Immune Network Vol. 1, No. 1, 2001

Generation and characterization of 1H8 monoclonal antibody against human bone marrow stromal cells

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= Abstract =

Background: Bone marrow stromal cells (BMSCs) express many cell surface molecules, which regulate the proliferation and differentiation of immune cells within the bone marrow.
Methods: To identify cell surface molecules, which can regulate cell proliferation through cell interaction, monoclonal antibodies (MoAbs) against BMSCs were produced. Among them, 1H8 MoAb, which recognized distinctly an 80 kDa protein, abolished myeloma cell proliferation that was induced by co-culturing with BMSCs.
Results: IL-6 gene expression was increased when myeloma or stromal cells were treated with 1H8 MoAb. In addition, the expression of IL-6 receptor and CD40 was up-regulated by 1H8 treatment, suggesting that the molecule recognized by 1H8 MoAb is involved in cell proliferation by modulating the expression of cell growth-related genes. Myeloma cells contain high levels of reactive oxygen species (ROS), which are related to gene expression and tumorigenesis. Treatment with 1H8 decreased the intracellular ROS level and increased PAG antioxidant gene concomitantly. Finally, 1H8 induced the tyrosine phosphorylation of several proteins in U266.
Conclusion: Taken together, 1H8 MoAb recognized the cell surface molecule and triggered the intracellular signals, which led to modulate gene expression and cell proliferation.

Key Words: stromal cells, myeloma cells, cell surface molecule, 1H8 monoclonal antibody

INTRODUCTION

Cellular interaction is achieved by cell surface molecules, and plays a pivotal role in the communication between multiple myeloma (MM) cells, which are malignant B cells, and bone marrow stromal cells (BMSCs) (1,2). Various surface molecules are involved in the growth, activation, and homing action of immune cells by regulating cell-cell interactions. The majority of myeloma cells are located in the bone marrow (BM), because BMSCs present a variety of surface molecules and trap myeloma cells. This strongly suggests that cell surface molecules are important for the growth and tumorigenesis of B cells.

The development of B lineage cells proceeds through several stages marked by the rearrangement and expression of the immunoglobulin genes. Mature B cells are generated by functional V(D)J rearrangement of the IgH and IgL genes in the bone marrow and target secondary lymphoid tissues (3). Mature B cells then differentiate into memory B cells and lymphoblasts by antigen stimulation. The lymphoblasts enter germinal centers, where they undergo intensive proliferation and somatic hypermutation in Ig genes, which leads to affinity maturation. Cells that have homed to the BM

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differentiate into long-lived memory cells. The proliferation and differentiation of B cells are regulated by a close interaction between hematopoietic cells and the BM microenvironments. Within the BM, these cells receive the signals, which lead to proliferation and terminal differentiation(4-6). BMSCs stimulate the proliferation and differentiation of myeloma cells via cell-cell interaction and the secretion of soluble mediators (2). The contact between granulocyte-macrophage progenitor and stromal cells is inhibited by antibodies, which suppress the functions of the cell surface molecules (7-9).

B cells are activated by several cytokines and differentiate into plasma cells, and the apoptosis of B cells is blocked by the stimulation via CD40 antigen in a germinal center. Durie *et al.* (10) demonstrated that CD40 triggers IL-6 secretion and promotes the cell survival of MM cells. The up-regulation of IL-6 in MM cells was found to be induced by CD40 and CD40 ligand interaction (11). The stimulation of CD40 antigen by the CD40 monoclonal antibody (MoAb) or CD40 ligand induced the up-regulation of IL-6 and promoted cell proliferation in IL-6-dependent myeloma cells (12).

To investigate new cell surface molecules, which regulate the functions of myeloma cells and BMSCs, MoAbs against BMSCs were generated and characterized during this study. 1H8 MoAb regulated cell proliferation and the gene expression of myeloma cells and BM stromal cells.

MATERIALS AND METHODS

1. Reagents

Anti-IL-6 polyclonal antibody, monoclonal anti-IL-6 receptor antibody (MP18), and IL-6 cDNA were kindly provided by Dr. T. Taga (Osaka University, Japan). Propidium iodide (PI), HAT (Hypoxanthine, Aminopterin, and Thymidine), DMEM, and RPMI 1640 were obtained commercially from GIBCO (Gaithersburg, USA). RNase and TUNNEL assay kits were purchased from Boehringer Mannheim (Mannheim, Germany). [-^{32}P]ATP was purchased from Amersham International (Braunschweig, Germany).

2. Cell culture

U266, IM-9 cells, WIL-2 NS, and CESS cell lines, were obtained from the American Type Culture Collection (Rockville, USA) and BMSCs were obtained as described previously (13). Cells were cultured at 37℃, 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA), 100 U/㎖ penicillin, and streptomycin (GIBCO). The cells were maintained in RPMI 1640 supplemented with 10% FBS.

3. Preparation of myeloma cells

Fresh myeloma cells (MMBs) were purified from bone marrow aspirates of MM patients as described previously (14). Briefly, mononuclear cells were isolated by differential centrifugations on Ficoll-Hypaque density solution (1.077 g/cm^{-2}). Mononuclear cells were washed twice with Hanks balanced salt solution (HBSS), without calcium and magnesium. Fresh myeloma cells were purified by removing T cells by rosetting with aminoethyl isothiouronium bromide (AET)-treated sheep RBC. Adherent cells were depleted by 2 h adherence to a 24 well culture plate (Costar, Cambridge, USA) at 37℃, in RPMI 1640 supplemented with 10% FBS. The percentage of fresh myeloma cells was evaluated by cytoplasmic immunofluorescence using anti- or anti-light chain antibodies directly coupled to fluorescein (DAKO, USA) (>90%). The cells were cultured for 3 days at a density of 1x10^6 cells/㎖ in RPMI 1640 supplemented with 5% FBS. Supernatants were then harvested and stored at -20℃. To prevent direct cell-to-cell contacts between stromal cells and fresh myeloma cells, inner membrane filter chambers (0.45 ㎛, Millicell CM, Millipore, Bedford, USA) were used within the 24-well culture plates. After 3 days of co-culture, fresh myeloma cells were harvested, and analyzed for IL-6 receptor expression by flow cytometry (Becton Dickinson, Sunnyvale, USA). To examine the effects of cell proliferation, 1× 10^4 cells were resuspended in 100 ㎕ of culture medium supplemented with 2.5% FBS, and incubated in a 96-well culture plate for 48h. Cell

proliferation was then measured by [^{3}H]thymidine incorporation.

