tumor with a black sheet to prevent the primary site from emitting high levels of photons.

2.6. Histological analysis

Samples were fixed in 10% buffered formaldehyde and then embedded in paraffin. Sections, 4 μM thick, were mounted onto glass slides and stained with hematoxylin–eosin (HE). To determine the number of vascular endothelial cells, immunohistochemical staining was performed with a primary antibody against α smooth muscle actin (SMA) (clone 1A4; Sigma, St. Louis, Missouri, USA). Diaminobenzidine served as a chromogen and the slides were counterstained with hematoxylin. Appropriate positive and negative controls were included in each staining procedure [23]. Ten random microscopic fields (200× magnification) were analyzed with the IP Lab™ software for Apple Macintosh (BD Biosciences).

2.7. Statistics

Results are expressed as mean ± SD. Student's *t*-test was used to determine the statistical significance of detected differences; *p* values were derived from two-sided tests and values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of ZOL on LM8 cell proliferation and prenylation

ZOL inhibited the growth of LM8 cells in a time- and dose-dependent manner (Fig. 1A). The IC_{50} value of ZOL after 48 h of exposure was 7.36 μM. To determine whether the mevalonate pathway participates in the growth inhibitory effects of ZOL, we examined whether 10 μM GGOH could prevent ZOL from inhibiting the growth of LM8 cells. Indeed, GGOH treatment partially reversed the inhibitory effect of 10 μM ZOL after 48 h, increasing cell viability from 38% to about 61% (Fig. 1B). Western blot analysis of these cells revealed that 10 μM ZOL-treated LM8 cells contained unprenylated Rap1A (Fig. 1C). Flow cytometry of LM8 cells after 24 or 48 h exposure to 10 μM ZOL revealed a decreased frequency of cells in the G_2/M phase and an increased frequency of cells in the S phase (Fig. 1D).

3.2. Effect of ZOL on LM8 VEGF production

To examine whether ZOL affects the angiogenic capability of LM8 cells, we measured the VEGF concentrations in the culture supernatants of ZOL-treated and -untreated LM8 cells. ZOL treatment for 48 h reduced VEGF production in a dose-dependent manner, with 50% reduction being

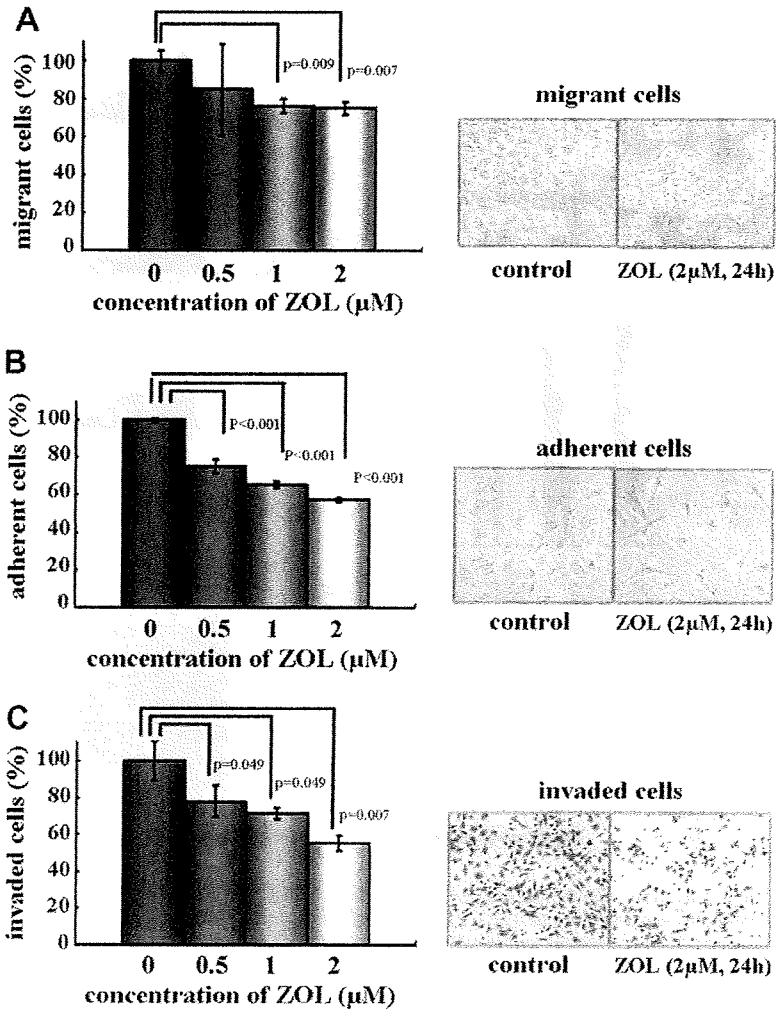

Fig. 2. ZOL prevents the migration, adhesion and invasion of OS cells. The effect of non-toxic doses of ZOL for 24 h on LM8 cell migration (A), adhesion (B) and invasion (C) was analyzed. The data shown are representative of three independent experiments.

achieved only with 2.55 μM ZOL (Fig. 1E, bars). It is notable that when the viability of the cells in this experiment was assessed with the MTT assay, a 50% reduction in viability was achieved with 7.36 μM ZOL (Fig. 1E, line plot).

3.3. Effect of ZOL on LM8 metastasis-related factors

To examine whether ZOL exerts anti-metastatic effects on OS cells, we investigated its ability to inhibit the migration, adhesion and invasion of LM8 cells during 24 h of culture. In this analysis, non-toxic doses were used as determined by examining LM8 cell viability after 24 h incubation with various concentrations of ZOL in serum-free medium. This revealed that ZOL did not exert a statistically significant effect on LM8 proliferation at concentrations up to 2.5 μM (data not shown). A migration assay performed with LM8 cells and various concentrations of ZOL up to 2 μM revealed that ZOL concentrations of 1 μM or higher significantly blocked LM8 cell migration towards a chemoattractant (Fig. 2A). In addition, ZOL concentrations of 0.5 μM or higher blocked both LM8 cell adhesion on collagen type I-coated plates (Fig. 2B) and LM8 cell invasion of Matrigel towards a chemoattractant (Fig. 2C).

