells obtained from MM patients and multiple myeloma cell lines were also measured using ELISA kit (R&D systems, Minneapolis, MN, USA). Bone marrow aspirate of MM patients were separated to plasma and bone marrow cells by centrifugation. After the centrifugation, supernatants were harvested and stored in -70 ℃. All cultured cells were maintained with serum free culture medium for indicated period and then cell free supernatants were harvested.

ELISA assay was performed in accordance with the manufacturer's instructions. The optical density of the samples was determined using a microplate reader set at 450nm.

Transfection for siRNA

Small interference RNA (siRNA) for siGENOME Human TNFR siRNA (M-005197-00) and siGENOME Non-Targeting 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 (5 μg) was added to 1 $\times 10^6$ 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.

Transfection for shRNA plasmid vector

The plasmid vectors containing shRNA sequence for target genes were obtained from OriGene Technologies, Inc (Rockville, MD20850, USA). CD44 targeted shRNA sequence were used. 1 set of shRNA constructs contains 4 different sequences of each shRNA expression vectors in pGFP-V-RS plasmid. Plasmids were amplified with transformation method using DH5α competent cell strain. In

transfection study, I used uni-dose mixture of different shRNA vectors for the same gene. Amaxa® Nucleofector® device and solution were used for electroporation method (Walkersville, MD 21793, USA). 2 X 106 cells were needed at one experiment. All processes were performed according to AMAXA manufactural protocol.

Animal model

6~8 week old NRG (NOD.Cg-Rag1$_{tm1Mom}$Ins2$_{Akita}$Il2rg$_{tm1Wjl}$/SzJ) mice purchased from the Jackson laboratory, Sacramento, USA were irradiated (400 rads) using a ^{137}Cs –irradiator source and mice received tail vein injections of 1 X 10^6 MOLP8 cell line that suspended in a total volume of 200 μl PBS / mouse. After 1 week, injected mice were treated with CKD-581 and/or Bortezomib. On the day of injections, CKD-581 and Bortezomib stock solution was diluted in normal saline buffer to indicated concentration (CKD-581: 40mg/kg, Bortezomib: 0.25rmg/kg) and administered by Intraperitoneal injection two times a week. Histological sections of tibia from MM cells injected mice were hematoxylin and eosin(H&E)－stained via standard methods.

Flow cytometry analysis

Briefly, cells (5 x 10^4 cells in 100 ml of RPMI 1640 media) were incubated for 10 min in the presence of 0.25 mg of propidium iodine. The sample was then diluted five times in PBS and analyzed on an

Epics XL (Beckmann Coulter, Villepinte, France). Propidium iodine (0.5 mg/ml) was detected on fluorescent channels 1 and 2, respectively. The percentages of living and dead cells were calculated from acquiring a total of 10000 events. Results are indicated as follows: percent of survival cells (B1) and dead cells (B2, B4) were quantified by using Coulter software. To measure apoptotic cells, analysis was performed on Ds-Red2-positive cells. Experiments were repeated three times**.**

Results

1. The expression levels of cytokines in the serum of bone marrow obtained from MM patients

1-1. The expression levels of cytokines in the MM microenvironment

To define the retained level of various cytokines in bone marrow serum of MM patients, ELISA assay was performed in a total of 80 multiple myeloma patients from my laboratory clinical sample cohort. As shown in figure 1, levels of FLT3L, IL-6, TNFα, TGF beta_1, HGF, DKK1 and OPN were elevated; however, cytokines, such as IL-2, IL-4 and IL-10, were too low to be detected by ELISA. Almost elevated cytokines, except FLT3L, were known as the relation of MM development, progression, and bone disease; yet, the correlation among cytokines and its acting mechanism have not cleared yet. This data indicated that inflammatory cytokine was highly expressed in bone marrow from MM patients. It suggested that constitutive activation of NF-kB contribute to the survival of MM cells during MM progressions.

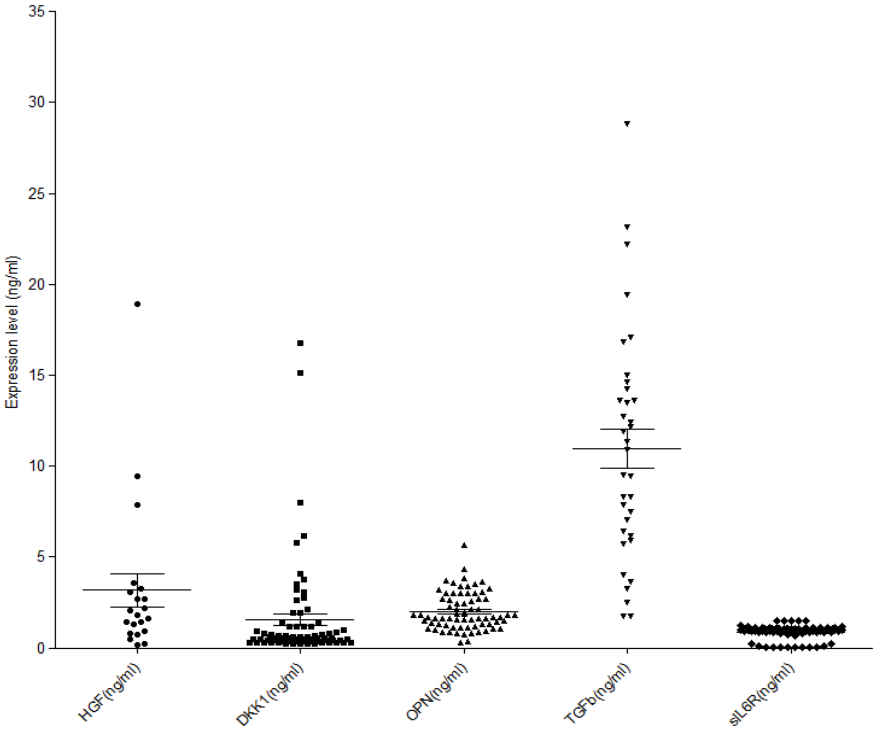
1-2. The correlation of cytokines in the serum of bone marrow obtained from MM patients

To address the association of examined cytokines, I calculated the r value. As shown in figure 2, the result demonstrated that IL-6 in MM was correlated with various cytokines existing in the bone marrow, which might have cross-talk between each other. These results suggest that the release of cytokines from bone marrow plasma might affect the expressions of other cytokines in an autocrine and/or paracrine manner.

Figures and Figure legends - 1

Figure 1. The expression levels of cytokines in serum of bone marrow

Bone marrow plasma was obtained from patients with multiple myeloma. The expression levels of IL-6, OPN, TNFa, TGFb, DKK1 and FLT3L, respectively, were measured using ELISA assays.


35
30
25
20
15
10
5
0
Expression level (ng/ml)
HGF(ng/ml)
DKK1(ng/ml)
OPN(ng/ml)
TGFb(ng/ml)
sIL6R(ng/ml)

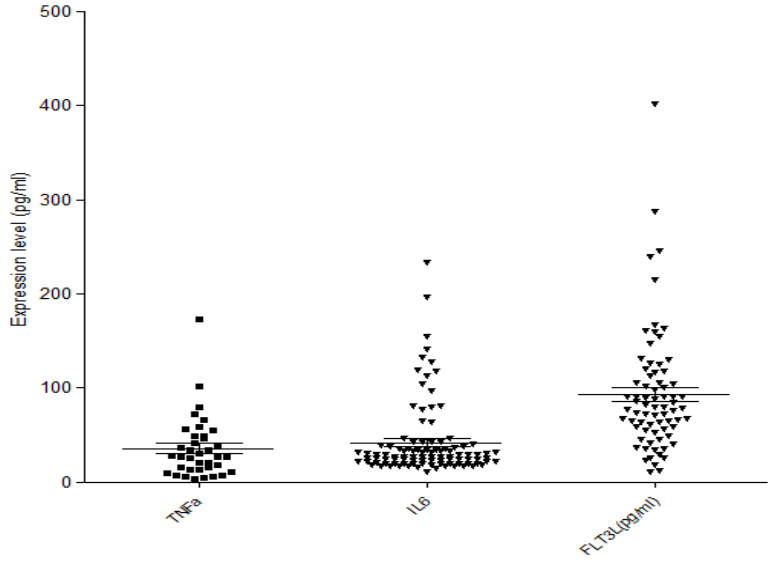
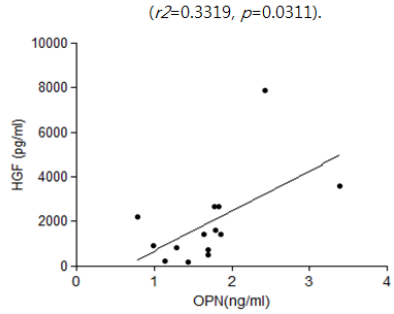
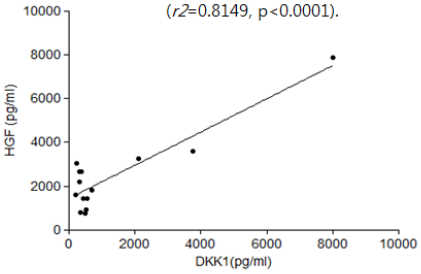
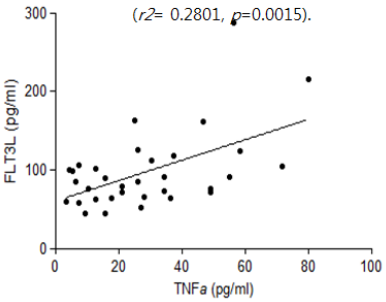

500
400
300
200
100
0
Expression level (pg/ml)
TNFa
IL6
FLT3L(pg/ml)

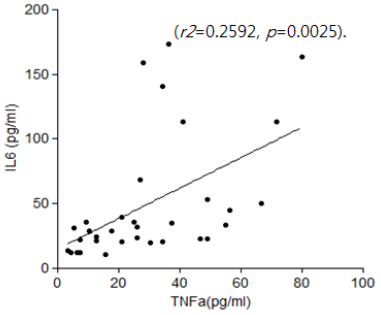
Figure 2. Correlation analysis between major cytokines retained in bone marrow of MM patients

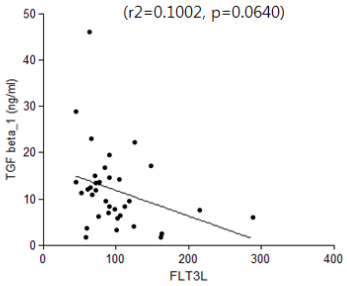
Bone marrow plasma were obtained from patients with multiple myeloma. The expression levels of IL-6, OPN, TNFa,TGFb-1, and FLT3L, respectively, were measured using ELISA assays. Correlation (r^2 value) between measured parameters was calculated by the Spearman's rank correlation coefficient. *p* values ≤0.05 were considered to be statistically significant.

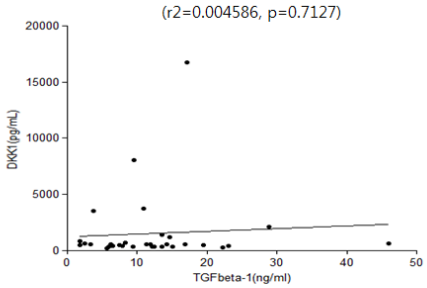

(r2=0.3319, p=0.0311).
HGF (pg/ml)
OPN(ng/ml)

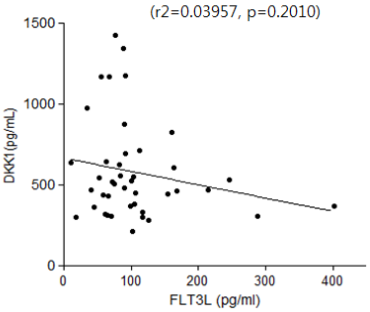

(r2=0.8149, p<0.0001).
HGF (pg/ml)
DKK1(pg/ml)

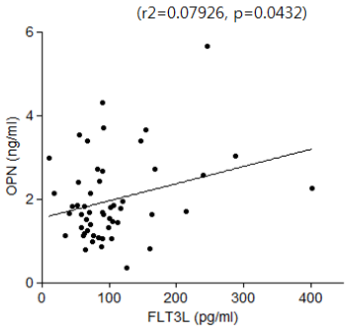

(r2= 0.2801, p=0.0015).
FLT3L (pg/ml)
TNFa (pg/ml)


(r2=0.2592, p=0.0025).
IL6 (pg/ml)
TNFa(pg/ml)


(r2=0.1002, p=0.0640)
TGF beta_1 (ng/ml)
FLT3L


(r2=0.004586, p=0.7127)
DKK1(pg/mL)
TGFbeta-1(ng/ml)


(r2=0.03957, p=0.2010)
DKK1(pg/mL)
FLT3L (pg/ml)


(r2=0.07926, p=0.0432)
OPN (ng/ml)
FLT3L (pg/ml)

2. Alternations of cytokine expression levels in MM cells co-cultured with bone marrow stromal cells

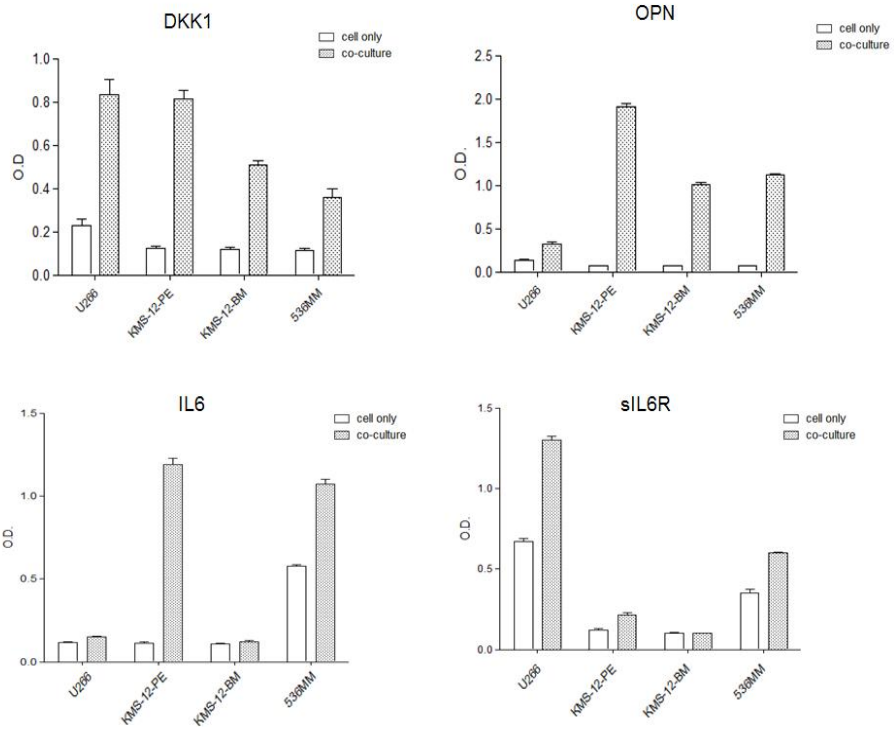
2-1. Up-regulation of IL-6, sIL-6R, DKK1, and OPN co-cultured with BMSCs obtained from MM patient's bone marrow in MM cell lines

To examine whether bone marrow stromal cells directly affect the production of cytokines, I examined the expression levels of cytokines in either MM cells only or MM cells co-cultured with BMSCs. As shown in figure 3, the expression of IL-6, sIL-6R, DKK1, and OPN was elevated when several MM cells, such as U266, KMS-12-BM, KMS-12-PE and 536MM, co-cultured with BMSCs. The result suggested that BMSCs triggered to produce cytokines from either MM cells or BMSCs.