4. Production of monoclonal antibody (1H8) to BMSCs

1) Immunization

Stromal cells ($1x10^7$ cells) isolated from the BM aspirates of MM patients were immunized into the intraperitoneum of a BALB/C female mouse (5 weeks old). Boost was twice performed every two weeks into the same site. Five days later, a little blood was collected from an eye and assayed by cell ELISA to determine the activity and amount of antibody.

2) Cell fusion

The splenocytes of the immunized mice were isolated by teasing and centrifuged at 1,200 rpm for 5 min in serum-free DMEM medium. Erythrocytes of the splenocytes were lysed in 0.17 M NH_4Cl at 37℃ for 5 min, and then washed twice with cold DMEM. These splenocytes were mixed with the SP2/0-Ag14 cell lines in the ratio of 5:1 and centrifuged. The pellet was carefully resuspended and kept at 37℃ for 5 min. 1 ㎖ of prewarmed 50% PEG was added drop by drop for 1 min and then 40 ㎖ of serum-free DMEM was added gently. This suspension was centrifuged at 1,200 rpm for 5 min. The cell pellet was diluted to $1-2x10^6$/㎖ with HAT (Hypoxantine, Aminopterin, and Thymidine) medium containing 10% FBS, dispensed into the wells of 96 well microtiter plates, and then incubated at 37℃ in a humidified CO_2 incubator for two weeks.

3) Single cell cloning of hybridoma

To obtain a clone of hybridoma cells, single-cell cloning by limiting dilution was performed. Hybridoma cell clones were diluted to 5 cells/㎖ with HAT medium containing 10% FBS and the 100 ㎕ each was added into the each well of a 96 well microtiter plate. 100 ㎕ of the splenocytes ($1-2\times10^5$ cells/㎖) of a nonimmunized mouse were added into each well as the feeder cells. One week later, the culture supernatant of host wells was concentrated 10-fold by ultrafiltration (Amicon).

4) Screening of monoclonal antibody (1H8) to BMSC

Screening of 1H8 monoclonal antibody was performed by cell ELISA. BMSCs were harvested and resuspended in serum-free RPMI1640. The cells were plated at a density of 5×10^4 cells/well and blocking buffer (1% of bovine serum albumin in PBS) added for 30 min. The plates were then washed three times by centrifugation and then with washing buffer (PBST; 0.05% Tween 20 in PBS, pH 7.4). 10 ㎕ of 2.5% glutaraldehyde was added and incubated for 30 min at room temperature (RT). After three washings with PBST, 100 ㎕ of the culture supernatant was serially diluted in dilution buffer (0.05 M Tris-Cl, 1 mM $MgCl_2$ $6H_20$, 0.15 M NaCl, 0.92% NaN_3, 1% BSA, and 0.05% Tween 20, pH 8.1) was added into the wells for 2 h at RT and then washed three times with PBST. 100 ㎕ of a solution containing goat anti-mouse IgG-alkaline phosphatase (1:1.000-diluted in dilution buffer) was added to each well for 2 h at RT. After three washings, 100 ㎕ of substrate buffer (0.05 M $NaHCO_3$, 10 mM $MgCl_2$ $6H_20$, pH 9.8) containing 1 ㎎/㎖ of p-nitrophenyl phosphate was added into each of the wells for 20-30 min. O.D at 495 nm was measured by an ELISA reader (Titertek Multiskan MCC/340, Finland) after the addition of 50 ㎕ of 1 N H_2SO_4.

5) Class determination of monoclonal antibodies

The class of monoclonal antibodies was determined by using the Ouchterlony double immunodiffusion assay followed. Culture supernatant from clones of hybridoma in 0.01% PBS was added to the center well of an 1% agarose gel plate and other standard antibodies (Sigma, IgA, IgM, IgG1, IgG2a, IgG2b, IgG3) were added into the outer rings of the plate. Culture supernatant and standard antibody were incubated in a humidified atmosphere overnight at RT. Staining was done using 0.5% Coomassie brilliant blue R-250 (Sigma) and destaining using 50% methanol/10% acetic acid.

6) Purification of monoclonal antibody

A 10 week old BALB/c mouse was used to obtain a large amount of monoclonal antibody from cloned cells,. Ten days after priming with an intraperitoneal injection of Freund's incomplete adjuvant (IFA), 5×10^6 cells of monoclone in 0.5 ㎖ PBS were intraperitoneal injected. As much ascitic fluid was drawn as possible with a 18G needle approximately 1-2 weeks after injecting the cells, when the ascitic fluid had built up. The fluid was incubated at 37℃ for 1 h and kept at 4℃ overnight to remove precipitates. The fluid was centrifuged at 5,000 × g for 20 min to separate the oil layer and the cell pellet. The fluid was mixed with PBS at a ratio of 1:1, and then purified using an anti-mouse IgM column. The eluted monoclonal antibody was identified by measuring O.D at 280 nm and concentrated.