3.4. Effect of ZOL on primary engrafted tumor growth

Before the initiation of mouse experiments, we confirmed that there was no significant difference in the cell characteristics such as proliferation and morphology between the $LM8^{Luc}$ transfected cells and the parent LM8 cell line (data not shown). Mice were injected in the subcutaneous soft tissue of the lateral lumbar region with $LM8^{Luc}$ cells and were either left untreated [group (i)] or were treated with 80 μg/kg ZOL once a week for 4 weeks [group (ii)] or with 80 μg/kg ZOL on the first 3 days of each week for 4 weeks [group (iii)]. We evaluated the efficacy of ZOL over time after inoculation by measuring both the photons of the primary site (Fig. 3A–C) and the tumor volume (data not shown because tumor volumes correlated well with the photons as previously observed [19,24]). By week 4 of treatment, while the lower dose of ZOL had not affected cell proliferation at the primary site, the higher dose strongly reduced the photons ($p = 0.010$; Fig. 3D). Histological analysis revealed that the primary tumors of group (iii) mice (Fig. 3F) showed many areas of necrosis and calcification, whereas this was not observed in the primary tumors of the control mice (Fig. 3E). Since ZOL inhibited LM8 VEGF production *in vitro* at clinically relevant concentrations (Fig. 1E), we also investigated whether ZOL inhibited the angiogenesis induced by LM8 cells *in vivo* by immunohistochemical analysis of SMA. The group (iii) primary tumors had significantly fewer SMA-positive areas (Fig. 3H and I) than the control tumors (Fig. 3G).

3.5. Effect of ZOL on lung metastasis

Spontaneous lung metastases were observed in all groups by the second week after LM8 cell inoculation (Fig. 4A and C). However, the growth of these metastases after 4 weeks of ZOL treatment was significantly inhibited in group (iii) (Fig. 4B and D). Interestingly, the lower dose of ZOL also reduced the growth of lung metastases (Fig. 4B), even though it barely affected the growth of the primary tumors (Fig. 3D).

4. Discussion

Based on preclinical studies [4,25], several randomized clinical trials have examined the anti-tumor effects of BPs, including the prevention of bone metastases, in breast cancer, and prostate cancer patients [26,27]. However, to our knowledge, clinical trials examining the anti-tumor effects of BPs on OS have not yet been conducted.

ZOL is an inhibitor of farnesyl pyrophosphate (FPP) synthase, and its administration depletes FPP and its downstream metabolite geranylgeranyl diphosphate (GGPP),

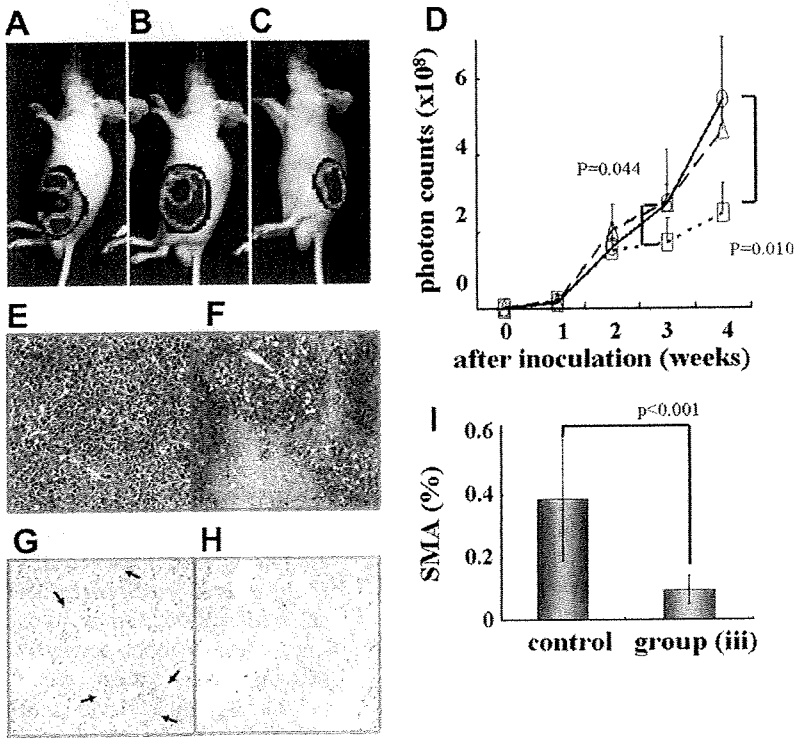

Fig. 3. Effect of ZOL on the *in vivo* growth of OS cells at the primary site. Luc-labeled OS cells ($LM8^{Luc}$) were implanted into the subcutaneous tissue of the lateral lumbar region and monitored by IVIS100. (A–C) IVIS images of mice that were left untreated (A) or were treated with ZOL once a week (B) or for three sequential days every week [C; group (iii)]. The images were taken 4 weeks after inoculation. (D) Mean real-time photon count curves (D) of the primary lesion. Untreated (○); treated with ZOL once a week (△); treated with ZOL on three sequential days a week (□). (E–H) Histological (E and F) and immunohistochemical (G and H) analyses of the primary tumor on week 4 after inoculation from untreated mice (E and G) and mice treated with ZOL for three sequential days a week (F and H). For histological and immunohistochemical analysis, the sections were stained with HE and SMA, respectively. The SMA-positive areas are indicated by the arrows. (I) The SMA-positive areas in G and H were calculated from 10 randomly chosen microscopic fields.