Figures and Figure legends - 2

Figure 3. Up-regulation of IL-6, sIL-6R, DKK1, and OPN in MM cells co-cultured with BMSCs obtained from MM patient's bone marrow

MM cell lines were co-cultured with MM patient's BMSC at diagnosis for 72 hour incubation. After 72h incubation, cultured soup were harvested and then passed them through 0.22um syringe filters. Then, the expression levels of IL-6, sIL-6R, DKK1, and OPN were measured using ELISA in cultured soups of U266 cells co-cultured with MM patient's BMSC. p values ≤0.05 were considered to be statistically significant.


DKK1
cell only
co-culture
O.D
1.0
0.8
0.6
0.4
0.2
0.0
U266
KMS-12-PE
KMS-12-BM
536MM
OPN
cell only
co-culture
O.D.
2.5
2.0
1.5
1.0
0.5
0.0
U266
KMS-12-PE
KMS-12-BM
536MM
IL6
cell only
co-culture
O.D.
1.5
1.0
0.5
0.0
U266
KMS-12-PE
KMS-12-BM
536MM
sIL6R
cell only
co-culture
O.D.
1.5
1.0
0.5
0.0
U266
KMS-12-PE
KMS-12-BM
536MM

3. TNFα mediated IL-6 secretion is regulated by JAK/STAT pathway in multiple myeloma cells

3-1. Effect of TNFα on IL-6 release from multiple myeloma

Previous report told that TNFα plays a key role in the facilitation of IL-6 secretion [52]. Figure 2 showed that there was a correlation between the IL-6 and TNFα levels in bone marrow aspirate samples of patients. Hence, I 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 did not show a drastic IL-6 secretion. As shown in Figure 4, level of IL-6 was 1.5-fold higher in conditioned media of TNFα-treated U266 cells compared with untreated cells. However, the induction of IL-6 by TNFα treatment was not detected in IM9 cells.

3-2. Activation of various signaling pathways by TNFα and suppression of IL-6 release with inhibitors

To evaluate the signaling mechanism of TNFα induced IL-6 secretion, I first examined activation of signaling molecules by TNFα treated U266 and IM9 cells by western blot analysis. After serum starvation, cells were stimulated with TNFα for 10 min. As shown in figure 5A, various signaling molecules were regulated by TNFα,

including Raf/MEK/Erk, JNK, and PI3K/AKT pathways in both cell lines. Because IM9 showed a slight difference in the level of IL-6 secretion despite TNFα stimulation, I opted to use the U266 cell line for further study because the aim of my study was to delineate the role of TNF alpha on the secretion of IL-6. Cells were pre-incubated with PD98059, LY294002, SB203580 and JNK inhibitor II for the suppression of the phosphorylation of p44/42MAPK, PI3K/AKT, p38 MAPK and JNK, then, they were stimulated with TNFα. As shown in figure 5B, the inhibitors were not potent to block the IL-6 secretion from U266 cells.

3-3. 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 cylin D1, downstream genes of JAK/STAT activation were examined in U266 cells. As shown in Figure 6A, TNFα induced phospho-STAT3 as well as increased the expression levels of cMyc and Cyclin D1. Therefore, I 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 the JAK/STAT pathway. As shown in Figure 6B, all inhibitors that target JAK/STAT signaling resulted in the suppression of

IL-6 secretion by TNFα. Similar suppression was also achieved by targeting the TNFα receptor using siRNA.

3-4. Suppression of IL-6 secretion by TPCK

Many studies revealed that IL-6 secretion by TNFα from multiple myeloma cells and bone marrow stromal cells were induced via the activation of NF-κB [53]. And it was known that NF-κB signaling was constitutively activated in MM. Hence, I examined whether the inhibition of NF-κB pathway affects the secretion of IL-6 by TNFα. Figure 7A 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 them with three different inhibitors. Figure 7B describes that none of the NF-κB inhibitors suppresses TNFα-induced phosphorylation of signaling molecules. Because TPCK did not directly inhibit the TNFα-induced p-STAT3, I can assume that TPCK action is not mediated by the STAT3 pathway, but by the downstream of the JAK/STAT pathway (that is, NF-κB pathway).

Figures and Figure legends - 3

Figure 4. 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 non-treated samples as a control were shown in graph. Bars represent the mean ± SEM from three independent experiments.

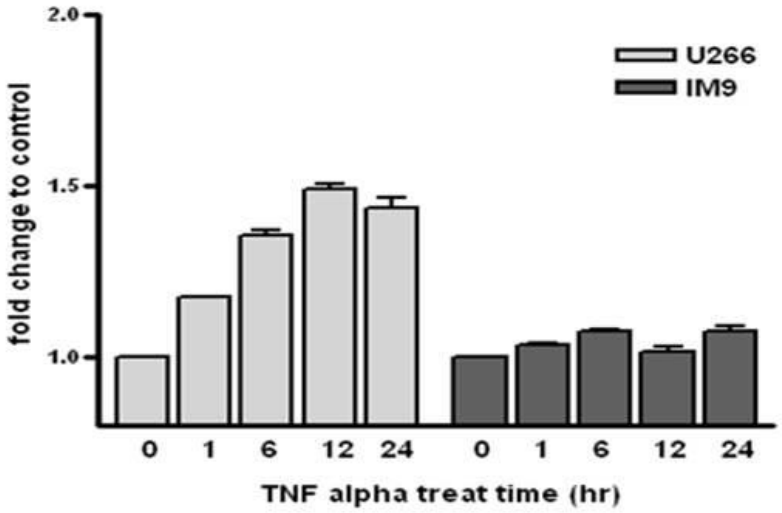

2.0
1.5
1.0
fold change to control
U266
IM9
0
1
6
12
24
0
1
6
12
24
TNF alpha treat time (hr)

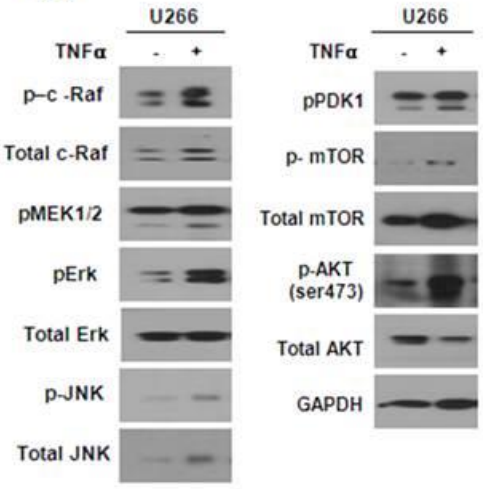
Figure 5. Effect of TNFα on activation of various signaling pathways and suppression of IL-6 secretion with indicated inhibitors

(A) U266, multiple myeloma cells were starved for 8h and then treated with or without 1ng/ml TNFα for 10min. Cells were harvested and prepared for immunoblotting. Up-regulation of MAPK, JNK, and PI3K/AKT by TNFα was examined with indicated antibodies. GAPDH was used to ensure equal loading.

(B) U266 cells were pre-treated with various signal inhibitors for 1h respectively. These cells were then stimulated with TNFα for 10min, 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 PD98059; 3: TNFα plus LY294002; 4: TNFα plus SB203580; 5: TNFα plus JNK inhibitor II

(A)


U266
TNFα - +
p–c -Raf
Total c-Raf
pMEK1/2
pErk
Total Erk
p-JNK
Total JNK
U266
TNFα - +
pPDK1
p- mTOR
Total mTOR
p-AKT (ser473)
Total AKT
GAPDH

(B)

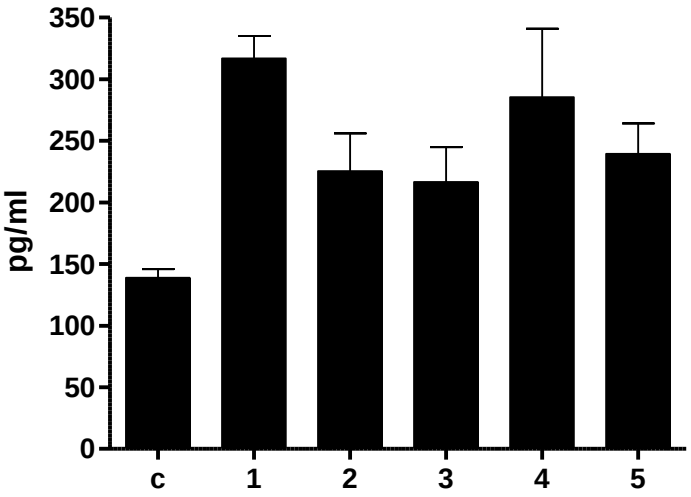
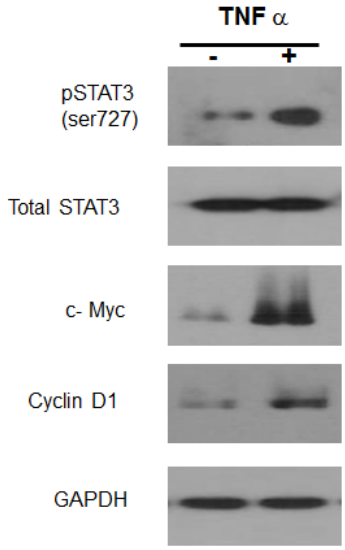

pg/ml
350
300
250
200
150
100
50
0
c
1
2
3
4
5

Figure 6. IL-6 secretion by TNFα regulated by JAK/STAT pathway

U266 cells were serum starved for 8h 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 AG490; 3: TNFα plus JAK inhibitor I; 4: TNFα plus JAK inhibitor II; 5: TNFα plus TNFR siRNA


TNF α
-
+
pSTAT3
(ser727)
Total STAT3
c- Myc
Cyclin D1
GAPDH

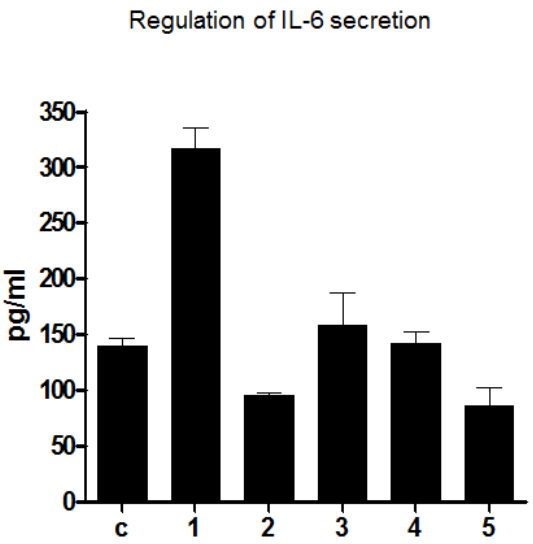
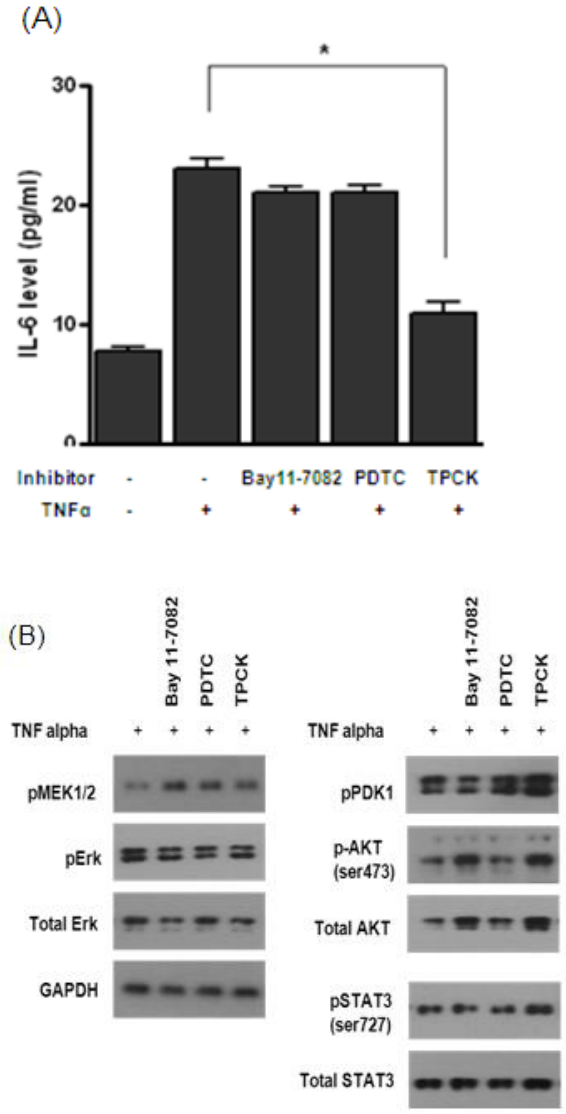

Regulation of IL-6 secretion
pg/ml
350
300
250
200
150
100
50
0
c
1
2
3
4
5

Figure 7. Effect of NF-κB inhibitors on TNFα induced IL-6 levels and signaling induction of its upstream pathways

U266 Cells were serum starved for 8hr and pre-treated with NF-κB inhibitors (10uM Bay 11-7082, 10uM PDTC, 20uM TPCK) for 1hr. These cells were then stimulated with 1ng/ml TNFα for 10min 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 meeans ± SEM of three independent experiments.


(A)
*
30
20
10
0
IL-6 level (pg/ml)
Inhibitor - - Bay11-7082 PDTC TPCK
TNFα - + + + +
(B)
Bay 11-7082
PDTC
TPCK
TNF alpha + + + +
pMEK1/2
pErk
Total Erk
GAPDH
Bay 11-7082
PDTC
TPCK
TNF alpha + + + +
pPDK1
p-AKT (ser473)
Total AKT
pSTAT3 (ser727)
Total STAT3

4. HGF mediated MAPK pathway in MM cells induced OPN, DKK1 and OPN induced MMP-9 expression via PI3K/AKT pathways in BMSCs

4-1. HGF induced activation of MAPK pathways in MM cell lines

To examine the role of HGF in MM, MM cell lines such as U266, RPMI8226 and IM9 were treated with HGF (20ng/ml) for 10 min, and the activation of PI3K/AKT and MAPK pathways related protein kinases was examined. As shown in figure 8A, the Raf, Erk and Elk1 involved in MAPK pathway were activated in U266, RPMI8226, and IM9 cells. PI3K/AKT pathway was activated (phosphorylation of PDK1 and AKT) in IM9 cells. And then, I examined the effect of several signaling inhibitors on activation of Erk mediated by HGF treatment in MM cells. As shown in figure 8B, MEK inhibitor (PD98059), PI3K inhibitor (LY294002), and JAK inhibitor II effectively inhibited Erk phosphorylation, however AG490 (JAK2 inhibitor) did not. It showed that HGF-activated MAPK pathways were related to several signaling transduction in MM cell lines.