5. Flow cytometric analysis

IL-6 receptor expression of U266 cells was analyzed by staining with anti-IL-6 receptor antibody (MT18) or anti-CD40 antibody in HBSS containing 3% FBS and 0.1% NaN_3, for 30 min at 4℃. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig was used as the secondary antibody. After incubation for 30 min on ice, the cells were washed and analyzed using a flow cytometer. The levels of IL-6 receptor and CD 40 expression were expressed as the mean fluorescence intensity (MFI).

6. Reverse transcription-polymerase chain reaction (RT-PCR)

Myeloma cells were harvested by centrifugation, washed with ice-cold phosphate buffered saline (PBS), and lysed with NP-40 lysis buffer. Total cytoplasmic RNA was isolated by acid guanidinium thiocyanate phenol-chloroform extraction. cDNA was produced for use in PCR using murine moloney leukemia virus reverse transcriptase (MMLV RT). PCR was carried out with Taq polymerase in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles of, 1min at 94℃, 1 min at 55℃, and 2 min at 72℃. The amplified PCR products were electrophoresed on a 0.8 % agarose gel (Sigma). The sequences of the oligonucleotides were as follows; for IL-6, sense oligonucleotide, 5'-ATGAA CTCCTTCTCCACAAGCGC-3'; antisense oligonucleotide, 5'-GAAGAGCCCTCAGGCTGGACTG-3'; for Proliferation-associated gene (PAG), sense oligonucleotide, 5'-CTTTGGTATCAGACCCGAAG-3'; antisense oligonucleotide, 5'-TT TGGCTTTGGGACATCA-3'; and for β-actin, sense oligonucleotide, 5'-GTGGGGCGCCCCAG GCACCA-3'; antisense oligonucleotide, 5'-CTCCTTAA TGTCACGCACGATTTC-3'.

7. Northern blot analysis

Myeloma cell total cytoplasmic RNA was isolated using the guanidinium isothiocyanate and cesium chloride method by ultracentrifugation. Samples were separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes (Genescreen Plus, NEN), and hybridized with a [^{32}P]-labeled PAG cDNA probe prepared by nick translation. The hybridization was detected by autoradiography.

8. Preparation of BMSC plasma membrane

BMSCs (1×10^8) were washed with PBS and lysed with lysis buffer (7.5 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM PMSF, 10 ㎍/㎖ leupeptin) for 10 min at 4℃. After centrifugation at 500 rpm for 5 min, the supernatant was centrifuged again at 15,000 rpm for 30 min. The pellet was collected and the concentration of protein was determined using Bradford reagent (BioRad).

9. Western blot analysis

The BMSC plasma membrane fraction was electrophoretically separated and then transferred from a gel to a nitrocellulose membrane (NCM), which was then incubated with blocking buffer (3% BSA in PBS) at RT for 1 h and incubated with monoclonal antibody at RT for 2 h. After three washings with TBST (50 mM Tris. pH 7.4, 150 mM NaCl, 0.05% Tween 20), 100 ㎕ of 1:5,000 diluted alkaline phosphatase conjugated goat-anti-mouse IgM was added at RT for 1 h. After three washings with TBST, NCM was mixed with the

substrates for alkaline phosphatase (66 μℓ of NBT, 33 μℓ of BCIP in 10 mℓ of alkaline phosphatase buffer).

10. IL-6 ELISA

Polystyrene 96 well microtiter plates were coated overnight at 4℃ with 100 μℓ of purified monoclonal anti IL-6 antibody (1 μg/mℓ). After three washings with PBST, the plates were blocked with 1% BSA in PBS at RT for 2 h, and after a further three washings with PBST, 100 μℓ of human IL-6 two-fold diluted in dilution buffer (1% BSA in PBS) was added for 2 h at RT. The plates were washed 3 times with PBST, 100 μℓ of biotinylated polyclonal antibody (250 ng/mℓ) was added and incubation was continued at RT for 2 h. Plates were then washed 3 times with PBST, 100 μℓ of 1:5,000 diluted streptavidin-HRP was added and incubation continued at RT for 2h. After three washings with a PBST, 100 μℓ of HRP substrate buffer (5 μℓ H_2O_2 and 4 mg Orthophenydiamine in 10 mℓ dilution buffer) was added and plates were re-incubated at RT for 20-30 min. The reaction was stopped by adding 20 μℓ of 1M H_2SO_4 solution and the O.D at 495 nm was measured.

11. IL-6 bioassay

IL-6 activity in the culture supernatants of the fresh myeloma cells, and in the sera of patients with MM was assayed using an IL-6 dependent murine hybridoma subclone, the B9.55 cell line. 5×10^3 B9.55 cells per