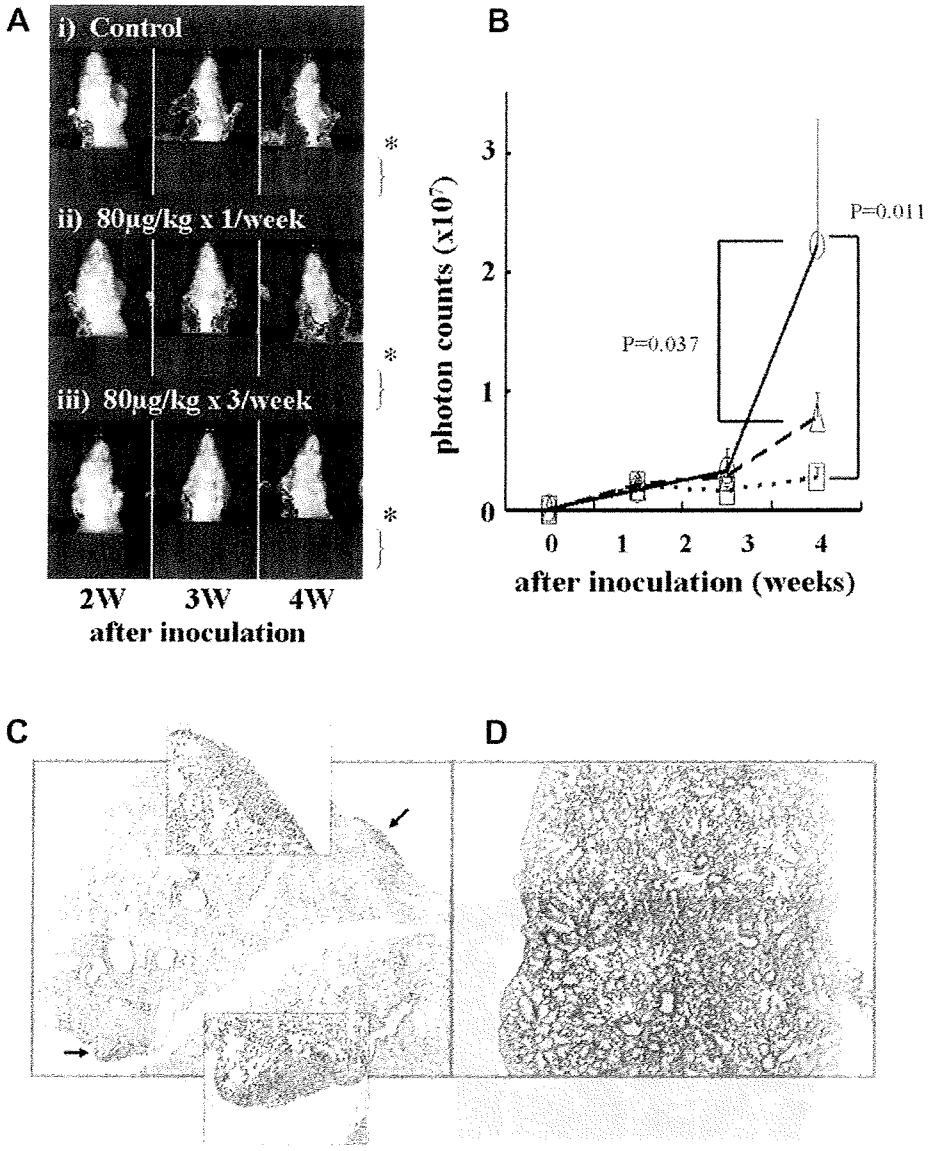

Fig. 4. Effect of ZOL on spontaneous lung metastasis. (A) Lung metastatic lesions in (i) untreated, (ii) treated with ZOL once a week, and (iii) treated with ZOL 3 days a week were monitored by IVIS100. (B) Mean real-time photon curves of metastasis. Untreated (○); treated with ZOL once a week (△); treated with ZOL 3 days a week (□). (C and D) Histological findings of the week 4 HE-stained lungs of untreated mice (C) and mice treated with ZOL for 3 days a week (D). The arrow pointing at the metastasis lesion, and the high power fields are shown in the control group. On the contrary, there is no obvious metastasis lesion in the excised lung of the mice treated with ZOL.

which are essential for the prenylation of small GTPases and thus the activation of Ras family proteins [28]. We showed that treatment of LM8 cells with 10 μM ZOL not only inhibited cell growth (Fig. 1A) and altered the cell cycle (Fig. 1D), but it also induced the accumulation of unprenylated Rap1A (Fig. 1C). Moreover, treatment with GGOH partially reversed the inhibitory effects of 10 μM ZOL on cell proliferation (Fig. 1B). These findings suggest that the anti-proliferative effects of ZOL against LM8 cells are due to interruption of the mevalonate pathway, thereby inhibiting prenylation of Ras family proteins. Similar observations have been made in other cancer cells [4,25].

The anti-proliferative IC_{50} value of ZOL for LM8 cells was less than that reported previously for other cancer cell lines [5–8,25]. BPs are non-hydrolyzable pyrophosphate analogs that strongly bind to hydroxyapatite, which is the main component of bone and teeth [29]. The sensitivity of different cell types to BPs probably depends largely on their ability to internalize sufficient amounts of BPs. Thus, OS, which is defined as an osteoid or bony matrix-producing malignant bone tumor [30], may be more sensitive to BPs than other cancer cells.

ZOL also might have another anti-tumor activity, since we found that it inhibited LM8 VEGF production. Significantly, this activity was exerted by 1–2 μM ZOL, which was lower than the 5–10 μM ZOL concentration needed to inhibit LM8 proliferation (Fig. 1E). VEGF is one of the most potent angiogenic factors known and its expression increases vascular permeability and promotes extravasation of proteins from tumor vessels [31]. Inhibition of VEGF

suppresses tumor growth *in vivo* [32]. BPs have been shown in a previous study to exert anti-angiogenic effects [33]. In the present study, we also found that non-toxic doses of ZOL inhibited not only VEGF production but also the migration, adhesion, and invasion of LM8 cells (Fig. 2). These findings indicate that even if the clinical serum concentration of ZOL does not reach the concentration needed to prevent OS proliferation at the primary site, ZOL may still be useful in preventing metastasis.

It is unclear which molecules consist of Ras family proteins such as Ras, Rho and Rap1, etc. mediate the anti-metastatic effects of ZOL. Since a Ras farnesylation inhibitor reduces cancer metastasis [34], and active Rho is required for variety of complex morphogenetic processes [35], the effects of ZOL on Ras and Rho may, at least in part, mediate its anti-metastatic effects. However, Ras and Rho inhibition only partly explains the anti-metastatic effects of ZOL because 1–2 μM ZOL did not induce a detectable accumulation of unprenylated Ras family protein (Fig. 1C), while this concentration was sufficient to inhibit metastasis-related factors (Fig. 2). Thus, other, non-Ras family protein, targets might be involved. Indeed, van Beek et al. suggested that pamidronate had an additional, as yet unidentified, molecular target in osteoclasts [36]. Furthermore, Monkonnen et al. recently reported that the inhibition of FPP synthase caused the accumulation of the upstream substrate isopentenyl pyrophosphate; this then became conjugated to AMP to form a novel ATP analog that inhibited mitochondrial adenine nucleotide translocase and induced osteoclast apoptosis [37]. Thus, comprehensive identification of the targets of ZOL will be needed to understand how ZOL inhibits metastasis.

Several studies in mouse models have shown that treatment with BPs can inhibit metastasis *in vivo* [9–11]. However, in two of these studies, the mouse models used involved the intravenous inoculation of cancer cells, which meant that the extravasation step from primary tumors was not needed for metastasis. In contrast, Hiraga et al. used a mouse model involving orthotopic inoculation of breast cancer cells. They showed that these cancer cells spontaneously metastasized to bone, liver and lung and that ZOL markedly inhibited this process. However, studies investigating the ability of BPs to prevent the lung metastasis of OS cells have not been performed until now (Fig. 4A).