4-2. OPN secretion induced by HGF was suppressed by the MEK inhibitor in U266 cells.

To examine whether HGF modulates the secretion of OPN via HGF-mediated cell signaling, HGF-induced OPN secretion was measured in

U266 cells treated with PD98059 using ELISA. As shown in figure 9, PD98059 sufficiently suppressed the HGF-induced OPN secretion from U266 cells. However, LY294002 did not. The level of OPN secreted from U266 cells that co-treated with HGF and PD98059 was 2.7-fold lower than that of U266 cells treated with HGF alone. Although OPN secretion was reduced by LY294002 in U266 cells treated with HGF, it was not statistically significant. Data indicated that the increasing levels of OPN by HGF was more significant correlated with activation of MEK-induced MAPK pathway in bone marrow plasma.

4-3. Regulation of RUNX2 mRNA expression by HGF in MM cells

OPN secretion from MM cells is regulated by exogenous growth factors, such as IL-6 and IGF-1, and growth factors induce the expression of RUNX2. To examine whether RUNX2 expression is regulated by HGF to induce the OPN secretion, I evaluated the effect of HGF on the regulation of RUNX2 in multiple myeloma. As shown in figure 10, U266, IM9, and RPMI8226 cells were treated with the indicated dose of HGF, and RUNX2 mRNA expression was subsequently quantified using real-time PCR. This method suggests that HGF mediated the induction of RUNX2 trigger in order to secrete the OPN from MM cells.

4-4. Effect of OPN and HGF on BMSCs derived from MM patients

To examine whether OPN directly affects to BMSCs via PI3K/AKT pathway, three different BMSC samples from MM patients were used. BMSCs were incubated in serum-free media for 1 h and then treated with OPN (10ng/mL) for 15 min. As shown in figure 11A, phospho-PDK1, phosphor-AKT and GSK3β were induced, showing that OPN activated PI3K/AKT pathways in BMSCs. As shown in figure 11B, I examined activation of PI3K/AKT pathway molecules mediated by OPN in MM cell lines. After serum starvation for 1 h, MM cells were stimulated with OPN (10ng/mL) for 15 min. As a result, OPN induced activation of PDK1, Raf (Ser259), and GSK3β in U266 cells. OPN also induced the phosphorylation of PDK1 and AKT (Thr 308) in RPMI8226 and IM9 cells.

4-5. Activation of PI3K/AKT signaling is associated with MMP-9 expression in BMSCs.

I further examined the regulation of MMP-9 expression in BMSCs. As shown in figure 12, the results indicated that MMP-9 expression was increased more than 2 folds by treatment of OPN in BMSCs. As I observed that OPN activates the PI3K/AKT pathway in BMSCs, I treated BMSCs with the PI3K inhibitor, LY294002 (20 μM), and MAPK inhibitor, PD98059 (10μM). The induction of MMP-9 expression by OPN was significantly inhibited by LY294002 (2.4 fold).

4-6. The expression of various types of CD44 isoforms in MM cell lines

To examine whether CD44 isoform exits in MM cell lines, I perform RT PCR using specific primers, which are able to detect different types of transcripts. As shown in figure 13A, several isoforms were detected. To investigate the expression of CD44s, I performed immunofluorescence staining using pan anti- CD44 antibody in U266 cells. As shown in figure 13B, CD44s were expressed and located to the cell surface in U266 cells. In addition, as shown in figure 13C, variant forms of CD44s were detected in CD138 positive cells of bone marrow cells derived from MM patients, but not in CD138 negative cells.

4-7. CD44 suppression regulated activation of MAPK pathway mediated by HGF

To examine the functional role of CD44 in HGF-mediated cell signaling in MM cells, CD44 shRNA was transfected into U266 cells; then the transfected cells were observed. As shown in figure 14A, CD44 expression was effectively down-regulated by CD44 shRNA. To examine the role of CD44 in HGF-mediated cell signaling, the following experiments were performed: U266 cells were transfected with 1ug mock vector or vector of CD44 shRNA. After starvation for 6 h, the transfected cells were stimulated with HGF during the indicated time.

Further, MAPK pathway-related proteins are then analyzed by the western blot. As shown in figure 14B, the level of p-c-Raf and p-ERK were increased in a time-dependent manner in the control cells. However, in CD44 knock down cells, the level of p-c-Raf and p-ERK were not changed. These results indicated that CD44 contribute to the activation of HGF-mediated MAPK pathway.

4-8. CD44 regulates HGF-mediated DKK1 expression in U266 cells.

HGF-mediated cell signaling regulates the expression of DKK1 associating with bone lesion. To examine whether CD44 mediates the induction of DKK1 by HGF, DKK1, respectively, was measured using ELISA in U266 cells transfected with CD44 shRNA. As shown in figure 15A, both levels of DKK1 were down-regulated when the levels of CD44 were low. As shown in figure 15B, similar results were found in the western blot. It suggests that HGF-mediated DKK1 expression was regulated by CD44 existing in the surface of U266 cells.

Figures and Figure legends - 4

Figure 8. Effect of HGF on the activation of MAPK and PI3K/AKT pathways and suppression of these signal molecules by inhibitors

(A) After starvation, U266, RPMI8226, and IM-9 cells were treated with HGF (20ng/mL) for 10 min. The expression of MAPK and PI3K pathways related molecules were analyzed by Western blot. MM cells were pre-treated with various signal transduction inhibitors for 1 h and then stimulated with HGF (20ng/mL) for 15 min. 30μg of whole-cell extracts were used to Western blot analysis for activating MAPK and PI3K/AKT pathway activation. (B) Cells were treated with various signaling inhibitors for 1h and then stimulated with HGF for 10min. Effect of inhibitors on ERK activation was examined by western blotting.

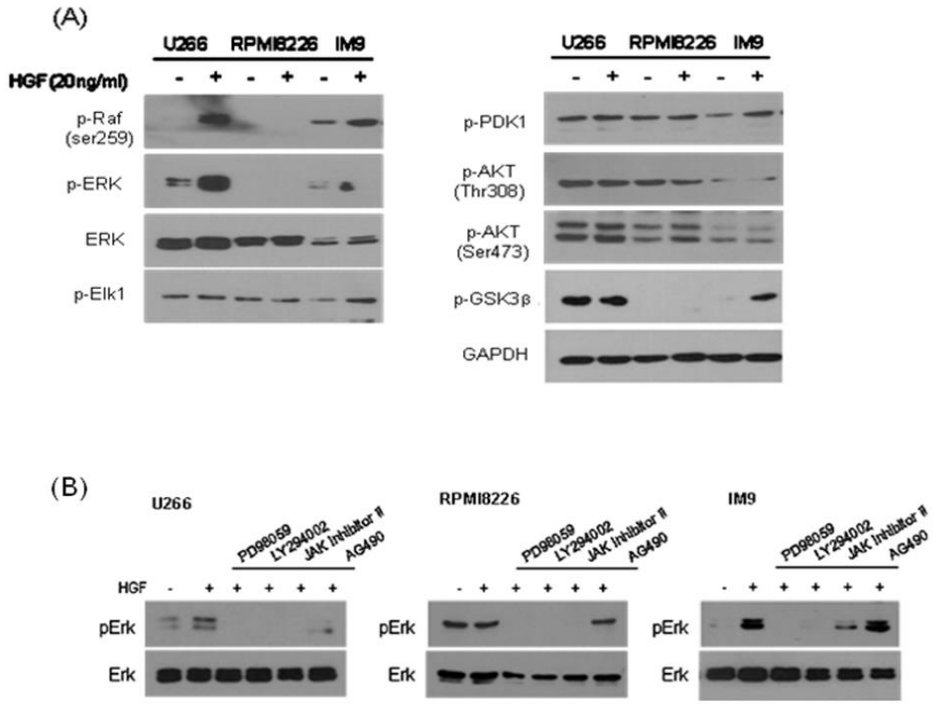

(A)
U266
RPMI8226
IM9
HGF (20ng/ml)
- + - + - +
p-Raf (ser259)
p-ERK
ERK
p-Elk1
U266
RPMI8226
IM9
- + - + - +
p-PDK1
p-AKT (Thr308)
p-AKT (Ser473)
p-GSK3β
GAPDH
(B)
U266
RPMI8226
IM9
PD98059
LY294002
JAK Inhibitor II
AG490
HGF - + + + + +
pErk
Erk

Figure 9. HGF induced OPN secretion from U266 cells, and its induction was suppressed by PD98059 treatment

After serum starvation, U266 cells were treated with signaling inhibitors such as PD98059 (10μM) and LY294002 (20μM) for 1 h and then stimulated with HGF (20ng/ml) for 10min. Cell supernatants were measured by ELISA for concentration of OPN. Bars represent the mean ± SEM from three independent experiments. * indicates statistical significance.

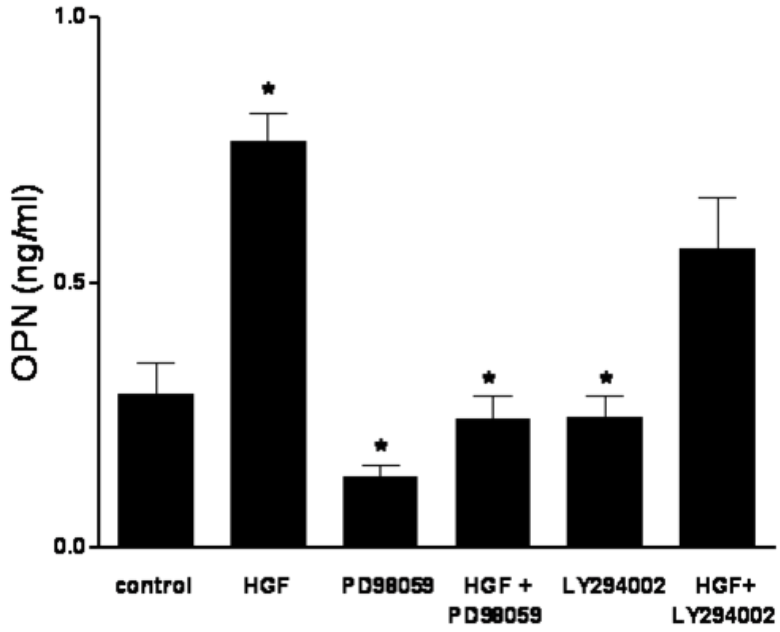

1.0
0.5
0.0
OPN (ng/ml)
*
*
*
*
control
HGF
PD98059
HGF + PD98059
LY294002
HGF+ LY294002

Figure 10. RUNX2 was up-regulated by exogenous HGF treatment in multiple myeloma cells

Cells were serum starved and treated with indicated dose of HGF. Then, cell pellets were subjected to measure RUNX2 mRNA level by qPCR analysis. The expression levels were normalized by β-actin and described as fold change to control sample. Bars represent the mean ± SEM from three independent experiments.

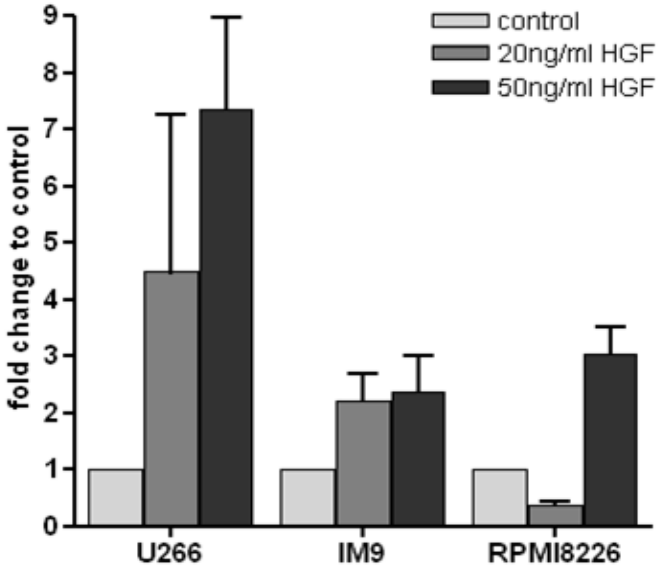
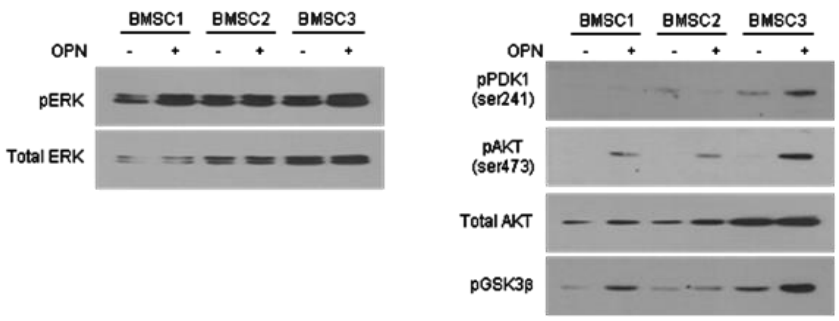

control
20ng/ml HGF
50ng/ml HGF
fold change to control
0
1
2
3
4
5
6
7
8
9
U266
IM9
RPMI8226

Figure 11. Effect of OPN on MAPK and PI3K/AKT pathways in BMSCs and MM cells

After serum starvation, BMSCs derived from three different patients with multiple myeloma (A) and MM cells, U266, RPMI8226, and IM-9 (B) were treated with 10ng/ml OPN for 15min, and then, the expression of MAPK and PI3K pathways related molecules were analyzed by Western blot.

(A)


BMSC1
BMSC2
BMSC3
OPN
- + - + - +
pERK
Total ERK
BMSC1
BMSC2
BMSC3
OPN
- + - + - +
pPDK1 (ser241)
pAKT (ser473)
Total AKT
pGSK3β

(B)

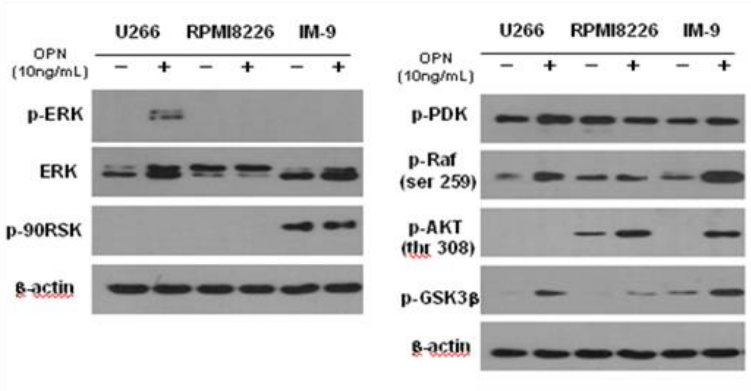

U266
RPMI8226
IM-9
OPN (10ng/mL)
– + – + – +
p-ERK
ERK
p-90RSK
ß-actin
U266
RPMI8226
IM-9
OPN (10ng/mL)
– + – + – +
p-PDK
p-Raf (ser 259)
p-AKT (thr 308)
p-GSK3β
ß-actin

Figure 12. MMP-9 induced by OPN was suppressed by MAPK and PI3K inhibitors

BMSCs were treated with PD98059 (10μM) and LY294002 (20μM) for 1 h and then stimulated with OPN (10ng/ml) for 15min. Cultured supernatants were collected to measure MMP-9 expression by ELISA. Bars represent the mean ± SEM from three independent experiments. * indicates statistical significance.