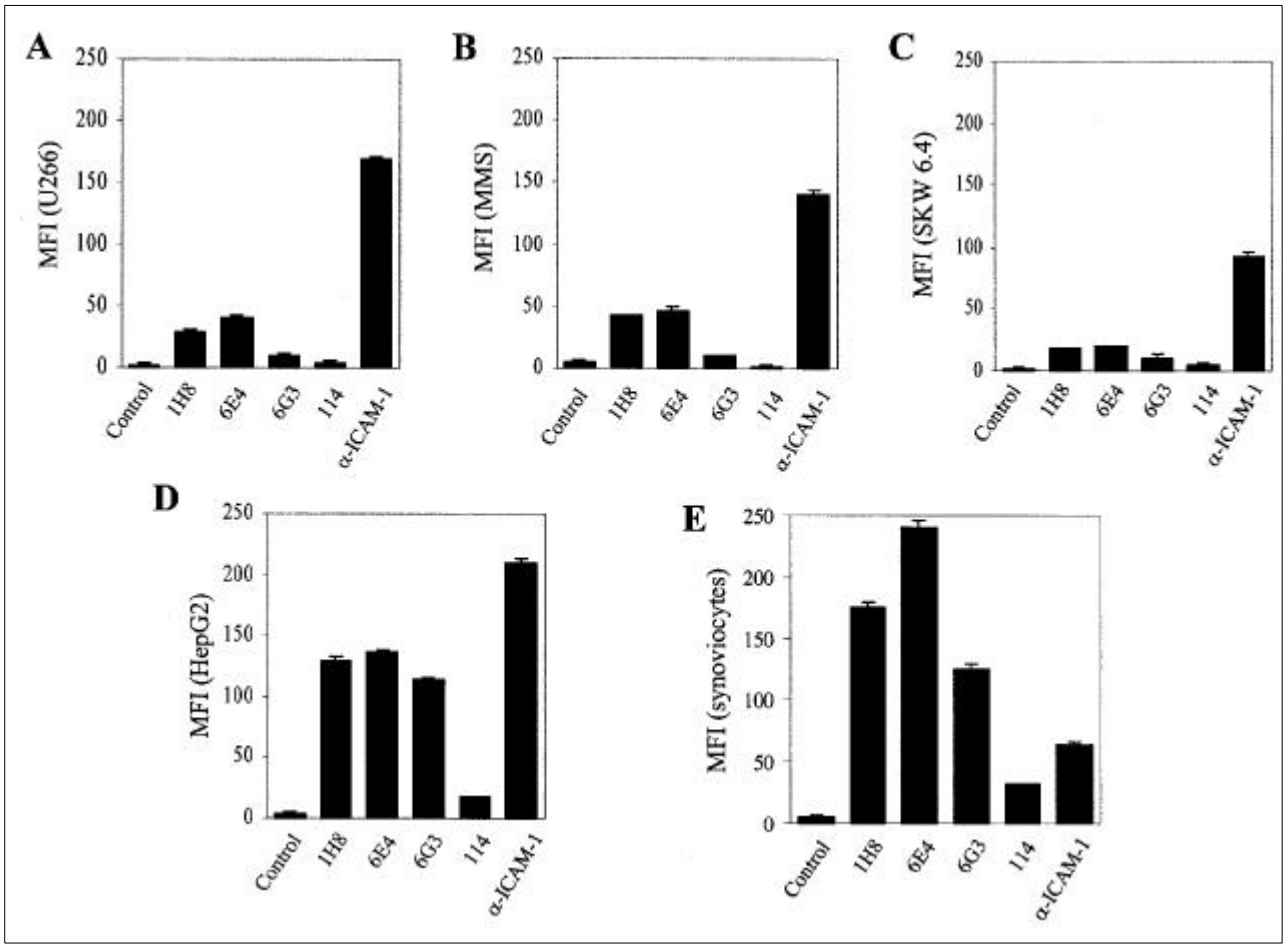


Fig. 1. The expression of 1H8 in various human cells by flow cytometric analysis. Various human cells were stained with MoAbs against BMSCs, and FITC conjugated anti-mouse Ig was used as a secondary antibody. After 30 min on ice, the cells were washed and analyzed by flow cytometry. MFI; mean fluorescence intensity, A; U266, B; BMSC from MM patients, C; SKW 6.4, D; HepG2, E; synoviocytes from rheumatoid arthritis patients.

well were seeded in 96-well flat-bottom microtiter plates in 100 μℓ of RPMI 1640 supplemented with 10% FBS, and 100 μℓ of serial dilutions of test samples were added. The plates were incubated for 72 h in a humidified 5% CO_2 incubator at 37℃. Cells were pulsed with 0.5 μCi of [^{3}H] thymidine (specific activity; 84.8 Ci/mmol, New England Nuclear, Boston, USA) per well for the last 6 h of the incubation time, and were harvested onto glass fiber filter papers using an automated cell harvester (Inotech, Brieger, Zurich). The amount of radioactivity incorporated into the DNA was determined using a liquid scintillation counter (Beckman, LS 6000A). In all assays, recombinant human IL-6 was used as the internal standard. One unit of IL-6 was defined as the amount inducing half-maximal proliferation.

RESULTS

1. Production and screening of 1H8 MoAb against BMSC

To investigate whether cell surface molecules promote cell proliferation through cellular interaction, a co-culture of human myeloma cells with BMSCs was performed. U266 cell proliferation increased on co-culturing with BMSCs compared with the culture of U266 cells alone, but the inner cell membrane chamber, which blocked direct cell-to-cell contact inhibited U266 cell proliferation (data not shown). This result suggested that the cell surface molecules of BMSCs and myeloma cells are involved in the proliferation of myeloma cells. To identify surface molecules, which may regulate cell growth, MoAbs against BMSCs obtained from an MM patient were raised. Screening the positive MoAbs using BMSC ELISA showed that four MoAbs (IgM) had high reactivity to BMSCs compared with the control (data not shown). Flow cytometric analysis was performed to assess whether these MoAbs stain other human cell lines, (Fig. 1). Among these, 1H8 MoAb highly stained

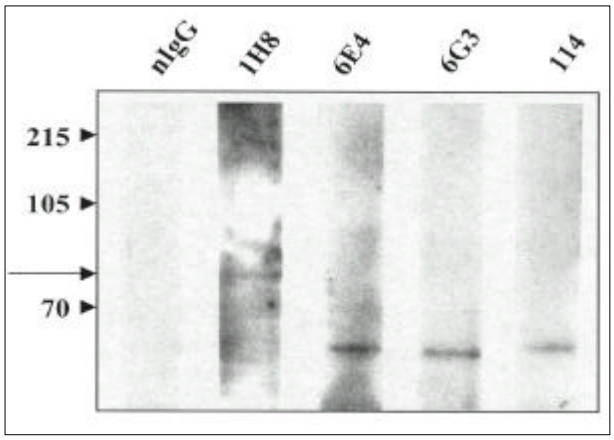


Fig. 2. Western blot of MoAbs against BMSC in U266 cells. The membrane fraction of BMSCs was electrophoretically separated and transferred from a gel to a nitrocellulose membrane (NCM), which was incubated with the MoAbs against BMSCs at room temperature for 2 h. After three washings, alkaline phosphatase conjugated goat anti- mouse IgM was added at RT for 1 h. After a further three washings, NCM was mixed with the substrates for alkaline phosphatase.