Although ZOL clearly has the potential to be an anti-tumor drug [5–11], it remains unclear whether the serum concentrations of this agent achieved in humans *in vivo* are sufficient to exert a clinically relevant anti-tumor effect. In humans, ZOL is administered once every 3–4 weeks at an approved dose of 4 mg/patient. This results in peak serum concentrations in the range of 1–2 μM [38]. The clinical ZOL dose of 4 mg per nominal 65 kg body weight is equivalent to approximately 100 μg/kg of the research grade disodium salt of ZOL [11]. Thus, mice given 80 μg/kg ZOL once a week for 4 weeks had a cumulative dose of 320 μg/kg per month, which is 3.2 times the human dose. We found that this dose of ZOL could not inhibit the growth and neovascularization of the primary LM8 tumor (Fig. 3). Only the higher dose of ZOL, namely, 80 μg/kg ZOL given three times a week for 4 weeks (9.6 times the human dose), could inhibit proliferation and neovascularization of subcutaneous OS. This indicates that, in humans, it may be difficult to achieve the high serum concentrations needed for ZOL to inhibit OS proliferation in soft tissue. However, ZOL may still be useful clinically because we found that the lower dose of ZOL could significantly prevent lung metastasis (Fig. 4). Moreover, Daubiné et al. showed (albeit in a mouse model that does not recapitulate all the steps required for spontaneous metastasis) that a cumulative dose of ZOL of 100 μg/kg per month significantly inhibited bone metastasis in a mouse model [10]. It is conceivable that the lower dose we used approximates to the dose used in humans since bone turnover in rodents is three to five times higher than that in humans. Thus, it could be argued that fivefold higher BP doses in animal models of bone metastasis will mimic the clinical situation in humans [39]. This conclusion is supported by the observation that BPs given at the standard 4 mg reduce circulating VEGF levels in cancer patients [40]. Thus, the standard dose of ZOL in humans may be theoretically able to inhibit OS lung metastasis.

In current clinical OS surgery, we are frequently obliged by necessity to salvage the limbs of OS patients and need neoadjuvant chemotherapy to prevent the risk of later metastasis despite incomplete effects. Since ZOL is safe and well tolerated and rarely causes irreversible adverse events, even upon long-term use, our results suggest that ZOL may be useful for preventing lung metastasis in OS patients when accompanied with surgical resection of the primary site. Moreover, the use of ZOL in combination with adjuvant therapy and anti-cancer agents may also improve the outcome of OS patients. Supporting this are the reports by us and others that ZOL can act synergistically with a variety of currently used anti-cancer agents, at least *in vitro* [13,41,42]. For example, Mitsiades et al. have recently reported that with androgen ablation-refractory metastatic prostate cancer patients under androgen ablation, ZOL combined with dexamethasone and a somatostatin analog achieves palliative clinical responses superior to those generated by ZOL alone [43].

In conclusion, the lower concentration of ZOL which is achievable in the clinic showed anti-metastatic effects *in vitro* on OS cells. *In vivo*, the high dose of ZOL inhibited both OS growth at the primary site and metastases from the primary site. Interestingly, a lower dose of ZOL which may be achievable in humans significantly prevented lung metastasis despite no effects at the primary site. These finding may provide a rationale to use ZOL for the prevention of lung metastasis when accompanied with surgical resection of primary OS.

Conflict of interest

All authors have nothing to declare about the conflict of interest.

Acknowledgements

We are grateful to Dr. Jonathan Green (Novartis Pharma AG, Basel, Switzerland) for critically reading the manu-

script and to Ms. Yoko Nakagawa for her excellent technical support. This study is partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (15790796 to H.M. and 18591056 to S.K.).

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β-Catenin Small Interfering RNA Successfully Suppressed Progression of Multiple Myeloma in a Mouse Model

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Abstract

Purpose: β-catenin is the downstream effector of the Wnt signaling pathway, and it regulates cell proliferation. β-catenin overexpression correlates positively with prognosis in several types of malignancies. We herein assessed its effects on growth of multiple myeloma cells using a xenograft model.

Experimental Design: We first investigated the expression of β-catenin in multiple myeloma cell lines and multiple myeloma cells obtained from patients. Next, we investigated the growth inhibitory effects of β-catenin small interfering RNA on the growth of multiple myeloma cells *in vivo*. Six-week-old male BALB/c *nu/nu* mice were inoculated s.c. in the right flank with 5×10^6 RPMI8226 cells, followed by s.c. injections of β-catenin small interfering RNA, scramble small interfering RNA, or PBS/atelocollagen complex twice a week for a total of eight injections.

Results: Significantly higher levels of β-catenin expression were observed in multiple myeloma cell lines and in samples from patients with multiple myeloma than those found in mononuclear cells obtained from healthy volunteers. In *in vivo* experiments, no inhibitory effects were observed following treatment with scramble small interfering RNA or PBS/atelocollagen complexes, whereas treatment with β-catenin small interfering RNA/atelocollagen complex significantly inhibited growth of multiple myeloma tumors ($P < 0.05$).

Conclusions: β-catenin small interfering RNA treatment inhibited the growth of multiple myeloma tumors in a xenograft model. To our knowledge, this is the first report showing that the treatment with β-catenin small interfering RNA produces an inhibitory effects on growth of hematologic malignancies *in vivo*. Because treatment with β-catenin small interfering RNA inhibited growth of multiple myeloma cells, β-catenin is the attractive novel target for treating multiple myeloma.

As we gain a better understanding of the pathogenesis underlying multiple myeloma, new molecular targeting agents can be developed. At present, multiple myeloma remains incurable, so it is important to continue to investigate new therapeutic agents that focus on the biology of multiple myeloma cells. β-catenin is the downstream effector of the Wnt signaling pathway, and it regulates the genes encoding cyclin D1 and c-myc (1–3). Activation of Wnt signaling is closely involved in the process of carcinogenesis (4), and β-catenin overexpression has been observed in several types of malignant tumors, including hematologic malignancies (5–9).

RNA interference is a powerful tool in postgenomic research, and recently, experimentally introduced small interfering RNAs have been used in cancer therapy. The success of small interfering RNA therapy depends upon the development of suitable delivery systems, and several useful drug delivery systems have been developed (10–13). Among the drug delivery systems, atelocollagen represents one of the most attractive nonviral carriers for gene delivery. It is obtained from calf dermis, following the removal of immunogenic telopeptides located at the N- and C-termini of collagen molecules. Because atelocollagen has a positively charged surface, it easily binds negatively charged molecules such as nucleic acids. The small interfering RNA/atelocollagen complex is also resistant to nucleases and is transduced efficiently, resulting in long-term gene silencing (14). Here,

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Received 5/23/08; revised 12/21/08; accepted 1/12/09; published OnlineFirst 4/7/09.