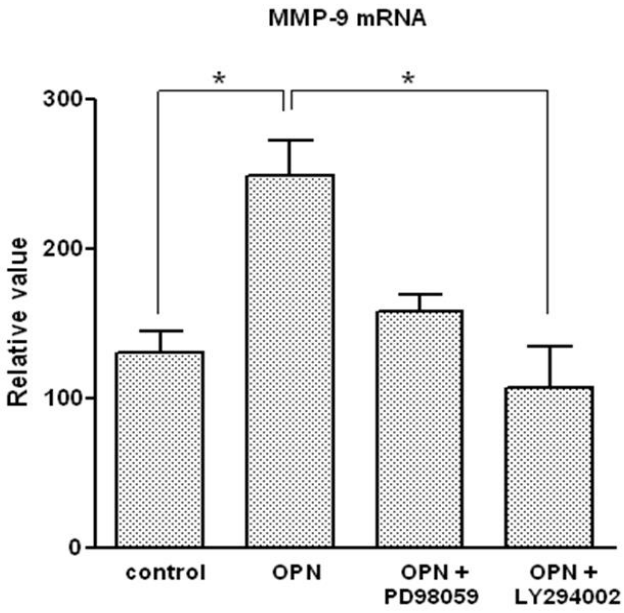

MMP-9 mRNA
*
*
300
200
100
0
Relative value
control
OPN
OPN +
PD98059
OPN +
LY294002

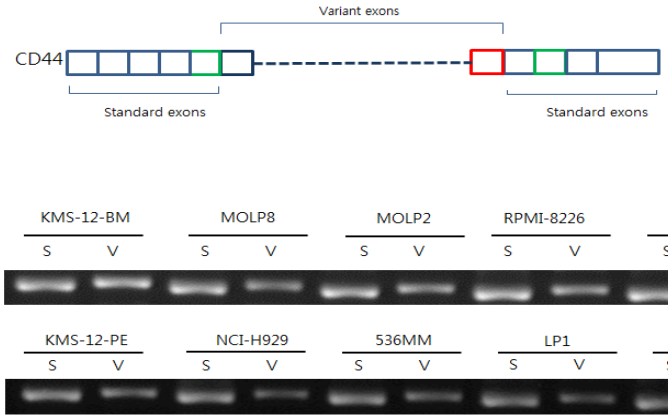
Figure 13. Effect of CD44 suppression on HGF-mediated MAPK activation in U266 cells

(A) GFP expression in U266 and IM9 cells transfected GFP tagged expression vector. Cells were transfected with the 1μg of mock vector or shRNA contained plasmid vector that targeted against CD44. Expression of CD44 in transfected MM cells was determined by RT-PCR.

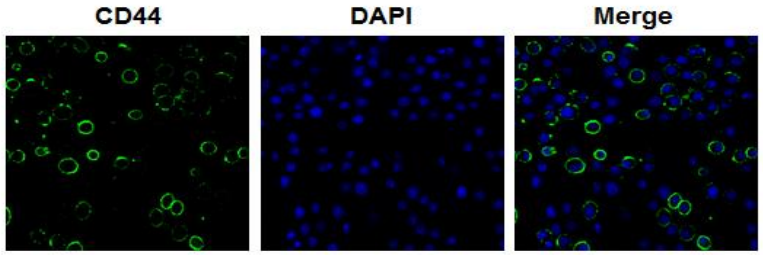
(B) U266 were transfected with 1ug mock vector or vector of CD44 shRNA. After starvation, transfected cells were stimulated with HGF(10ng/mL) for indicated time. MAPK pathway related proteins are analyzed by western blot analysis. cell lysate was analyzed for p-c-raf, Total-c-raf, pErk, Total Erk and GAPDH. Lane 1 : non-treated control, Lane 2 : treated with HGF for 30min, Lane 3 : treated with HGF for 1 h

(c) Total RNA was prepared from MM patients samples. Buffy coat samples were separated by CD138, one of general MM cell maker. Total RNA was analyzed by RT-PCR. Lane P : fraction of CD138 positive cells, N : fraction of CD138 negative cells, S : standard isoform of CD44, Lane V : variant isoforms of CD44

(A)


Variant exons
CD44
Standard exons
Standard exons
KMS-12-BM
MOLP8
MOLP2
RPMI-8226
IM9
S V S V S V S V S V
KMS-12-PE
NCI-H929
536MM
LP1
U266
S V S V S V S V S V

(B)


CD44
DAPI
Merge

(C)

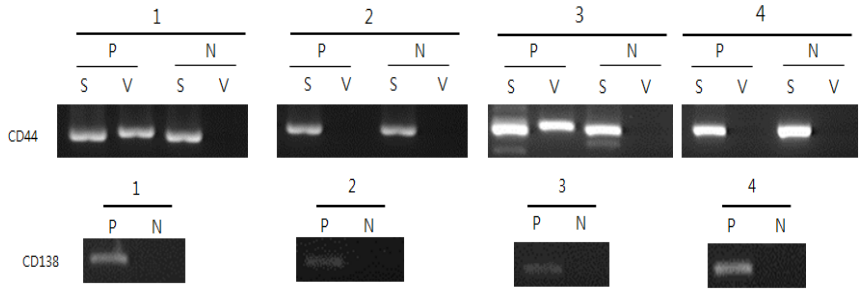
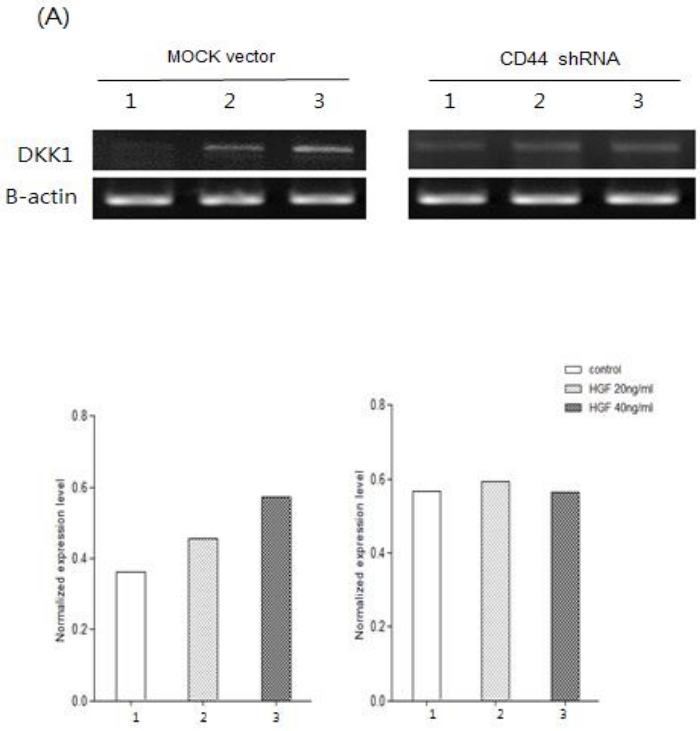

1
2
3
4
P N P N P N P N
S V S V S V S V S V S V S V S V
CD44
1
2
3
4
P N P N P N P N
CD138

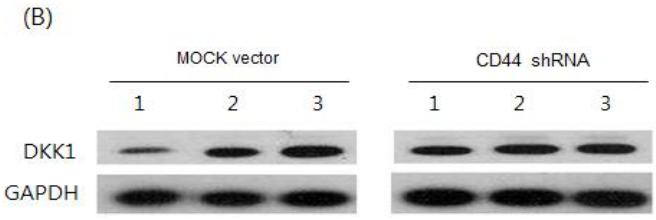
Figure 14. Effect of CD44 suppression on DKK1 expression in U266 cells

U266 were transfected with 1ug mock vector or vector of CD44 shRNA. After starvation, transfected cells were stimulated with HGF for indicated doses during 1 h.

(A) Total RNA was extracted and analyzed by RT-PCR. DKK1 and beta-actin are analyzed. on the bar graphs, all values were normalized with value of internal control, b-actin.

(B) DKK1 and GAPDH are analyzed by western blot analysis. Lane 1 : non-treated control, Lane 2 : treated with 20ng/ml HGF for 1h, Lane 3 : treated with 40ng/ml HGF for 1h.


(A)
MOCK vector
CD44 shRNA
1
2
3
1
2
3
DKK1
B-actin
control
HGF 20ng/ml
HGF 40ng/ml
Normalized expression level
0.0
0.2
0.4
0.6
0.8
1
2
3
Normalized expression level
0.0
0.2
0.4
0.6
0.8
1
2
3


(B)
MOCK vector
CD44 shRNA
1
2
3
1
2
3
DKK1
GAPDH

5. The role of Flt3-ligand in the progression of multiple myeloma

5-1. The level of Flt3-ligand in BM plasma derived from patients with various hematologic malignancies

Even though Flt3L is one of the crucial cytokines in the differentiation of hematopoietic stem cells, the role of Flt3L in the progression of MM still remains unknown. To detect the secreted level of FLT3-ligand in the microenvironment of patients, I measured the BM serum of patients with various hematologic malignancies that were included in my laboratory clinical sample cohort using the ELISA assay method. As shown in figure 16, I found that Flt3L levels were higher when compared to the case controls. The results demonstrated that the steady-state level of Flt3-ligand in MM patients (n = 80) was higher than the case-control groups, such as AML, CML, and ALL. It suggests that Flt3L may be associated with the MM progression.

5-2. Association of Flt3-ligand level in MM with prognostic parameters

The association between the levels of Flt3-ligand and clinical parameters of MM patients was examined. I statistically performed a data analysis to investigate the relation of Flt3L level and several poor

prognostic parameters. As shown in figure 17, the statistical analysis revealed a difference between the two groups of IgH spilt (14q32 FISH). The Flt3L plasma concentrations of group with IgH spilt showed a higher level than Flt3L plasma concentrations of groups without IgH spilt in MM. Subsequently, I analyzed the data for investigating the relation with Flt3L level and MM progression.

5-3. Activation of FLT3 mediated by FLT3L and its relation of relapse after autologous bone marrow transplantation

To investigate the signaling activation mediated by Flt3L in MM patients, I examined the Flt3 activity in the two patient groups that were either in relapse or not. At first, I measured the level of Flt3 and phosphorylated Flt3 in the protein obtained from patients' buffycoat cells using the ELISA assay; then I compared the p-Flt3/Flt3 ratio between each MM patients' group. In this experiment, the average duration of the CR status for the used non-relapsed patients' samples is 10.1 months, and that for the used non-relapsed patients' sample is 10.8 months. As shown in figure 18A, there was no significant difference between the relapse and non-relapse patient groups after BMT. However, the ratio of phosphor-FLT3 versus FLT3 was significant between the two groups in figure 17B.

To further examine whether the expression of Flt3L induces in MM cells co-cultured with BMSCs, its expression was measured using

ELISA. As shown in figure 18, neither MM cells nor BMSCs were expressed Flt3L. Its expression was significantly increased when MM cells were co-cultured with BMSCs. This suggests that the dynamic interplay between MM cells and BMSCs trigger in order to express Flt3L in bone marrow niches.

5-4. Induction of DKK1 secretion mediated by Flt3 activation via JAK/STAT and AKT signaling pathways

In the presence of Flt3-ligand in U266 cells and MOLP8 cells, the phosphorylation of AKT and STAT5 was dose-dependently increased; further, Flt-3 ligand-induced DKK1 secretion was observed. Because DKK1 expression was regulated by wnt-signaling, I then examined a set of genes related to wnt signaling after the U266 cells and MOLP8 cells were treated with Flt3-ligand for 2 hours. As shown in figure 19, I found that Flt3 ligand effectively activated wnt signaling and that induction of DKK1 was detected in both cells treated with Flt3-ligand. Partial knockdown of Flt-3 expression by short interfering RNA also resulted in the inhibition of DKK1 secretion. These results indicated that Flt-3 signaling plays a role in the growth of MM cells as well as in the regulation of DKK1 secretion.

Figures and Figure legend - 5

Figure 15. The level of Flt3-ligand in multiple myeloma is higher than the case control group (ALL, AML, CML)

The level of Flt3-ligand in bone marrow plasma samples derived from ALL, AML, Multiple Myeloma and CML patients was examined using Flt3-ligand ELISA assay .

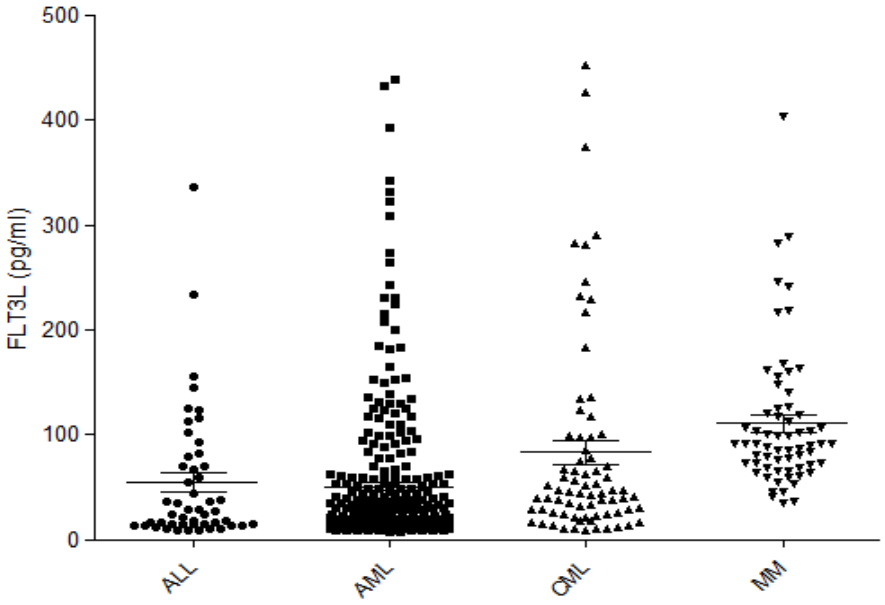
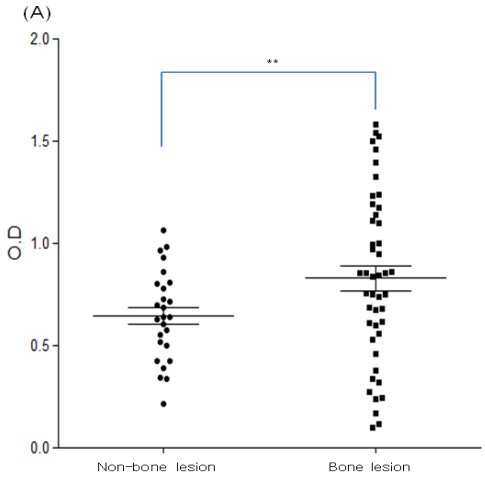

500
400
300
200
100
0
FLT3L (pg/ml)
ALL
AML
CML
MM

Figure 16. The level of Flt3-ligand in BM plasma is associated with poor prognostic clinical parameters in multiple myeloma

Plasma samples obtained from MM patients were examined using Flt3-ligand ELISA assay and all results were statistically analyzed. These data showed that Different expression level of Flt3-ligand between bone lesion (A) and IgH split (B). Levels of Flt3-ligand were higher in patients who have bone lesion and IgH split than those of normal.