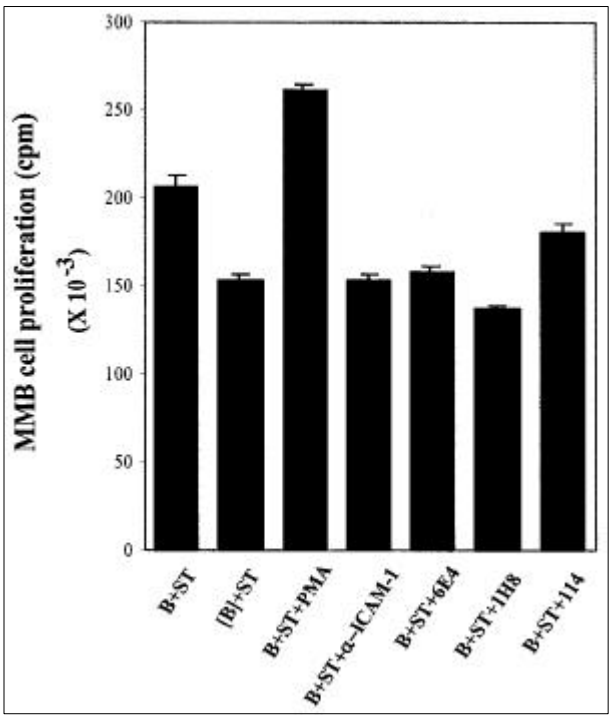


Fig. 3. Effects of MoAbs against BMSCs on fresh myeloma cell proliferation after co-culture with BMSC. BMSCs (ST) were plated at a density of 2x104 cells/mℓ. After 12 h incubation, fresh myeloma cells (B) were added at a density of 5x104 cells/mℓ. Coculture was used in the absence (B) or presence ([B]) of an inner cell membrane filter chamber. After 3 days, cell proliferation was measured using[3H]-thymidine incorporation.

synoviocytes and HepG2 cells, and was moderately reactive to SKW 6.4, BMSCs, and U266 cells. ICAM-1 MoAb was used as positive control expressed in all cell lines analyzed. However, the staining patterns of the various cells with ICAM-1 MoAb was obviously different from those stained with 1H8, indicating that 1H8 did not recognize ICAM-1. Immunoblots were preformed using BMSC extract to identify the specific proteins recognized by these anti-BMSC antibodies (Fig. 2). 1H8 recognized distinctly an 80 kDa molecule, indicating that it is different from opther antibodies.

2. Effects of 1H8 on cell proliferation and IL-6 production of human myeloma cells

Next, to assess whether the antibodies could inhibit myeloma cell proliferation induced by BMSCs, these antibodies were added to primary myeloma cells, which were co-cultured with BMSC. Blocking the co-culture between these cells with an inner cell membrane chamber ([B]) inhibited fresh myeloma cell proliferation (Fig. 3). It is been known that IL-6 is one of the growth factors involved and that the up-regulation of IL-6 in fresh myeloma cells is induced via CD40 stimulation [11].