Grant support: Aki Horinouchi Research Grant from the International Myeloma Foundation Japan and by a Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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doi:10.1158/1078-0432.CCR-08-1350

Translational Relevance

In this study, we showed that β-catenin small interfering RNA treatment successfully inhibited the growth of myeloma cells *in vivo* and we revealed that β-catenin is an attractive target for multiple myeloma. These findings not only show evidences for efficacy of targeting therapies for Wnt/β-catenin pathway but also encourage the possibilities of small interfering RNA therapies against multiple myeloma.

we use a xenograft model to show the inhibitory effect of the β-catenin small interfering RNA/atelocollagen complex on growth of multiple myeloma cells.

Materials and Methods

Cell lines and human samples. The human AMO-1, RPMI8226, NCI-H929, U226, OPM-2, KMS-12-BM, EJM, LP-1 myeloma cell lines, and IM-9 Epstein-Barr virus–transformed cell line derived from multiple myeloma patient were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The IM-9, OPM-2, RPMI8226, NCI-H929, and U266 were cultured in RPMI1640 (Gibco) containing 10% heat-inactivated FCS (Invitrogen), 2 mmol/L L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). The AMO-1 and KMS-12-BM cell lines were cultured in RPMI1640 containing 20% FCS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. The EJM and LP-1 cell lines were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 10% FCS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. All cell lines were maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

Six bone marrow samples, one ascites sample, and two pleural effusion samples were obtained from five multiple myeloma patients. Three bone marrow samples were obtained from healthy volunteers. In accordance with the Declaration of Helsinki recommendations, all procedures were approved by the institutional review board at Kyoto Prefectural University of Medicine, and written informed consent was obtained from every participant.

Expression of β-catenin in multiple myeloma cells. We used Western blotting analysis to investigate the expression of β-catenin in nine human multiple myeloma cell lines, as well as primary multiple myeloma cells. Ficoll-Hypaque density centrifugation was used to separate mononuclear cells from each participant's samples. A magnetic cell sorting separation system (Miltenyi) and anti-CD138 antibody (Miltenyi) were used to enrich multiple myeloma cells and normal plasma cells from bone marrow samples. Cells were analyzed by FACS Calibur using the Cell Quest software (BD Bioscience). The purity of enriched CD138+ cell populations was ≥90%. Multiple myeloma cells from ascites and pleural effusion were shown to express CD138. Cells were lysed with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4; 0.25 mol/L NaCl; 5 mmol/L EDTA; 20 mmol/L NaF; 1% NP-40) containing freshly prepared phenylmethylsulfonylfluoride (1 mmol/L) and protease inhibitor (10 μg/mL). Cell suspensions were cleared by centrifugation at 14,000 × *g* for 30 mins at 4°C. Nuclear and cytoplasmic protein fractions were obtained using by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology) according to the manufacturer's instruction. The supernatant (total cell lysate, nuclear, and cytoplasmic protein fractions) was either used immediately or stored at -80°C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Immunoblotting was done as described previously (15). Samples of cell extracts containing 20 μg of protein were analyzed. As the primary antibodies, we used a mouse monoclonal anti–β-catenin antibody (BD Pharmingen), a mouse anti–dephosphorylated β-catenin monoclonal antibody (Alexis Biochemicals), a mouse anti–phosphorylated β-catenin monoclonal antibody (Sigma-Aldrich), a rabbit polyclonal anti–cleaved caspase-3 antibody (Cell Signaling Technology), a rabbit polyclonal anti–caspase-3 antibody (Cell Signaling Technology), a rabbit polyclonal anti–Oct-1 (Santa Cruz Biotechnology), and a rabbit polyclonal anti-actin antibody (Sigma-Aldrich). Horseradish peroxidase–coupled anti–mouse and anti–rabbit immunoglobulin G (Amersham Biosciences) were used as

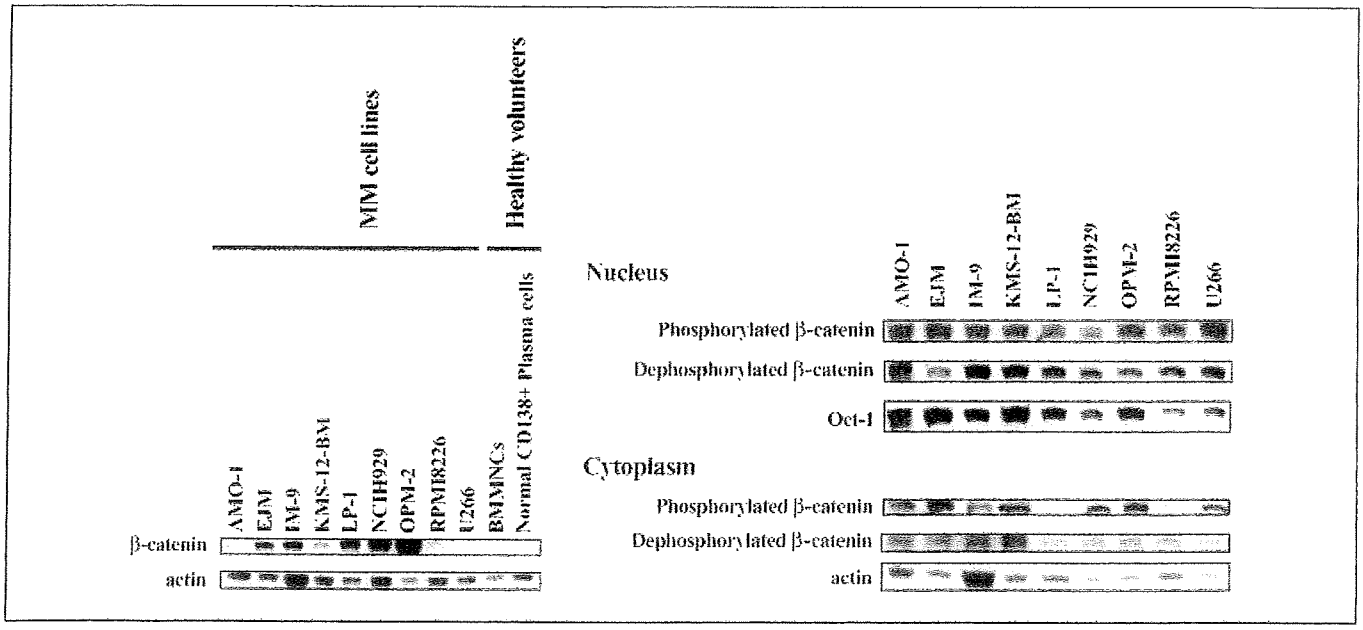

Fig. 1. β-catenin expression in human myeloma cell lines. β-catenin expression was examined by Western blotting. Left, β-catenin was overexpressed in myeloma cell lines and in multiple myeloma patient samples compared with bone marrow mononuclear cells and normal CD138+ plasma cells obtained from healthy volunteers. Actin expression was used as a loading control. Right, phosphorylated and dephosphorylated forms of β-catenin were expressed in multiple myeloma cell lines.