(A)
2.0
1.5
1.0
0.5
0.0
O.D
**
Non-bone lesion
Bone lesion

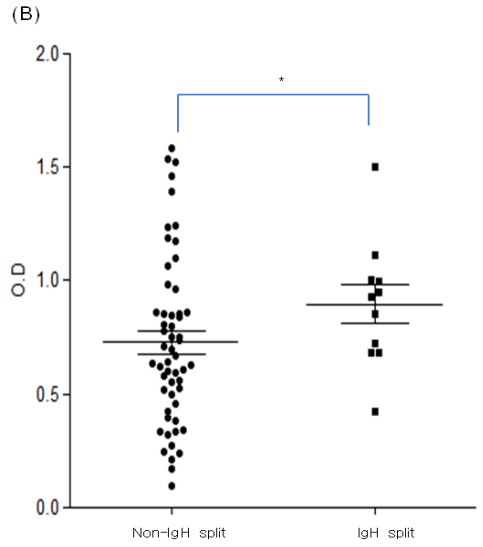

(B)
2.0
1.5
1.0
0.5
0.0
O.D
*
Non-IgH split
IgH split

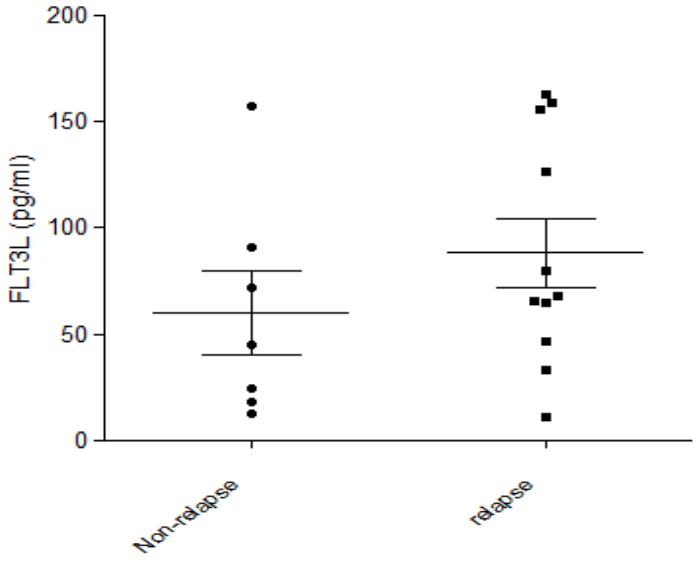
Figure 17. The activation rate of Flt3 in MM cells is related to relapse after Auto-PBSCT and the level of Flt3-ligand also illustrated a relapse-associated-pattern in same patients groups

In this results, Iinvestigated relation rate of Flt3 activation with relapse of Auto-PBSCT patients and released level of Flt3-ligand in plasma was compared with non-relapse and relapse group of MM patients.

(A)This result showed different level of Flt3-ligand on group of relapse and non- relapse after Auto-PBSCT. All Plasma samples were examined using Flt3-ligand ELISA assay.

(B) In BM samples, buffy coat layer was separated by Ficoll density gradient centrifugation. All cells of buffy coat layer were lysed and whole lysate was extracted. The level of p-Flt3 and Flt3 were measured using ELISA assay in whole lysate.

(A)


200
150
100
50
0
FLT3L (pg/ml)
Non-relapse
relapse

(B)

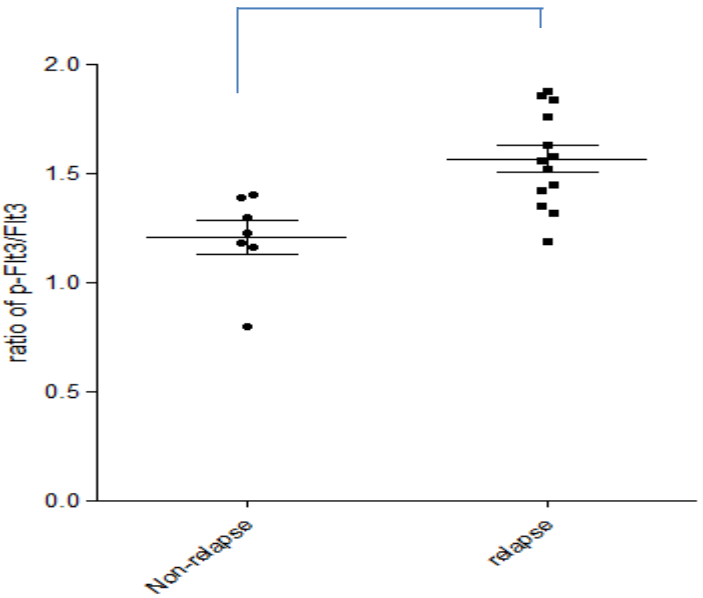

2.0
1.5
1.0
0.5
0.0
ratio of p-Flt3/Flt3
Non-relapse
relapse

Figure 18. Induction of FLT3 ligand in MM cell lines co-cultured with BMSCs from MM patients

Four different MM cell lines were co-cultured with BMSCs and secreted FLT3 was then measured in the cultured soup after MM cell lines were co-cultured with BMSCs for 48 hours.

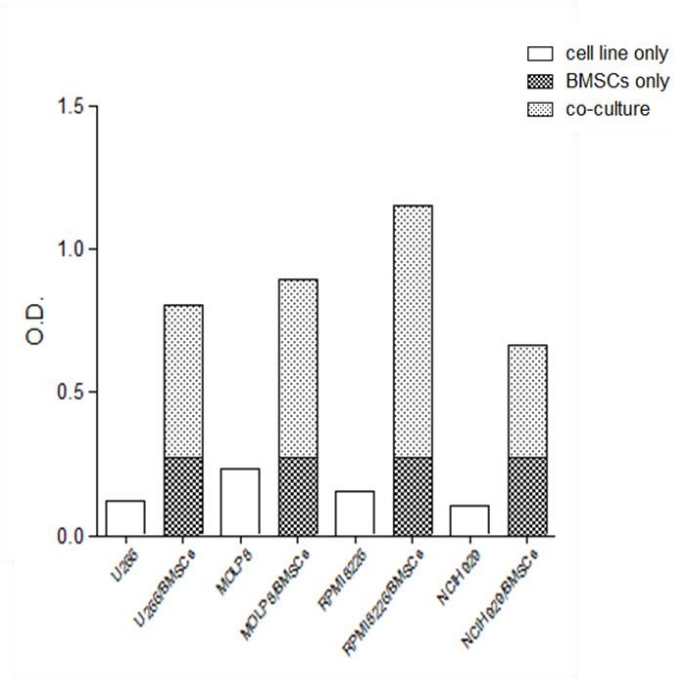
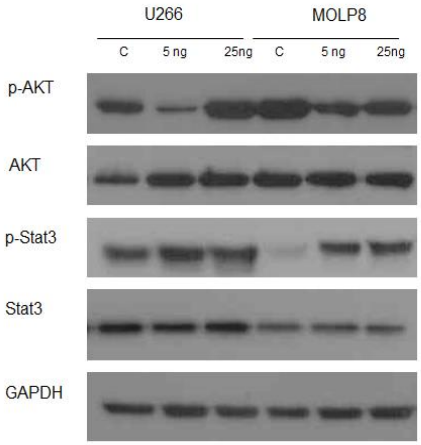
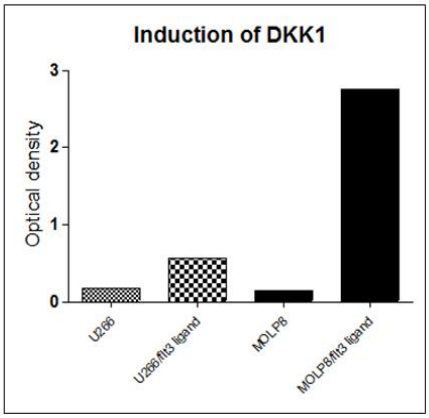

cell line only
BMSCs only
co-culture
O.D.
1.5
1.0
0.5
0.0
U266
U266BMSCs
MOLP8
MOLP8BMSCs
RPMI8226
RPMI8226BMSCs
NCIH929
NCIH929BMSCs

Figure 19. Flt3-ligand is an effectively induced activation of AKT, STAT3 and expression of DKK1

To investigate expression and activation of AKT and WMT signaling related molecules, whole cell lysates were extracted and analyzed by western blot in parental and Flt3-ligand treated cells. After serum starvation, MM cells were treated with rhFlt3-ligand (5, 20 ng/mL) for 1h. In this set, DKK1 was measured in the cultured soup after treatment of rhFlt3-ligand using ELISA assay


U266
MOLP8
C
5 ng
25ng
C
5 ng
25ng
p-AKT
AKT
p-Stat3
Stat3
GAPDH


Induction of DKK1
Optical density
3
2
1
0
U266
U266/flt3 ligand
MOLP8
MOLP8/flt3 ligand

6. The novel pan-HDAC inhibitor, CKD-581 reveals apoptotic effects in multiple myeloma

HDAC inhibitor has been reported as a one of the most potent ant-MM chemotherapeutic drugs. As shown in figure 20, HDAC inhibitor induces apoptosis of MM cells as well as regulates the activation of NF-kB mediated cell signaling. Based on those evidences, HDAC inhibitor, as a combination drug, is a useful anti-MM drug with Bortezomib. In this study, I examine the efficacy of HDAC inhibitor in MM cell lines in the in vitro and in vivo model. In addition, the role of HDAC inhibitor was examined in the regulation of NF-kB-mediated cell signaling.

6-1. Effects of pan-HDAC inhibitor, CKD-581, on the growth of MM cell line and patient's derived cells

To examine whether CKD-581 effectively inhibits the growth of MM cells, cell proliferation assay was performed using 6 different MM cell lines. CKD-581 effectively inhibits most MM cell lines; yet, the rate of growth inhibition was different. As shown in figure 21A, three cell lines (KMS-12-BM, PE, and MOLP8) were more effective than the other three cell lines. LP-1 cells among other cell lines showed strong resistance (4 folds) against CKD-581 treatment. The effect of CKD-581

on MM cell lines, histone acetylation patterns, was examined using the western blot. As shown in figure 21B, CKD-581 effectively induced histone acetylation regardless of the type of MM cell lines. Also, the cytotoxic effects were not related to the induction of histone acetylation. As shown in figure 22C, the induction of histone acetylation in U266 and EJM was lower than that in others. Moreover, cleaved PARP and cleaved Capase-3 were observed in KMS-12-BM and U266 cells.

Furthermore, I treated the indicated dose of CKD-581 to the buffy coat cells obtained from MM patients' BM samples. The results of the cell proliferation assay with whole buffy coat cells did not show a significant effect of CKD-581.I further examined CD138 positive cells, known as MM cells, using the FASC analysis. It is shown that CD138 positive cells included in the buffy coat dramatically reduced in the FASC analysis. It suggested that CKD-581 might affect to MM cells selectively in bone marrow. (Fig.22)

6-2. Global expression profiles in MM cells treated with CKD-581

To examine which genes were involved in CKD-581 mediated apoptosis, I performed microarray using the total RNA of KMS-12-BM cells. These results indicated that 4375 genes with a FDR-adjusted p-value of < 0.05 were identified. As shown in figure 23A, according to functional categories, the classification of analyzed genes indicated that 14% were involved in transcription factors, 8% were involved in

cell signaling transduction, 3.8% participated in cell cycle, and 3.5% were involved in apoptosis. As shown in figure 23B, IL-6Ra, JAK2, NFKB1, cMYC and cMyb genes, which were associated with MM progression, were decreased by CKD-581 treatment, whereas Caspase9, Apaf1, p15, p19, and p21 were significantly increased. As shown in figure 23C, the expression of p19 and p21 was confirmed by RT-PCR.

6-3. Effects of CKD-581 and bortezomib in co-cultured MM cells with BMSCs

To confirm the efficacy of CKD-581 and Bortezomib in the condition supported by MM microenvironment factors, I performed MTT assay using co-cultured MM cells with bone marrow stromal cells derived from MM patients. U266 and KMS-12-BM were co-cultured with BMSCs for 24 h, and the indicated doses of CKD-581 and Bortezomib were treated for 24 h. The effects of drugs differed depending on the cell type. In the U266 cell line, effects of CKD-581 and Bortezomib to co-cultured cells were similar with the control cells. However, the co-cultured KMS-12-BM cells were less sensitive to drugs than the control cells. (Fig.23)

6-4 Effects of combined CKD-581 and Bortezomib on DKK1

expression

To define the released level of DKK1 released from MM cells and co-cultured MM cells, ELISA assay was performed. As shown in figure 25, the level of DKK1 was decreased by drug treatments in U266 cells. Further, the combined treatment of CKD-581 and Bortezomib reduced DKK1 secretion more than the single drug treatment. In the co-culture condition, the level of DKK1 was also decreased slightly, and the combined treatment showed a more effective reduction of DKK1 secretion compared to the single drug treatment. This data indicated that DKK1, one of the major factors related to bone lesion in MM, was affected to the combined treatment of CKD-581 and Bortezomib.

6-4. In vivo efficacy of CKD-581 and Bortezomib

To determine whether CKD-581 could inhibit both survival and progression of MM cells in vivo, I examined the effects of CKD-581 using the MM orthotopic mice model. In this experiment, I used NRG mice. Mice were irradiated (400 rads) using a ^{137}Cs–irradiator source and MOLP8 cells were implanted intravenously in mice. I divided the mice into two groups with either CKD-581 treatment or not. After one week, in order to detect BM engraftment of MOLP8 cells, peripheral blood of all mice was sampled; further, I also examined the secretion of human light chain γ produced by MOLP8 cells using ELISA assy. In this

experiment, all MOLP8 injected mice showed a higher level of light chain γ compared with non-injected mice. Next, CKD-581 at a concentration of 40mg/kg, Bortezomib at a concentration of 0.25mg/kg and the mixture of both drugs were administered by intraperitoneal injection two times a week in 20 mice. In figure 26A, the survival curve indicated that CKD-581 significantly increased the survival rate of the treated mice group compared to the control mice; however, Bortezomib did not. Furthermore, the combined treatment of CKD-581 and Bortezomib showed a slight benefit of MM mouse survival, but without statistical significance. In figure 26B, I confirmed the MM cells that had been engrafted into the bone marrow using H&E staining in this experiment. Mice treated with CKD-581 and combined CKD-581 as well as Bortezomib showed a reduction of engrafted MM cells in the bone marrow.

