Reveromycin A, a novel acid-seeking agent, ameliorates bone destruction and tumor growth in multiple myeloma

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doi:10.3324/haematol.2019.244418

Supplementary information

Materials and Methods

Ethics Statement

All experiments with animals were performed according to the guidelines for animal protection in University of Tokushima, and approved by the Institutional Review Board for animal protection. All procedures involving human samples from patients were performed with written informed consent in accordance with the Declaration of Helsinki and a protocol approved by the Institutional Review Board for human protection at University of Tokushima (Permission number: 240).

Reagents

The following reagents were purchased from the indicated manufacturers: concanamycin A from Funakoshi (Tokyo, Japan); rabbit anti-cleaved caspase-8 antibody, rabbit anti-cleaved caspase-9 antibody, rabbit polyclonal anti-Sp1 antibody, rabbit anti-Pim-2 antibody, rabbit anti- antibody, rabbit anti-cMyc antibody, horseradish-peroxidase-conjugated goat anti-rabbit IgG, horseradish-peroxidase-conjugated goat anti-mouse IgG and bortezomib from Cell Signaling Technology (Beverly, MA, USA); mouse anti-β-actin antibody from Sigma (St Louis, MO, USA); Z-lle-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (Z-IETD-FMK) from TONBO biosciences (San Diego, CA, USA); and metformin hydrochloride from Sigma (St Louis, MO, USA).

Cells and cultures

Human MM cell lines, RPMI8226, OPM2 and MM.1S were obtained from the American Type Culture Collection (Rockville, MD). The MM cell line INA6 was kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany). Primary MM cells were purified from bone marrow mononuclear cells from patients with MM by positive selection using anti-CD138 microbeads and Miltenyi magnetic cell sorting system (Miltenyi Biotec, Auburn, CA), in accordance with the manufacturer's instructions.

MM cells were cultured in RPMI1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin G and 100 μg/mL streptomycin (Sigma). The pH levels in culture media were adjusted by adding lactic acid (Wako, Osaka,

Japan).

Osteoclast (OC) formation

Whole bone marrow cells were harvested from the femur of 10-day-old Japanese white rabbit (CLEA, Tokyo, Japan). The cells were cultured on bovine bone slices in Eagle's minimal essential medium alpha modification (α-MEM; Sigma-Aldrich) supplemented with 10% FBS, L-glutamine and 50 μg/mL penicillin/streptomycin. Soluble RANKL (R&D Systems, Minneapolis, MN) was added at 20 ng/mL for 4 days to generate OCs. After culturing for 4 days, non-adherent cells were removed by washing the well with PBS. TRAP-positive cells were detected with a Leukocyte Acid Phosphatase Assay kit (Sigma-Aldrich). TRAP-positive cells containing three or more nuclei were counted as mature OCs under a light microscope. (BX50, Olympus, Tokyo, Japan).

Cell viability

Viable cell numbers were measured by cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Kishida Chemical, Osaka, Japan). After the incubation, the absorbance of each well was measured at 450-655 nm with iMarkTM microplate reader (Bio-Rad Laboratories, Hercules, CA). To assess apoptotic cells, cells were stained with an annexin V-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to the manufacturer's instruction, and analyzed by flow cytometry. PKH26 labeled MM cell death was also assessed by flow cytometry using PKH26 Red Fluorescent Cell Linker Kits and 7-AAD ready made solution (Sigma-Aldrich).

Lactate production assay

After cultured MM cell lines 24 hours in RPMI1640, the cell culture supernatant was assessed for lactate content by L-lactate assay Kit (Colorimetric) (Abcam, Cambridge, MA).

Western blot analysis

Whole cell lysate was lysed in RIPA buffer, and nuclear extract was lysed by NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Rockford, IL, USA). These lysates were supplemented with 1 mmol/L phenylmethylsulfonyl fluoride

and protease inhibitor cocktail solution (Sigma). Cell lysates were electrophoresed in a 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing and addition of a horseradish-conjugated secondary antibody for 1 hour. The protein bands were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

RT-PCR

Total RNA was extracted from cells using TRIZOL reagent (Gibco BRL, Rockville, MD). For reverse transcription-polymerase chain reaction (RT-PCR), 2 μg of total RNA was reverse-transcribed with Superscript II (Gibco) in a 20-μL reaction solution. One tenth of the RT-PCR products were used for subsequent PCR analysis with 24–30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The following primers were used: The primers used for RT-PCR were as follows: human *SP1* sense 5'-TTGAAAAAGGAGTTGGTGGC -3' and anti-sense 5'-TGCTGGTTCTGTAAGTTGGG -3'; human GAPDH sense 5'-TGTCTTCACCACCATGGAGAAGG-3' and anti-sense 5'-GTGGATGCAGGGATGATGTTCTG-3'.

MM mouse model and measurement of serum soluble human IL-6 receptor levels

The SCID-rab MM mouse model was prepared as previously described[1]. Briefly, femora and tibiae from 4-week-old Japanese white rabbits (Kitayama Labes, Nagano, Japan) were implanted subcutaneously into six-week-old male CB-17 SCID mice (CLEA Japan, Tokyo, Japan). After allowing bone engraftment for 4 weeks following the implantation, 1 x 10^6 INA-6 cells in 50 μL of phosphate-buffered saline (PBS) was inoculated directly into the bone marrow cavity of the rabbit bones. The treatment with RM-A or bortezomib was initiated 4 weeks after the inoculation of INA6 cells. Saline was injected as a vehicle. Serum levels of soluble human IL-6 receptor derived from INA6 cells were used as a marker for tumor burden as previously described[1]. Mouse sera were collected and serum levels of soluble human IL-6 receptor were measured with an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). X-ray photographs and μCT images of the rabbit bones were taken before and after the treatment. μCT images were

taken with Latheta LCT-200 (Hitachi, Tokyo, Japan). The rabbit bones were then collected, fixed in 10% phosphate-buffered formalin, and decalcified with 10% EDTA. The samples were further embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H.E.) and TRAP for histopathologic examination.

Statistical analysis

Statistical significance was determined by a one-way analysis of variance (ANOVA) with Scheffe post hoc tests. The minimal level of significance was a p value equal to 0.05.

Reference

1. Takeuchi K, Abe M, Hiasa M, Oda A, Amou H, Kido S, et al. TGF-beta inhibition restores terminal osteoblast differentiation to suppress myeloma growth. PLoS One. 2010 Mar 25;5(3):e9870.