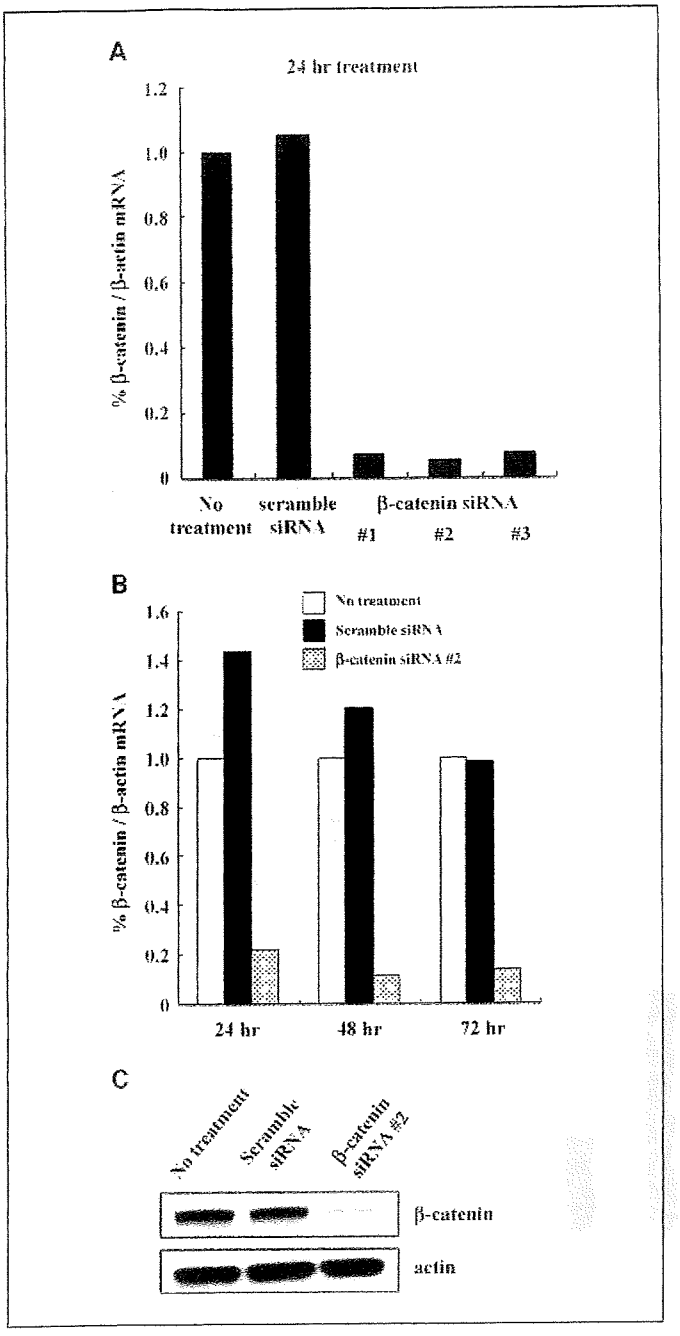

Fig. 2. Effects of β-catenin small interfering RNA *in vitro*. *A*, RT-PCR analysis. All three β-catenin small interfering RNAs decreased *β-catenin* mRNA levels in SW480 cells after 24-h treatment. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. *B*, real-time PCR analysis. Treatment of A549 cells with β-catenin small interfering RNA 2 leads to a decrease in *β-catenin* mRNA levels over a period of 72 h. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. *C*, Western blotting analysis of A549 cell lysates. There was a decreased expression of β-catenin small interfering RNA after 72-h treatment with β-catenin 2. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. Actin expression was used as a loading control.

secondary antibodies, and signal detection was done with an enhanced chemiluminescence kit (Amersham Biosciences).

Effects of knockdown with β-catenin small interfering RNA. Three types of β-catenin (Gene Bank accession number NM_001904) small interfering RNA and one scramble small interfering RNA with the following sense and antisense sequences were used: β-catenin small interfering RNA 1, 5′-CCAGGAUGAUCCUAGCUAUTT-3′ (sense), 5′-AUAGCUAGGAUCAUCCUGGTT-3′ (antisense); β-catenin small interfering RNA 2, 5′- GUAUUUGAAGUAUACCAUATT-3′ (sense), 5′-UAUGGUAUACUUCAAAUACTT-3′ (antisense); β-catenin small interfering RNA 3, 5′- CGAUUACAACUCUCCACAATT-3′ (sense), 5′-UUGUGGAGAGUUGUAAUGGTT-3′ (antisense); and scramble small interfering RNA 2, 5′- GGAAGAUAAUCUUUUCUAATT-3′ (sense), 5′-UUAGAAAAGAUUAUCUUCCTT-3′ (antisense). All small interfering RNAs were synthesized by Takara Bio, Inc. We first examined the effects of small interfering RNA-mediated knockdown using real-time reverse transcription-PCR (RT-PCR) and Western blotting analysis. Following transfection of β-catenin small interfering RNA into SW480 colon cancer cells and A549 lung cancer cells with Lipofectamine 2000 (Invitrogen), total RNA was extracted using the Micro-to-Midi Total RNA Extraction Kit (Invitrogen) and then subjected to reverse transcription (16). The levels of human *β-catenin* mRNA were analyzed using the LightCycler System (Roche Diagnostics) and FastStart DNA Master SYBER Green I (Roche). Amplicons were validated by melting curve and gel electrophoresis. The expression levels of the target mRNAs were normalized to those of the housekeeping gene *β-actin*. The specific primers used for amplification were as follows: *β-catenin*, 5′-GCTTGGTTCACCAGTGGATT (forward) and 3′- CCTTCCAGAGGAACCCTGAG (reverse); and *β-actin*, 5′-GGACTTCGAGCAAGAGATGG (forward) and 3′-GACATGCGGTTGTGTCACGA (reverse). Transfected cells were also examined using Western blotting analysis as described above.

In vivo effects of β-catenin small interfering RNA on myeloma tumors. After 3 Gy irradiation, specific pathogen-free 6- to 7-wk old male BALB/c *nu/nu* mice (SLC) were inoculated s.c. in the right flank with 5×10^6 RPMI8226 myeloma cells in 100 μL PBS. Palpable tumors (100 mm^3 in volumes) developed within 3 or 4 wks. Mice were then treated with s.c. (around tumors) injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%), scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex, β-catenin small interfering RNA (2.5 μmol/L)/PBS, or PBS/1% atelocollagen, twice a week for a total of eight injections. Tumor size was measured in two dimensions using a caliper, and tumor volume (mm^3) was calculated as $a^2 \times b/2$ mm^3 (a, minor axis; b, major axis).