Figures and Figure legends - 6

Figure 20. Schematic picture of molecular mechanism mediated by HDAC inhibitors on Multiple Myeloma cells

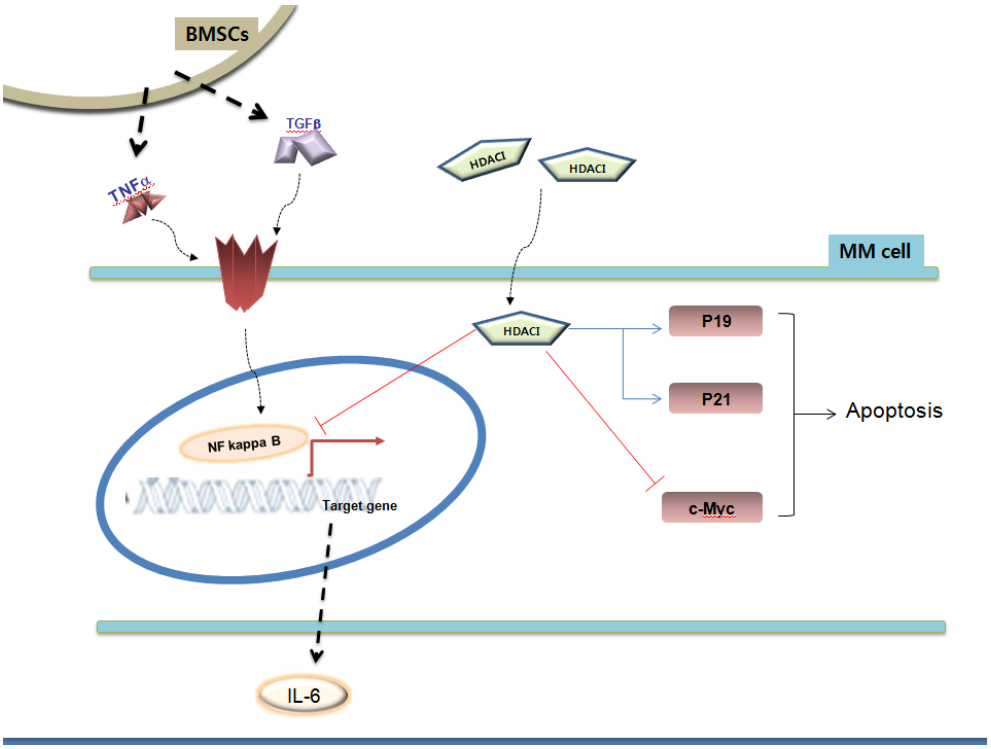


Figure 21. Effect of CKD-581 on Multiple Myeloma cells

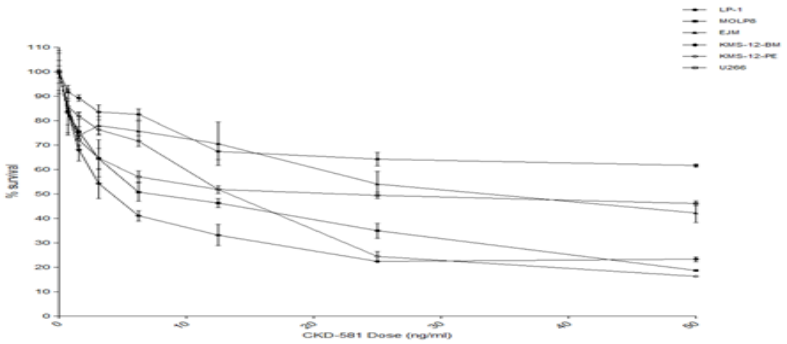
MM cell lines (LP-1, MOLP8, EJM, KMS-12-BM, KMS-12-PE and U266) were treated with indicated concentrations of CKD-581.

(A) 6 MM cell lines were treated with CKD-581 for 72 h and cell proliferation was measured using CCK-8 cell proliferation assay kit. Data shown are the means ± SEs of 3 independent experiments.

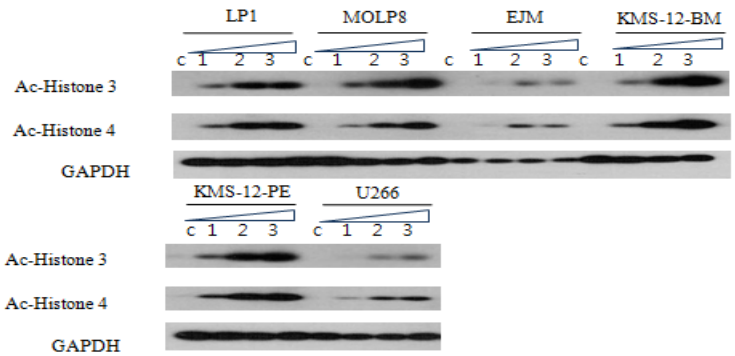
(B) 6 MM cell lines were treated with indicated doses of CKD-581 for 24 h. Acetylated histone 3 and histone 4 were determined by Western blot analysis. GAPDH was used as a loading control. Lane C : non-treated cells , 1: cells treated with 31.25ng/ml of CKD-581, 2: cells treated with 125ng/ml of CKD-581, 3: cells treated with 500ng/ml of CKD-581

(C) KMS-12-BM and U266 cells were treated with 10, 30, 90ng/ml of CKD-581 for 24 h. Apoptotic molecules (PARP, Caspase3, Cytochrome C) and acetylated histone 3 were determined by Western blot analysis. GAPDH was used as a loading control.

(A)


LP-1
MOLP8
EJM
KMS-12-BM
KMS-12-PE
U266
% survival
CKD-581 Dose (ng/ml)

(B)


LP1
MOLP8
EJM
KMS-12-BM
c 1 2 3
Ac-Histone 3
Ac-Histone 4
GAPDH
KMS-12-PE
U266

(C)

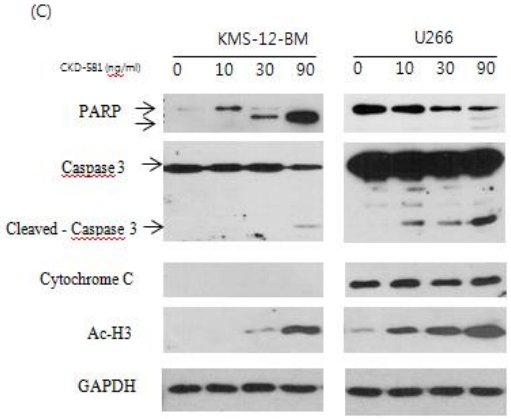

KMS-12-BM
U266
CKD-581 (ng/ml)
0 10 30 90
PARP
Caspase 3
Cleaved - Caspase 3
Cytochrome C
Ac-H3
GAPDH

Figure 22. Response to CKD-581 treatment in MM patient's primary cells

(A) Whole buffy coat cells obtained from BM of MM patients were treated indicated dose of CKD-581 for 24 h. And viability of buffy coat cells was determined by MTT method using CCK-8 cell proliferation kit. (B) Non-treated buffy coat cells and 50ng/ml CKD-581 treated cells were stained with CD138 antibody and examined by FACS analysis for detection CD138 positive cells. Data shown are the means ± SEM of independent experiments.

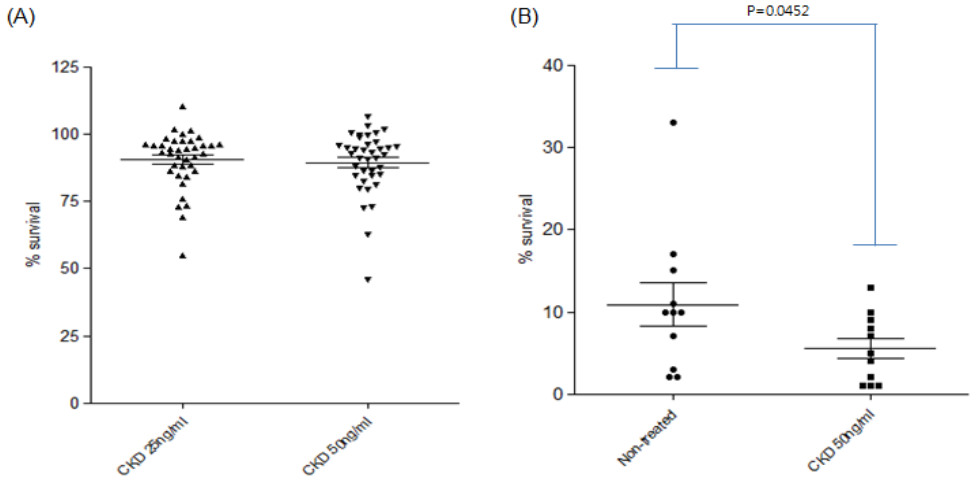

(A)
125
100
75
50
25
0
% survival
CKD 25ng/ml
CKD 50ng/ml
(B)
P=0.0452
40
30
20
10
0
% survival
Non-treated
CKD 50ng/ml

Figure 23. Functional analysis of alterative expressed genes in KMS-12-BM cells following CKD-581 treatment

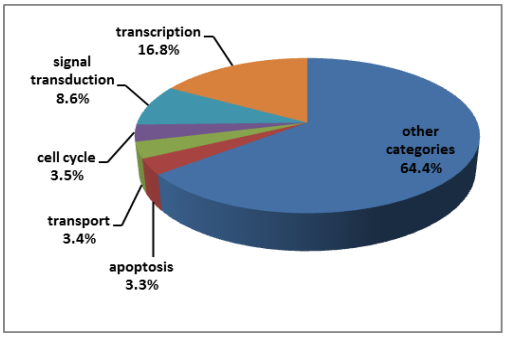
The analysis of GO terms for the alternative expressed genes using the R language package software showed various functional categories that involved input genes. KMS-12-BM cells were treated with indicated dose of CKD-581 for 6 h. RNA was extracted and cDNA was examined microarrays as described in Materials and Methods.

(A) Genes increased more than 2-fold or decreased less than -1.5-fold were categorized by different biological functions.

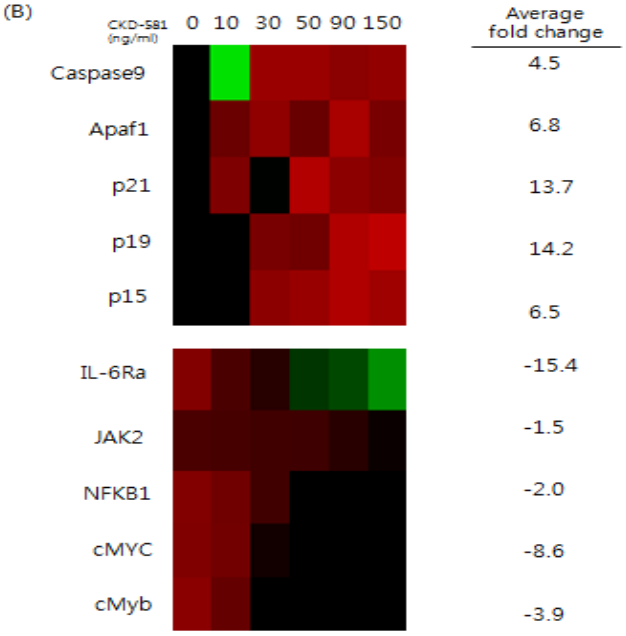
(B) Tree view of major genes involved in signaling transduction related MM progression, cell cycle and apoptosis, these genes were also well-known as genes regulated by HDAC inhibitors.

(C) KMs-12-BM cell were treated with indicated dose of CKD-581 for 6 h, and the mRNA expression of genes related with cell cycle on (B) was analyzed by RT-PCR. C: non-treated control cells, 1: cells treated with 10ng/ml of CKD-581, 2: cells treated with 30ng/ml of CKD-581, 3: cells treated with 50ng/ml of CKD-581, 4: cells treated with 90ng/ml of CKD-581, 5: cells treated with 150ng/ml of CKD-581

(A)


transcription
16.8%
signal
transduction
8.6%
cell cycle
3.5%
transport
3.4%
apoptosis
3.3%
other
categories
64.4%

(B)


CKD-581
(ng/ml)
0 10 30 50 90 150
Average
fold change
Caspase9
Apaf1
p21
p19
p15
IL-6Ra
JAK2
NFKB1
cMYC
cMyb
4.5
6.8
13.7
14.2
6.5
-15.4
-1.5
-2.0
-8.6
-3.9

(C)

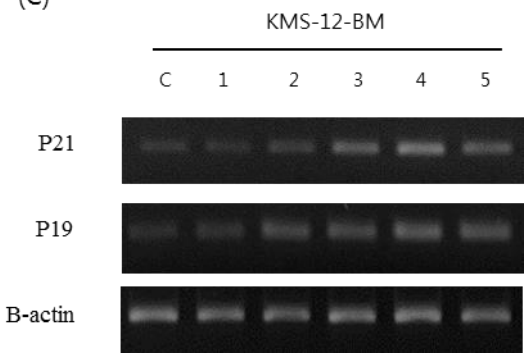
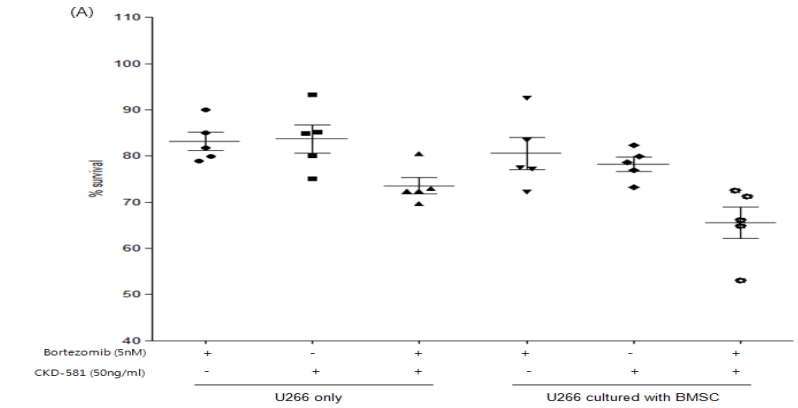
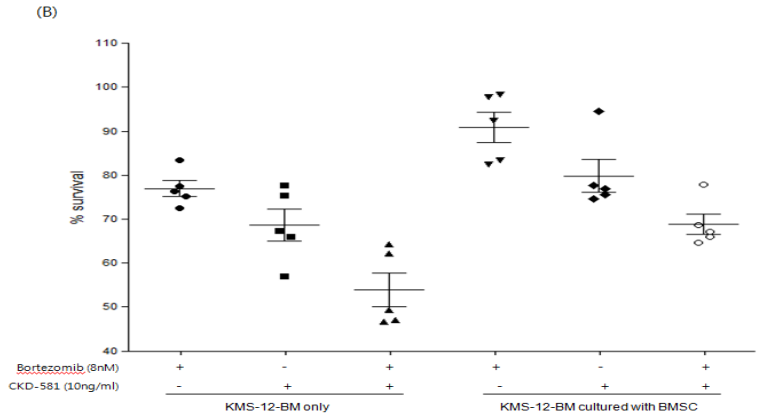

KMS-12-BM
C 1 2 3 4 5
P21
P19
B-actin

Figure 24. Survival rates of U266 cells against treatment of bortezomib and/or CKD-581

All cells of experimental sets were treated with indicated doses of bortezomib and CKD-581 for 24 h. BMSCs derived from MM patients were used in co-culture experiments individually. After 24 h incubation MM cells with BMSCs, all experimental sets were treated with indicated doses of CKD-581 and/or bortezomib for 24 h. Cell viability was determined by MTT method using CCK-8 cell proliferation kit. Cell viability was determined by MTT method using CCK-8 cell proliferation kit. (A) U266 cells were examined. Cells were treated with 50ng/ml CKD-581 and/or 5nM bortezomib. (B) KMS-12-BM cells were examined. Cells were treated with 10ng/ml CKD-581 and/or 8nM bortezomib. (C) Five BMSCs derived from MM patients were examined. Cells were treated with indicated concentrations of CKD-581 and/or Bortezomib that treated with U266 and KMS-12-BM cells.