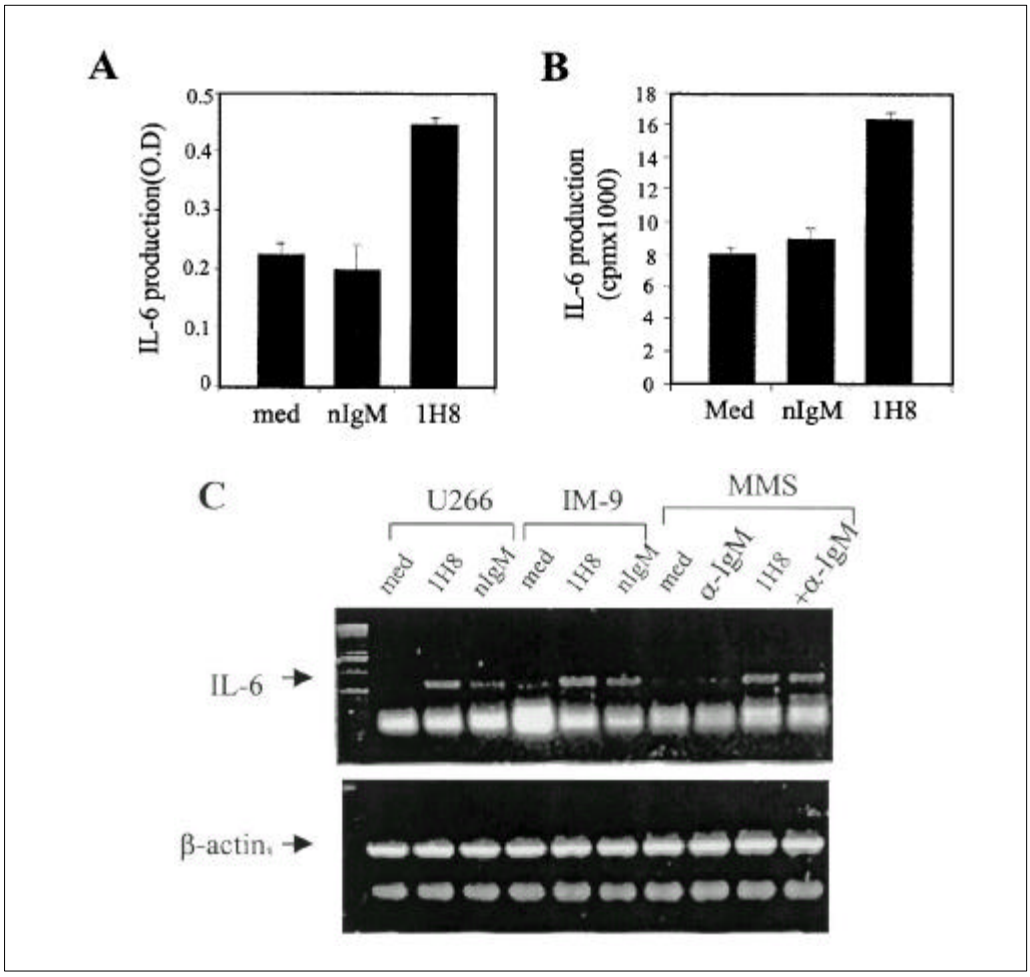
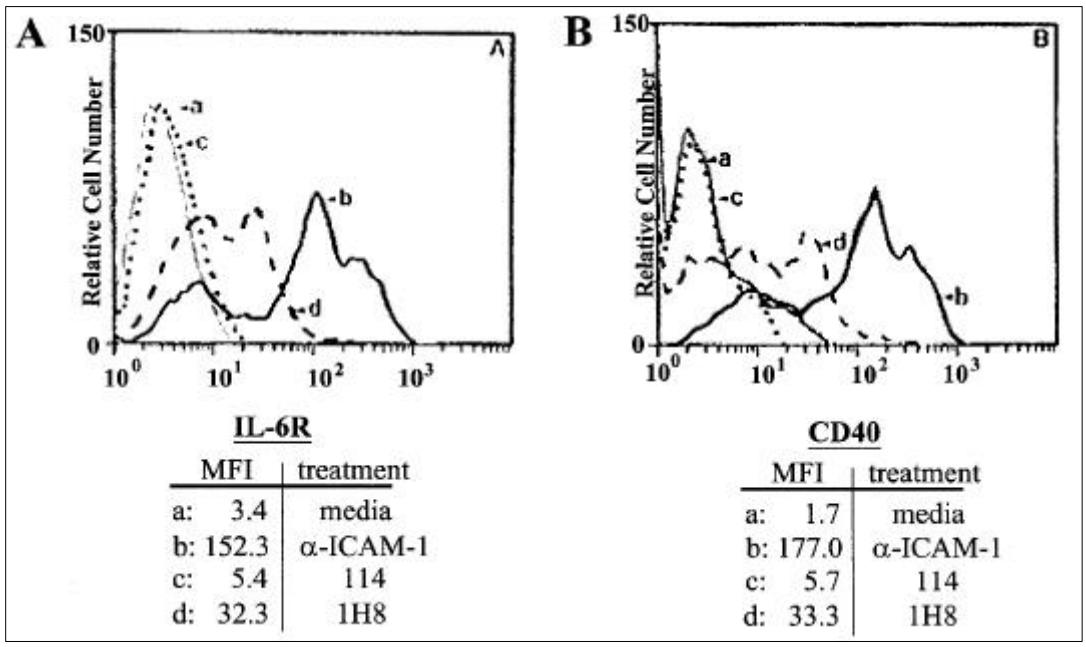


Fig. 4. Effects of 1H8 on IL-6 expression in myeloma cells and BMSCs. 1 ug/㎖ of 1H8 or normal mouse IgM (nIgM, isotype control) were coated in 24-well culture plate with PBS at RT for 1 h, and then washed with PBS three times. U266 or IM-9 cells were added and cultured for 48 h. The culture supernatants were harvested and assayed for IL-6 production using an IL-6 ELISA (A) and an IL-6-dependent B9.55 bioassay (B). U266, IM-9 or BMSCs were stimulated by 1H8 alone or by additional cross-linking with anti-IgM antibody (+-IgM) for 6 h, and cytoplasmic RNA was isolated. RT-PCR was performed as described in Materials and Methods (C).



IL-6R

MFI	treatment
a: 3.4	media
b: 152.3	α-ICAM-1
c: 5.4	114
d: 32.3	1H8

CD40

MFI	treatment
a: 1.7	media
b: 177.0	α-ICAM-1
c: 5.7	114
d: 33.3	1H8

Fig. 5. Effects of 1H8 on the expression of IL-6 receptor and CD40 on U266 cells. U266 cells were cocultured with BMSCs in the absence (media) or presence of MoAbs (114 and 1H8) against BMSCs. After 3 days of coculture, U266 cells were stained with anti-IL-6 receptor antibody (MT18) and anti-human CD40 antibody. FITC-conjugated anti-mouse Ig was used as a secondary antibody.

As shown in Fig. 4, the effects of 1H8 on the IL-6 expression of BMSCs and myeloma cells were investigated. IL-6 production by U266 cells was significantly increased by 1H8 treatment as determined by IL-6 ELISA (Fig. 4-A) and IL-6 bioassay (Fig. 4-B). The IL-6 gene expressions of U266, IM9, and BMSCs were also increased by 1H8 treatment (Fig. 4-C). In addition, IL-6 receptor and CD40 expression in U266 cells was analyzed by flow cytometry. As shown in Fig. 5, 1H8 treatment increased the IL-6 receptor and CD40 expression of U266 cells.

3. 1H8 inhibits ROS production from human myeloma cells

Many reports have shown that ROS modulates cellular functions including gene expression, survival, apoptosis, and tumorigenesis (15-19). Previous data including the regulation of proliferation and IL-6 gene expression of myeloma cells suggest that 1H8 can modulate ROS level in myeloma cells. The intracellular ROS levels of myeloma cells were compared to those of other B cells. As shown in Fig. 6, endogenous ROS levels were much higher in U266 (A) or IM-9 (B) than in WIL-2 NS (C) or CESS cells (D). Interestingly, 1H8 treatment suppressed the ROS level of U266 cells (E). Intracellular ROS levels are known to be controlled by an intracellular antioxidant pool, and PAG, which is well-known as a thio-specific antioxidant gene, is fairly ubiquitouslyexpressed and induced to higher levels by serum stimulation and oxidative stress. The expression of PAG was determined in U266 cells, and PAG was found to be induced by 1H8 in U266 cells, and that this was increased by cross-linking 1H8 with anti-mouse IgM (Fig. 7).