Real-time RT-PCR and immunohistochemical examinations were used to examine the effects of β-catenin small interfering RNA–mediated knockdown in s.c. multiple myeloma tumors. Real-time RT-PCR was done as described above. For immunohistochemical examinations, paraffin-embedded tumor sections were immunolabeled with primary antibodies; that is, mouse β-catenin or anti–c-myc monoclonal antibodies (Santa Cruz Biotechnology), or rabbit polyclonal anti–cleaved caspase-3 antibody (Cell Signaling Technology). Primary antibodies were visualized using the conventional avidin-biotin-peroxidase complex method (VECTASTATIN Elite ABC kit, Vector Laboratories, Inc.). Sections were counterstained with hematoxylin and mounted. Detection of apoptosis was done using the terminal uridine deoxynucleotide nick end labeling (TUNEL) method and an ApopTag plus peroxidase *in situ* apoptosis detection kit (Millipore), according to the manufacturer's instructions. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Statistical analysis. The *in vivo* effects of small interfering RNA treatment were analyzed using the Student's t test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Expression of β-catenin in myeloma cells. Firstly, we examined human multiple myeloma cell lines, all of which expressed

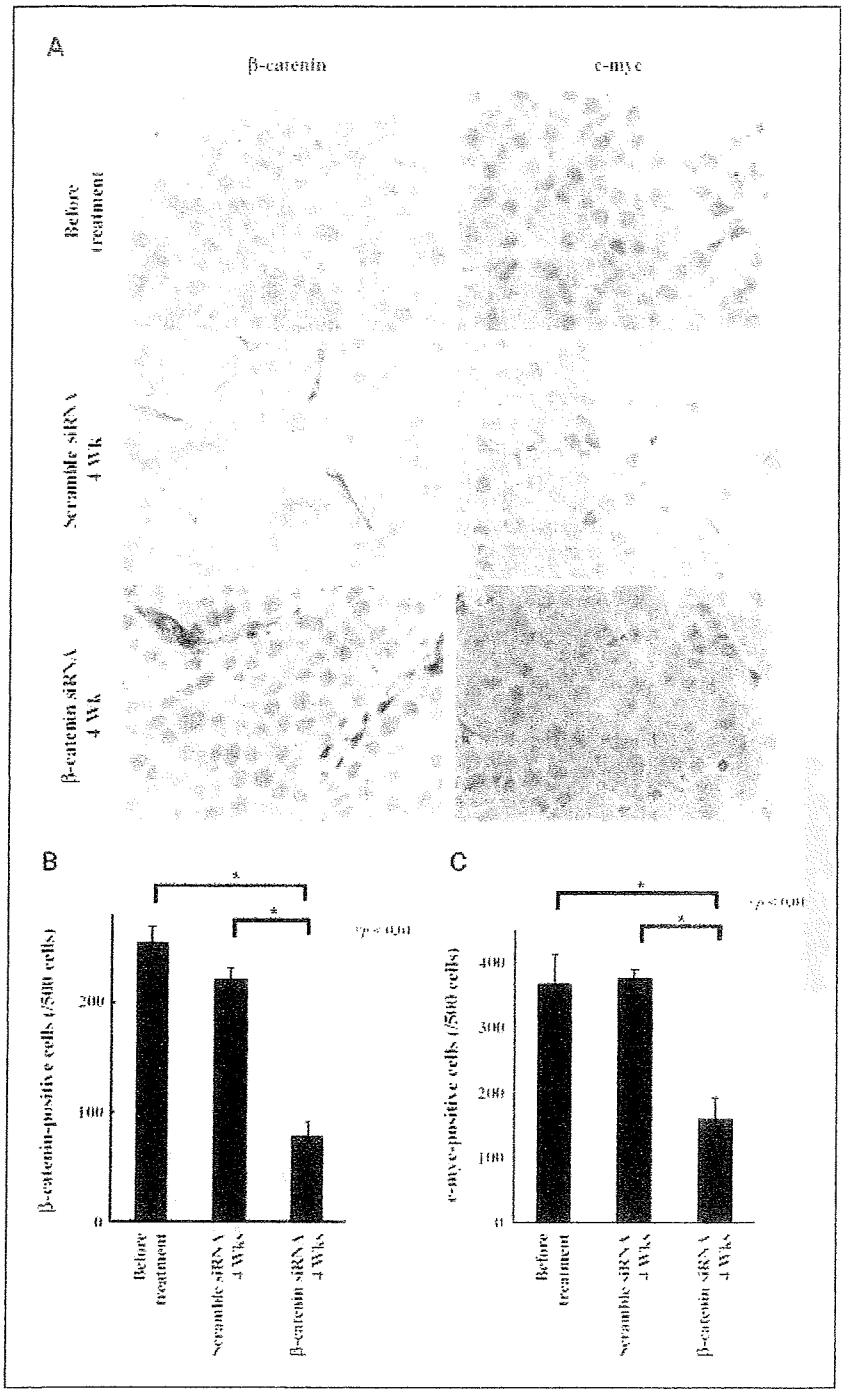

Fig. 3. Changes to β-catenin expression in myeloma tumors following treatment with β-catenin small interfering RNA. *A*, mice ($n = 3$ per group) were treated with s.c. injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%) or scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex. Immunohistochemical studies revealed that β-catenin and c-myc expression diminished following 4 wks of treatments with β-catenin small interfering RNA/atelocollagen complex relative to before treatment and with the scramble small interfering RNA/atelocollagen complex. *B*, β-catenin small interfering RNA/atelocollagen complex treatment significantly decreased β-catenin expression in multiple myeloma tumors relative to no treatment and treatment with the scramble small interfering RNA/atelocollagen complex. Results are means ± SEs. *C*, β-catenin small interfering RNA/atelocollagen complex treatment significantly decreased c-myc expression in multiple myeloma tumors relative to no treatment and treatment with the scramble small interfering RNA/telocollagen complex. Results are means ± SEs.

significantly higher β-catenin levels than normal human mononuclear cells and normal plasma cells (Fig. 1, *left*). We investigated phosphorylated and dephosphorylated forms of β-catenin. Both forms of β-catenin were expressed in multiple myeloma cell lines and primary multiple myeloma cells (Fig. 1, *right*). Total β-catenin levels in the cell lines did not correlate with phosphorylated and dephosphorylated β-catenin levels in the nuclear and cytoplasmic fractions. We speculate that the localizations of both β-catenin forms are different and that the degradation rates caused by proteasome are different in various cell lines. Moreover, we found significantly elevated expression of both forms of β-catenin in myeloma cells obtained from patients, relative to cells obtained from healthy volunteers (Supplementary Fig. S1).