(A)
% survival
110
100
90
80
70
60
50
40
Bortezomib (5nM)
CKD-581 (50ng/ml)
U266 only
U266 cultured with BMSC


(B)
% survival
110
100
90
80
70
60
50
40
Bortezomib (8nM)
CKD-581 (10ng/ml)
KMS-12-BM only
KMS-12-BM cultured with BMSC

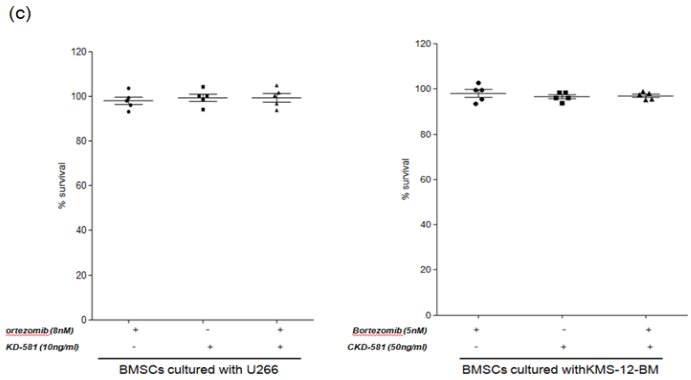

(c)
% survival
120
100
80
60
40
20
0
ortezomib (8nM)
KD-581 (10ng/ml)
BMSCs cultured with U266
Bortezomib (5nM)
CKD-581 (50ng/ml)
BMSCs cultured withKMS-12-BM

Figure 25. Effects of combined CKD-581 and Bortezomib on DKK1 expression

U266 cells were cultured alone or with MM patient's BMSC for 48 hour incubation. After 48h incubation, cultured soup were harvested and then passed them through 0.22um syringe filters. Then, the expression level of DKK1 was measured using ELISA in cultured soups of U266 cells and co-cultured U266 cells with MM patient's BMSC.

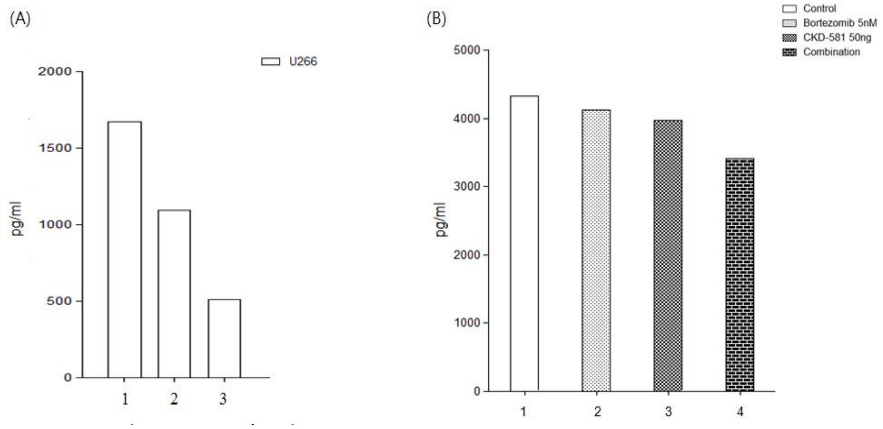

(A)
U266
2000
1500
1000
500
0
pg/ml
1
2
3
(B)
Control
Bortezomib 5nM
CKD-581 50ng
Combination
5000
4000
3000
2000
1000
0
pg/ml
1
2
3
4

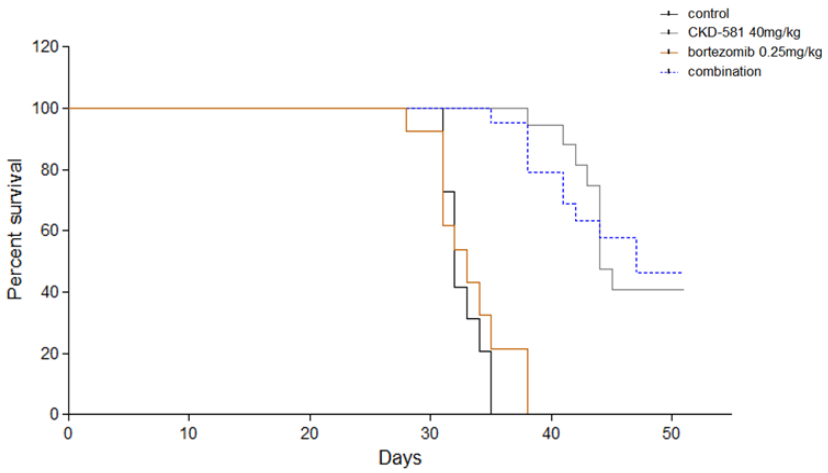
Figure 26. Effect of CKD-581 and bortezomib to survival duration of MM mouse model

MOLP8 cells were injected Intravenous 10^6 cells of NOD-Rag1null IL2rgnull, CB-17 SCID (Jackson Laboratories Inc., PA, USA). Mice were assigned to 4 treatment groups receiving either the vehicle alone (control), CKD-581 alone, bortezomi alone, and bortezomib plus CKD-581. All drugs were given Intraperitonealy twice a week.

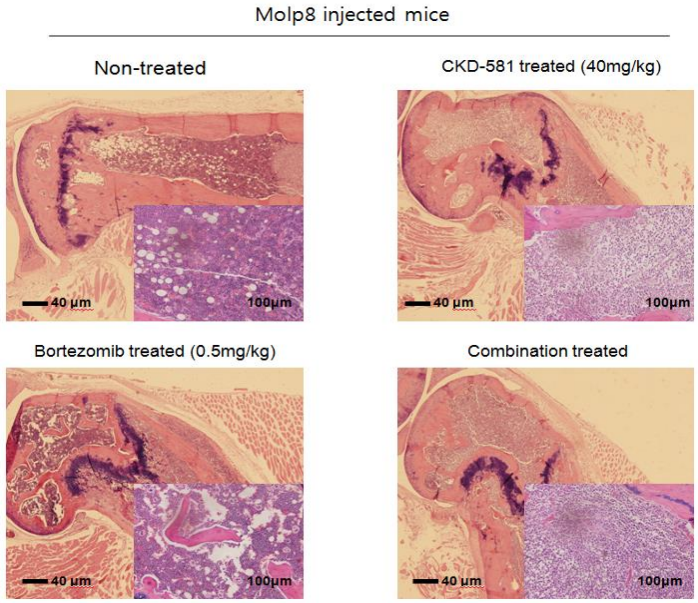
(A) Survival curves according to drug treatments in MOLP8 mouse model

(B) Hematoxylin and eosin (H&E) staining of the tibia bone marrow of in MOLP8 mouse model (original magnification ×25; scale bar = 40 and 100 μm). Arrow indicates the infiltrated MOLP8 cells into bone marrow.

(A)


control
CKD-581 40mg/kg
bortezomib 0.25mg/kg
combination
Percent survival
120
100
80
60
40
20
0
0
10
20
30
40
50
Days

(B)


Molp8 injected mice
Non-treated
CKD-581 treated (40mg/kg)
Bortezomib treated (0.5mg/kg)
Combination treated
40 μm
100μm

Discussion

The dynamic interplay between MM cells and BMSCs plays a critical role in the progression of MM. Various factors, such as cytokines, chemokines, and growth factors, are involved in the response of chemotherapy and stem cell transplantation. Even though the importance of bone marrow niches have been addressed through increasing evidences, the role of various factors existing in bone marrow niches still remains to be unknown. It has been reported that multiple myeloma is a heterogeneous disease, with some patients' cases showing aggressive disease and other cases having well responding disease to treatment [20]. It also suggests that the response of chemotherapeutic effects is regulated by bone marrow niches [4]. The roles of various factors existing in bone marrow niches should help us to understand the pathogenesis of MM. Subsequently, the identification of potent factors, to determine the biologic behaviors of MM cells showing chemo-resistance and relapse after autologous stem cell transplantation, is needed.

Various factors associated with disease progression were examined in bone marrow serums obtained from MM patients. The results indicated that the expression levels of IL-6, sIL-6R, HGF, TNFα, TGFβ, DKK1, Flt3L and OPN, such as well-known factors in MM, were detectable, whereas cytokines, such as IL-2, IL-4 and IL-10, were extremely low (Data is not shown). The results indicated that the release of cytokines

from bone marrow plasma might affect the expressions of other cytokines in an autocrine and/or paracrine manner. This implies that the interaction of MM cells and BMSCs are processed by the regulation of releasing factors. Also, the signaling mechanisms of each factor have complicated relationships in MM progression. Subsequently, the roles of HGF, OPN, TNFα, DKK1 and Flt3L were assessed with regard to their ability to control the signaling pathways in MM cells and their effects on the progression of MM.

In the aspirates of patients with multiple myeloma, elevated TNFα level has been observed in numerous studies, and this is correlated with poor prognosis [30,32]. There are also multiple lines of evidence suggesting that TNFα is predictive of progression-free survival after thalidomide therapy in MM patients [54,55]. Consistent with previous reports, I found that not only TNFα, but also the IL-6 level, was highly present in the aspirates of patients with multiple myeloma as well as 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 [56]. It is known to be responsible for promoting osteoclastogenesis and inhibiting osteoblast function. Thus, I analyzed the correlation patterns between TNFα levels and bone lesion development. Patients having higher levels of TNFα in bone marrow aspirate developed bone lesions more easily by

almost a 1.5-fold than those having lower levels of TNFα (data not shown), consistent with the previous report [57]. 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 the NF-κB dependent IL-6 secretion. TNFα potently stimulates IL-6 secretion by stromal cells and osteoblasts, and the accumulated IL-6 stimulates the growth of multiple myeloma cells. Furthermore, it mediates the effects of other inflammatory cytokines on osteoclast formation, such as IL-1 and TNFα again [15]. Recently, it was shown that it can induce apoptosis of mature osteoblast as well as inhibit the proliferation of mesenchymal stem cells [58]. I found that some of the myeloma cells, including U266 cells, showed a stronger response to exogenous IL-6 (data not shown); further, these cells also secreted more IL-6 in response to TNFα in vitro. Hence, I hypothesized that TNFα is one of the major factors that regulate IL-6 secretion from multiple myeloma cells. First, I examined the signaling pathways that may be the candidate bridge between TNFα and IL-6. I also found that TNFα could regulate cell proliferation, survival and anti-apoptosis by inducing various signaling pathways, such as PI3K/AKT, JNK, MAPK and JAK/STAT pathways. Thus, I blocked the above TNFα-induced molecules in order 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 a considerable decrease in the IL-6 level. This indicated that IL-6 secretion by TNFα was mainly dependent on the JAK/STAT pathway. Moreover, I demonstrated that TNFα up-regulates cyclin D1 as well as c-Myc. These are well known for cell proliferation that is related with cell cycle progression and anti-apoptosis. NF-κB is known to be correlated with drug-resistant activity [59] as well as 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 in order to support MM cell growth [60]. Therefore, NF-κB activation in BMSCs enhances a positive loop with adherent multiple myeloma cells by the 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 [61,62,63]. Among the three compounds that inhibit NF-κB pathway, these data indicated that only TPCK suppressed IL-6 secretion significantly from multiple myeloma cells as well as BMSCs. Previous reports demonstrated that TPCK, known as chymotrypsin-like protease, could be used as a NF-κB pathway inhibitor. It targets the serine and cysteine activation loop of IKK beta, resulting in the blocking of NF-κB binding to DNA in HeLa cells [64]. In

addition, Wang et al. (1999) reported that TPCK abolishes constitutive RelA activity and uPA overexpression in pancreatic tumor cell lines with dexamethasone [65]. Although the previous report demonstrated that TPCK inhibits TRAIL-mediated Caspase activity and PDK/AKT signaling in human prostatic carcinoma cell lines [66], my 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 supports the potential of TPCK as a NF-κB inhibitor, especially regulating IL-6 secretion in multiple myeloma cells. Taken together, I 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.

HGF is significantly related with MM proliferation, apoptosis and promotion of migration of MM cells [67]. HGF is known to be released by BMSCs and affects MM cells in a paracrine manner [68]. HGF stimulates with c-Met receptor. which activates multiple downstream signals, including PI3K/AKT, MAPK, and Src through two major

adaptor molecules Grb2 and Gab1 [69]. A recent study revealed that HGF inhibits BMP-induced osteoblastogenesis and may be involved in the development of osteolytic bone disease in MM patients [70].

Previously, in bone marrow plasma obtained from MM patients, I found that HGF expression was correlated with OPN expression. It was well-known that both HGF and OPN play an important role in the progression of MM. OPN is regarded as a multifunctional extracellular matrix molecule that has a key role in tumor growth, progression, and metastasis. The plasma level of OPN is increased in patients with MM, and this correlates with disease progression and bone destruction [71]. I conveyed HGF-induced activation of MAPK signal pathways in MM cells. Moreover, when examining the different types of MM cells, somewhat different patterns of cell signal pathway activation by HGF were shown. However, in all three cell lines, the activation of RAF and ERK involved in MAPK signal pathways was observed. Further, PD98059, a specific MEK inhibitor, significantly reduced the secretion of OPN compared to other signaling pathway inhibitors. It is possible that HGF elicits an invasive phenotype in MM cells by the stimulation of OPN production through ERK-dependent mechanism. In addition to previous results, the release of OPN is related with the regulation of RUNX2, as a transcription factor, which is involved in MM progression and OPN expression in tumor cells. My data showed that HGF could increase RUNX2 mRNA level in multiple myeloma cells. It means

HGF-mediated OPN expression may be enhanced via transcription induced by RUNX2 in MM cells.