4. 1H8 induces protein phosphorylation of human myeloma cells.

Protein phosphorylation plays a crucial role in the regulation of cell growth, cytokine expression, and cell cycle (20,21). U266 were treated with 1H8 and cell extracts were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblot with same antibody.

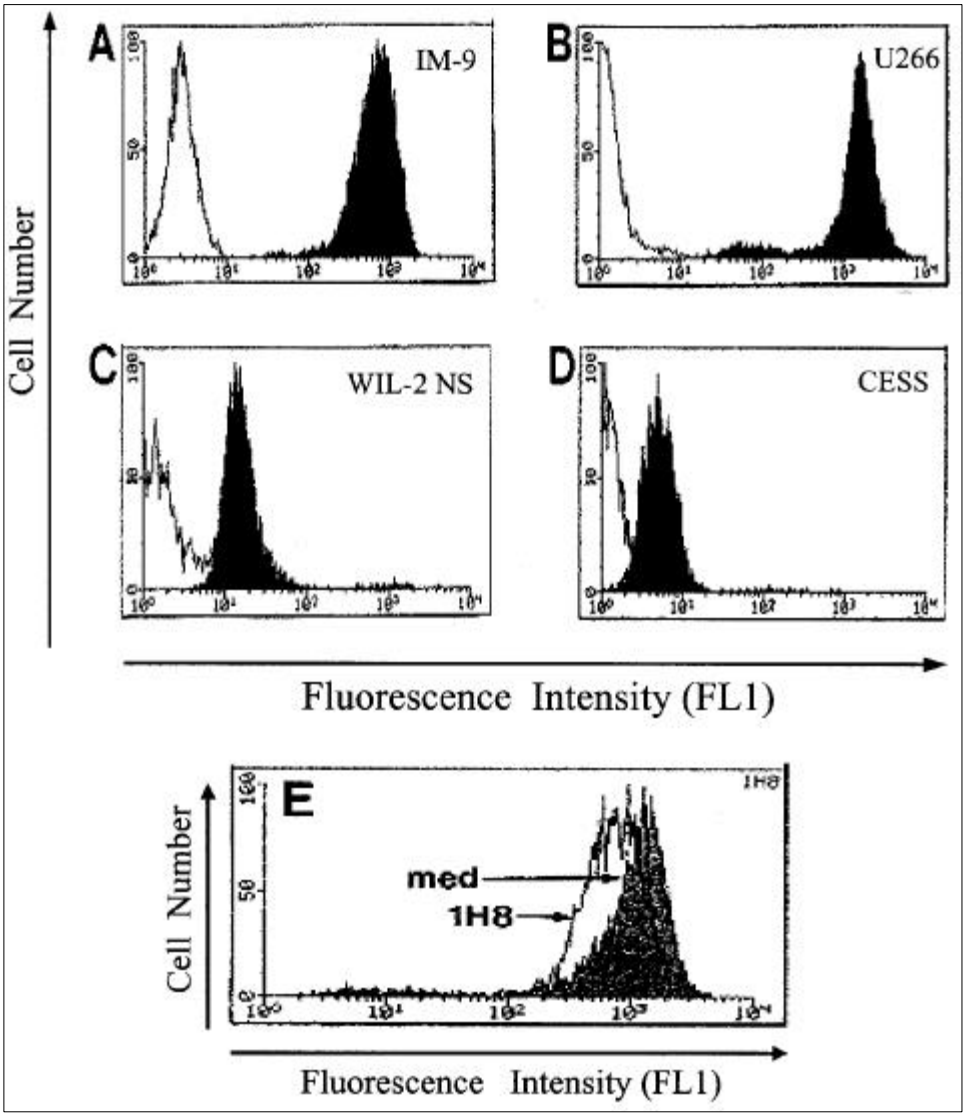


Fig. 6. Effects of 1H8 on intracellular ROS levels. Human B cells were incubated with 50 M of 2', 7'-dichloro- fluorescein diacetate (DCFH-DA) for 5 min at 37℃. The cells were washed twice and analyzed by flow cytometry. A, U266; B, IM-9; C, Wil-2 NS; D, CESS. Open and closed histograms represent unstained and stained cells, respectively. U266 Cells were incubated with 1H8 for 20 min (E) and intracellular ROS levels were analyzed as described above.

Immunoblot showed that several phosphoproteins such as 60, 65, and >100 kDa proteins were tyrosine phosphorylated time-dependently in 5 to 30min (Fig. 8), confirming that 1H8 delivers intracellular signaling through its binding molecule.

DISCUSSION

BMSCs produce soluble factors and express cell surface molecules, which regulate the proliferation and differentiation of hematopoietic cells. Thus, these cell surface molecules play a central role in the communication between myeloma and BMSC. Most malignant plasma cells are bound to BMSCs via cell surface molecules, which are locally located in the BM and rarely circulate in the peripheral blood. Moreover, the expression of cell surface molecules in malignant plasma cells is related to the regulation of immune cells. This

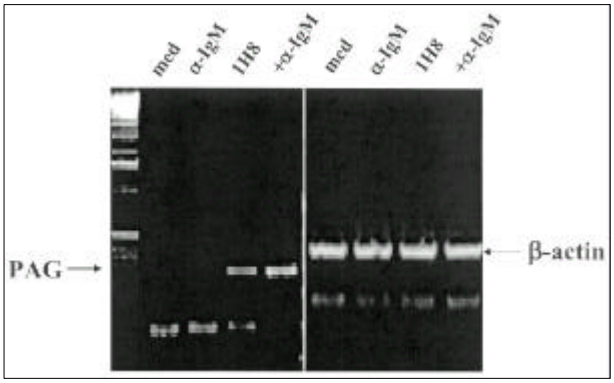


Fig. 7. Effects of 1H8 on the expression of PAG in U266 cells. U266 cells were incubated with 1 ug/㎖ of 1H8 for 20 min, and washed with PBS, and then 1 ug/㎖ of anti-mouse IgM was added. After 6 h incubation, the cells were harvested and cytoplasmic RNA was isolated. PAG gene expression was analyzed by RT-PCR.