Furthermore, I examined whether increasing levels of OPN expression by HGF were associated with both the invasion and migration of MM cells. OPN induced the PI3K/AKT signaling activation and led to the increase in the expression of MMP-9 in BMSCs, indicating the promotion of cell migration and tumor invasion. Also, OPN-mediated MMP-9 induction was suppressed by PI3K inhibitor, LY294002. This suggested that OPN-mediated MMP-9 expression was regulated by the PI3K/AKT pathway. These results demonstrated that the release of OPN by HGF primarily involves MAPK pathways in MM cells, and the blockage of these signaling by targeted signal transduction inhibitors effectively abolished HGF-induced OPN expression. In addition, exogenous HGF induced MAPK and PI3K/AKT pathways in BMSCs (data not shown), which suggests that the release of HGF by BMSCs activates MAPK and PI3K/AKT signal pathways and acts as an autocrine inducer. I also found that OPN induced activation of PI3K/AKT pathways in MM cells. These data implied that HGF induced OPN expression mediated by RUNX2 and OPN could contribute to MM progression via OPN-mediated PI3/AKT pathway, which, in turn, modulates the biologic behaviors of BMSCs.

Several adhesion molecules are present on BMSCs and MM cells, including the known signaling receptors, such as CD44 variants and

integrins [72]. HGF interaction with these receptors induced the activation of downstream signaling pathways and the activation of various transcription factors. First of all, I showed CD44s expression in MM cells. The results depicted that CD44s were detected in the MM cell line and CD138 positive cells were obtained from the bone marrow. In U266 cells, CD44s were stained in the cell membrane. Such result means that CD44s were respectively expressed in MM cells, which may relate to its biological behavior as a cell signaling molecule. In my data, the level of p-c-Raf and p-ERK were not changed by HGF stimulation in CD44 knock down U266 cells. In the immunostaining result of U266 cells, CD44 protein is located in the cell surface. This implies that CD44, as a signaling receptor or cofactor, can contribute to the activation of MAPK pathway induced by HGF stimulation. Moreover, CD44 can also contribute to the development of bone erosion via regulation of DKK1 expression in MM cells.

The FLT3L has roles in multi-lineage differentiation on the hematopoietic cell. In several hematologic malignancies, FLT3, as a major FLT3L receptor, have been defined in many studies. Further, FLT3L is one of the major factors for early B-cell differentiation. Thus, I further examined the function of Flt3L. The level of Flt3-ligand in MM patients was higher than the case-control groups, such as AML, CML, and ALL. These data mean that the activation of signaling pathways by

those cytokines could be involved in the progression of MM.

Genetic aberrations of FLT3, such as internal tendem repeat and some point mutations are well known as poor prognostic factors in acute myeloid leukemia occurring in approximately 20–30% of patients [73]. These mutations induced conserved activation of FLT3 mediated signaling. Today, some papers insisted that over-expressed FLT3L could induce FLT3 mediated signaling to mutated FLT3 as well as wild type. Moreover, FLT3L led to a further activation of FLT3 mutants and was particularly important in elevated FL levels in acute myeloid leukemia patients in response to chemotherapy [74]. This suggested that a high level of FLT3L could affect cancer development and progression via abnormal cell signaling activation. I analyzed the level of FLT3L in several kinds of hematologic malignancies. As a result, the level of FLT3L in MM was higher than the level of FLT3L in AML, CML, and ALL. To define the relation with MM and the level of FLT3L, I compared the level of FLT3L with the clinical parameters of the MM samples. Next, I found that the secretion of FLT3L was related with relapse after BMT. Both groups were divided with relapse have a similar duration to the CR status (about 10 month) after BMT. The level of FLT3L seemed to be related with relapse, but was not significant. The FLT3L level of the non-relapse group showed a lower pattern than the relapsed group. Furthermore, to detect the activation level of FLT3 in each sample, I measured the activated FLT3 (p-FLT3) and FLT3

level in mononuclear cells included in the BM using ELISA assay. Further, I compared the two groups using the p-FLT3/FLT3 ratio. The results showed that the p-FLT3/FLT3 ratio of the non-relapse group were statistically lower than that of the relapsed group. This suggested that the activation of FLT3/FLT3L signaling could be significantly related with early relapse in MM patients. In vitro, human MM cell lines increased the activation of AKT and STAT5 by the stimulation of FLT3L in a dose dependant manner. Also, the stimulation of FLT3L also induced DKK1 expression in MM cells, which was related to bone formation. These data imply that a high level of FLT3L is significantly related with MM progression and it can be a potent target for therapy in MM..

Standard therapeutic agents, alkylating agents, nucleoside analog, and stem cell transplantation are currently used in the treatment of MM [49]. Recently, pan-HDAC inhibitor has been reported as a potent anti-cancer drug [75]. HDAC inhibitor has been reported as a one of the most potent ant-MM chemotherapeutic drugs. As shown in figure 21, HDAC inhibitor induces the apoptosis of MM cells as well as regulates the activation of NF-kB-mediated cell signaling [76,77]. In this study, I present the complex biological and molecular activity of CKD-581, a novel pan-HDAC inhibitor, and provide insights on the mechanisms of the recently reported HDAC inhibitor activities [75,78]. My data showed

anti-proliferative activity of CKD-581 against MM cell lines in vitro, with IC_{50} in the nanomolar ranges. Similar to the previously reported pan-HDAC inhibitor, CKD-581 increase the acetylation of lysine residues on histone tails, thereby resulting in the expression of cyclin-dependent kinase p21 or p19. In the microarray analysis, cMYC expression was down-regulated. I found that its anti-proliferative activity also involved the regulation of several oncogenic pathways, including Caspase activation and induction of apoptosis.

To define the efficacy of CKD581 in a BM microenvironment condition, I examined the efficacy of CKD-581 in MM cells co-cultured with MM patient's BMSCs. my data indicated that MM cells cultured with BMSCs grow faster than MM cells alone, and that CKD581 effectively inhibits the growth of MM cells growing with BMSCs. Such data indicated that certain cytokines or growth factors inhibited anti-cancer drug-mediated apoptosis. Hence, I suggested that CKD-581 directly affected MM cells itself and inactivated cell signaling by the cytokines secreted from BMSCs. The results presented here conveyed that MM cells co-cultured with BMSCs grew faster at 24 h, but that CKD-581 inhibited MM cell growth, with or without the presence of co-cultured BMSCs. This result suggested that CKD-581 could affect MM cells within microenvironment factors. Interestingly, cell viability of the IL-6 dependent cell line U266 was enhanced by BMSCs, indicating that the survival of U266 cells was considerably influenced by the cells'

interaction with BMSCs, most likely due to the release by BMSCs of several growth factors that promote MM cell growth. To further investigate a selective anti-MM activity effect of CKD-581, I examined its activity in primary cultured mononuclear cells obtained from MM patient's bone marrow. After the ex vivo cultured mononuclear cells were treated with CKD-581, FASC analysis was examined using anti-CD138 antibody. In the cell proliferation assay using CCK-8, the reduction in the number of mononuclear cell was not decreased. However, the population of CD138 positive cells dramatically reduced in the FASC analysis using anti-CD138. Based on my data, CKD-581 selectively inhibited the growth of CD138 positive cells indicating MM cells.

Various combined chemotherapies have been proposed for MM treatment. Recently, pan-HDAC inhibitors in combination with Bortezomib have shown synergic efficacy in clinical studies of MM [79,80,81]. It suggested that the synergic activity of pan-HDAC inhibitors plus Bortezomib is due in part of the role played by pan-HDAC inhibitors in the regulation of therapeutic targets. These findings lead us to develop novel HDAC inhibitors [82]. In this regard, in vitro studies are essential in the search for optimal drug combinations to exhibit significantly better efficacy than that observed with Bortezomib treatment. As a result, combined treatment with CKD-581 and bortezomib induced a more effective response on MM cell lines as well

as co-cultured cells than a single drug treatment.

Furthermore, I confirmed these results in the orthotopic MM mice model. As a result, CKD-581-treated mice survived much longer than non-treated control mice. CKD-581 and Bortezomib combined treated mice showed a slight benefit of survival duration compared to CKD-581 single treated mice. In conclusion, the activity of CKD-581 in the mice MM models suggests that the therapeutic effects can be achieved systemically in the bone marrow and moreover, CKD-581 can be a candidate drug for a combined treatment with Bortezomib.

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국문 요약

다발 골수종(Multiple Myeloma)과 골수 간질 세포(Bone Marrow Stromal Cells – BMSCs) 사이의 동적인 상호 작용은 직접적으로 질병의 진행에 영향을 미친다. BMSCs로 부터 분비되는 다양한 사이토카인과 케모카인은 다발 골수종 세포가 악성의 생물학적 형질로 변화하도록 유도한다. 이러한 사이토카인들의 역할을 이해하기 위해, 먼저, 본 연구자는 진단 및 재발시에 수집된 MM 환자의 골수 혈청을 이용하여 다양한 사이토카인의 발현 정도를 확인하였다. 그 결과 IL-6, sIL-6R, HGF, TNFα, TGFβ, DKK1, Flt3L 그리고 OPN과 같은 다발 골수종에서 발현이 잘 알려진 여러 인자들을 측정할 수 있었다. 위 인자들의 발현 정도에 따라 상관 관계 분석을 진행하였고, 일부의 사이토카인은 오토크라인 혹은 파라크라인 작용을 통하여 다른 사이토카인들의 발현에 영향을 미칠 수 있다는 것을 확인하였다. 본 연구는 다발 골수종의 진행에 있어 골수 간질 세포로부터 분비되는 TNFα, HGF, OPN 그리고 Flt3L와 같은 사이토카인의 역할과 상호작용을 연구하였다.

TNFα에 의해 다발 골수종 세포에서의 IL-6의 생산이 증가된다는 사실은 이미 보고된 바가 있다. 그러나 TNFα에 의한 다발 골수종 세포의 IL-6 분비를 촉진시키는 신호 전달 기작은 많은 부분이 밝혀지지 않았다. 본 연구자는 다발 골수종 세포에 TNF

를 처리하였을 때 MEK, STAT3, AKT의 인산화가 증가한 것을 확인하였다. 이런 TNFα에 의한 IL-6 생산 촉진은 JAK2와 IKK 그리고 TNF receptors (TNFR)의 siRNA에 의해 억제되었으나, MEK, p38, PI3K inhibitor들에 의해서는 억제되지 않았다. 이것은 다발 골수종에서 TNFα가 JAK/STAT pathway를 통한 NF-κB 활성화에 의해 IL-6의 분비를 조절한다는 것을 보여준다.

다발 골수종 환자의 골수 혈청에서 HGF의 발현 정도는 OPN의 발현 정도와 상호 연관성을 가지고 있다. HGF는 MAPK, PI3K/AKT pathway를 활성화 시키는데 HGF에 의한 OPN 발현 유도는 MEK inhibitor (PD98059) 에 의해 억제되었지만, PI3K inhibitor (LY294002)를 처리했을때는 억제되지 않았다. 또한 본 연구자는 BMSCs에 OPN 처리했을때 MMP-9의 mRNA 발현이 증가하는 것을 관찰하였다. 그러나 이 MMP-9의 mRNA 발현은 PI3K inhibitor에 의해 억제되었다. 이 결과는 HGF가 MAPK pathway를 통하여 OPN의 발현을 유도하며, OPN에 의해 유도되는 PI3K/AKT pathway은 MMP-9의 발현을 활성화시켜 BMSCs의 생물학적 작용을 조절한다는 것을 보여준다.

CD44는 림프구의 homing과 백혈구의 활성에 관여하는 다양한 생물학적 작용에 널리 관여한다. 특히, HGF에 의해 유도되는 신호 전달은 CD44 동형 단백질에 의존적이며, CD44와 CD44 동형 단백질에 의해 유도된 receptor-tyrosine kinase의 활성화는 다발 골

수종 세포를 생존케하고 질병을 진행되게 한다고 보고된 바가 있다. 본 연구자는 다발 골수종 세포에서 CD44s의 발현을 확인하였다. 그 결과 다양한 다발 골수종 세포에서 CD44s의 발현이 확인되었고, 환자 골수에서 얻은 CD138 발현 세포에서도 CD44s의 발현을 확인할 수 있었다. U266 세포주에 shRNA vector를 transfection하여 CD44s의 발현을 감소시키자, HGF에 의해 유도되는 MAPK pathway의 활성과 DKK1의 발현이 감소하였다. 이는 다발 골수종 세포의 CD44s가 HGF에 의해 발생하는 뼈 병면의 발생에 관여할 수 있다는 것을 보여준다.

Flt3 ligand (Flt3L)는 혈액 세포와 미성숙 B-cell 분화에 영향을 미친다고 알려져 있다. 그러나 다발 골수종에서의 Flt3L 역할은 아직 알려져 있지 않다. 본 연구자는 다발 골수종 환자의 골수 혈청에 있는 Flt3L의 발현 정도와 임상적 악성 인자와 관련이 있음을 발견하였다. 또한 다발 골수종 환자의 골수 샘플에서의 Flt3L에 의한 Flt3의 인산화 증가와 골수 자가이식 후 다발골수종 재발은 상호관련성이 있었다. 게다가 Flt3의 활성화는 JAK/STAT, AKT 신호전달 체계를 통하여 DKK1의 분비를 유도하였다. 이 결과는 Flt3L의 높은 발현이 다발 골수종 진행에 영향을 미친다는 것을 말해준다.

다발 골수종 환자의 치료 효과를 증대시키기 위해, 새로운 표적 약제를 개발하기 위한 다양한 연구가 시도되고 있다. 새로운 HDAC inhibitor인 CKD-581의 다발 골수종에서의 효과를 확인하기 위해

여러 다발 골수종 세포주에서 그 효과를 확인하였다. 그 결과, CKD-581은 다양한 다발 골수종 세포주에서 항암 효과를 보였고, 다발 골수종 세포주에서 bortezomib과 병합 처리했을 경우 시너지 틱하게 bortezomib에 의한 세포 고사를 증가시켰다. 또한 BMSCs와 공동 배양한 다발 골수종 세포에서도 CKD-581 단독 및 bortezomib과 병합 처리에서 세포 단독에서의 결과와 비슷한 양상의 항암 효과를 보였다. 이것은 CKD-581이 항암제 치료에서 BMSCs에 의한 방해를 극복할 수 있음을 보여준다. 환자의 buffy coat를 이용한 ex vivo 연구에서, 전체 세포에 미치는 영향은 미미하였으나, FACS 분석 결과 CD138 발현 세포군을 확연히 감소시켰다. 동물 모델 실험에서도, CKD-581을 처리한 군은 control 군에 비하여 확연히 길어진 생존율을 확인하였다. 또한 CKD581과 Bortezomib을 병합 처리한 마우스 모델에서 길어진 생존율을 확인하였으며, CKD-581 처리군과 비교하여 약간의 증가를 보였다. 이러한 결과는 다발 골수종에서 CKD-581이 생체 내에서도 항암효과를 가지고 있으며, bortezomib과의 병합 효과를 가질 가능성을 보여준다.

주요어 : 다발 골수종, 체내 미세 환경, 골수 기질 세포, 사이토카인, 히스톤 디아세틸레이즈 억제제

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