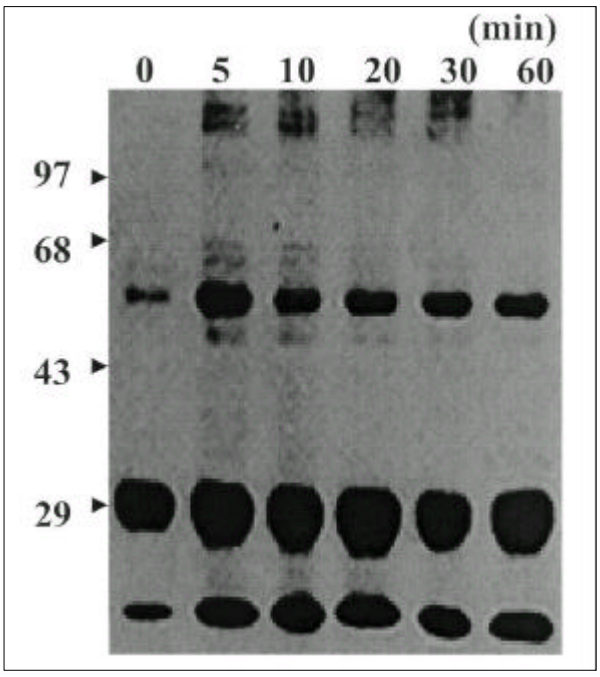


Fig. 8. Effects of 1H8 on the phosphorylation of several proteins of U266 cells. U266 cells were stimulated with 1 ug/㎖ of 1H8 at various times. The cell lysates were then immunoprecipitated with anti-phosphotyrosine antibody. Phosphorylated proteins were separated on a 10% SDS-PAGE and detected by immunoblot using anti-phosphotyrosine antibody.

means that cell-cell interactions via cell surface molecules plays a crucial role in the proliferation of malignant plasma cell (1).

In an attempt to identify the cell surface molecules of BMSCs, we generated several monoclonal antibodies against intact BMSCs. These antibodies recognized different cell surface molecules based on Facscan and immnoblot analysis. In addition, these antibodies blocked B cell proliferation induced by co-culturing with BMSCs. Among these antibodies, we characterized a 1H8 monoclonal antibody, which recognized an 80 kDa molecule.

Multiple myeloma is a B cell neoplastic disease, which results from malignant transformation of a single clone of plasma cells in the BM. Many soluble factors such as IL-3, IL-6, IL-8, TGF-β (22), and GM-CSF (23) are known to be involved in the progression of MM. In addition, adhesion molecules including CD54, CD56, CD29, VLA-4, and LFA are also known to have some role in cell proliferation and in the trapping of myeloma cells in the BM environment (6). IL-6 is a well-known growth factor for myeloma cells, although there have been some controversial reports on the mode of its action i.e., paracrine (14) or autocrine (24,25).

To test whether 1H8 regulated the functions of myeloma cells and stromal cells, the regulation of IL-6 expression by 1H8 was examined. 1H8 increased IL-6 expression in myeloma cells and BMSCs. In addition, it increased IL-6 receptor and CD40 expression in U266 cells cocultured with BMSCs. Urashima *et al.* (11) demonstrated that the up-regulation of IL-6 was induced by the stimulation of CD40 in MM cells, and Westendorf *et al.* (12) reported that the activation of CD40 induced the up-regulation of IL-6 to and stimulated IL-6-dependent myeloma cell proliferation. However, 1H8 inducing IL-6 production showed inhibitory effects on myeloma cell proliferation in the case of co-culturing with MMSCs. This may have been due to the epitope of IH8 being different from the binding site for the antigen for 1H8 and its ligand. Alternatively, the stimulating effects of co-culturing with MMSCs may require multiple interactions between different surface molecules including the antigen for 1H8, which by itself can induce IL-6 production. The

identification of antigen for 1H8 and the physiological ligand for the antigen are necessary to resolve this structural and functional issue.

Intracellular ROS level is believed to be important for tumorigenesis, cell survival and gene regulation. Recently, it has been reported that ROS is also a critical factor for cellular signaling. Endogenous ROS levels in other B lymphocytes such as WIL-2, SKW6.4, are CESS were different from those of U266 cells, suggested that elevated ROS function is a regulatory factor of cell survival and gene expression in myeloma cells. 1H8 can modulate ROS level by regulating PAG antioxidant gene expression. However, it did not affect cell apoptosis at whatsoever (data not shown).

Immunoblot with anti-phosphotyrosine antibody indicated that 1H8 can induce and deliver transient (5-10 min) and sustained (>30 min) signals into the myeloma cells, suggesting that 1H8 can induce diverse intracellular signals. Identification and studies upon the functional roles of these phosphoproteins are required to understand the intracellular signaling pathways triggered by 1H8.

In summary, 1H8 effects intracellular signaling by modulating protein phosphorylation, ROS level, and gene expression. It provides a tool for the studying the regulation of B cell proliferation and B cell function.

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FOOTNOTES

1. This work was supported by a grant from the HAN project (HSM0100033) at MOST, Republic of Korea.
2. Abbreviations used: BM, bone marrow; BMSC, bone marrow stromal cells; MM, multiple myeloma; MoAb, monoclonal antibody; ROS, reactive oxygen species; PAG, Proliferation-associated gene