Effects of knockdown with β-catenin small interfering RNA. We then examined knockdown of endogenous level of β-catenin protein levels using the three types of β-catenin small interfering RNA. Following transfection β-catenin or scramble small interfering RNA (100 nmol/L) into SW480 cells and A549 cells, we examined β-catenin expression using real-time RT-PCR and Western blot analysis. SW480 cells were examined 24 hours after treatment with the three β-catenin

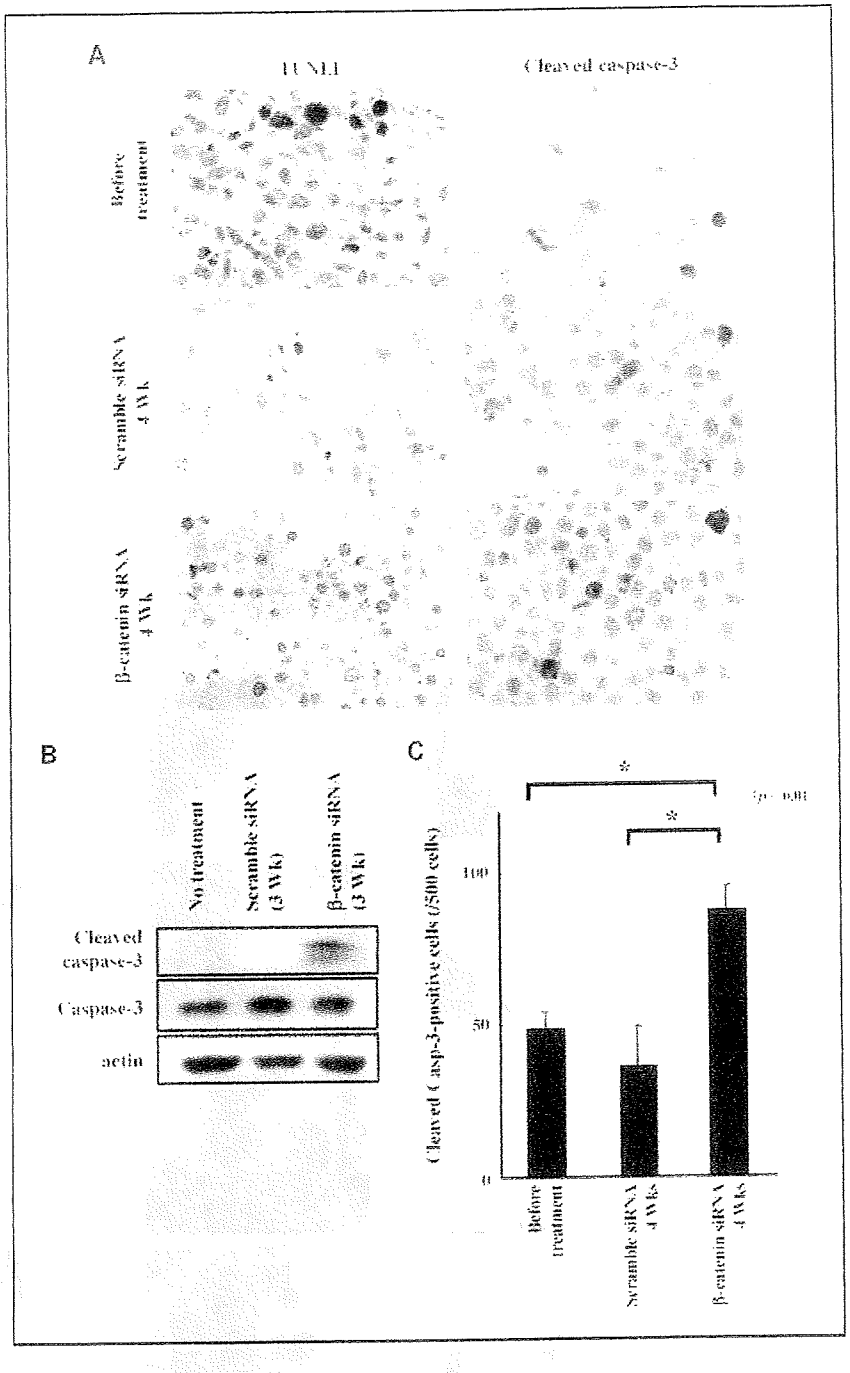

Fig. 4. Apoptotic cells and cleaved caspase-3 expression in myeloma tumors following treatment with β-catenin small interfering RNA. *A*, apoptotic cell (TUNEL-positive cell) numbers and cleaved caspase-3 expression increased following 4 wks of treatment with the β-catenin small interfering RNA/atelocollagen complex relative to before treatment and with the scramble small interfering RNA/telocollagen complex treatment. *B*, the expression of cleaved capsase-3 was increased in tumors after 3-wk treatment with β-catenin small interfering RNA/atelocollagen complex. *C*, cleaved caspase-3 positive cell numbers significantly increased in tumors of mice treated with β-catenin small interfering RNA/atelocollagen complex compared with those before treatment and those treated with scramble small interfering RNA/atelocollagen complex. Results are means ± SEs.

small interfering RNAs. In comparison with no treatment or treatment with scramble small interfering RNA, all three β-catenin small interfering RNAs caused a marked decrease in *β-catenin* mRNA levels (Fig. 2A). There were no significant differences in knockdown effects among three small interfering RNAs; therefore, β-catenin small interfering RNA 2 was used in further experiments. In A549 cells treated with β-catenin small interfering RNA 2, expression of *β-catenin* mRNA was reduced even at 72 hours after treatment, whereas no reduction was observed in non- or scramble small interfering RNA–treated cells (Fig. 2B). Similarly, β-catenin protein levels decreased after 72-hour treatment, whereas no reduction was observed in non- or scramble small interfering RNA–treated cells (Fig. 2C). These data showed that our β-catenin small interfering RNAs can diminish β-catenin expression successfully. Next, we investigated the antimyeloma effects of β-catenin small interfering RNA using a xenograft model.

In vivo *effects of β-catenin small interfering RNA on myeloma tumors.* We assessed β-catenin small interfering RNA–mediated growth inhibition *in vivo* using a mouse model (n = 3 per group). We administered β-catenin and scramble small interfering RNA/atelocollagen complexes twice a week for a total of eight injections and then compared expression of β-catenin and c-myc, as well as relative numbers of apoptotic

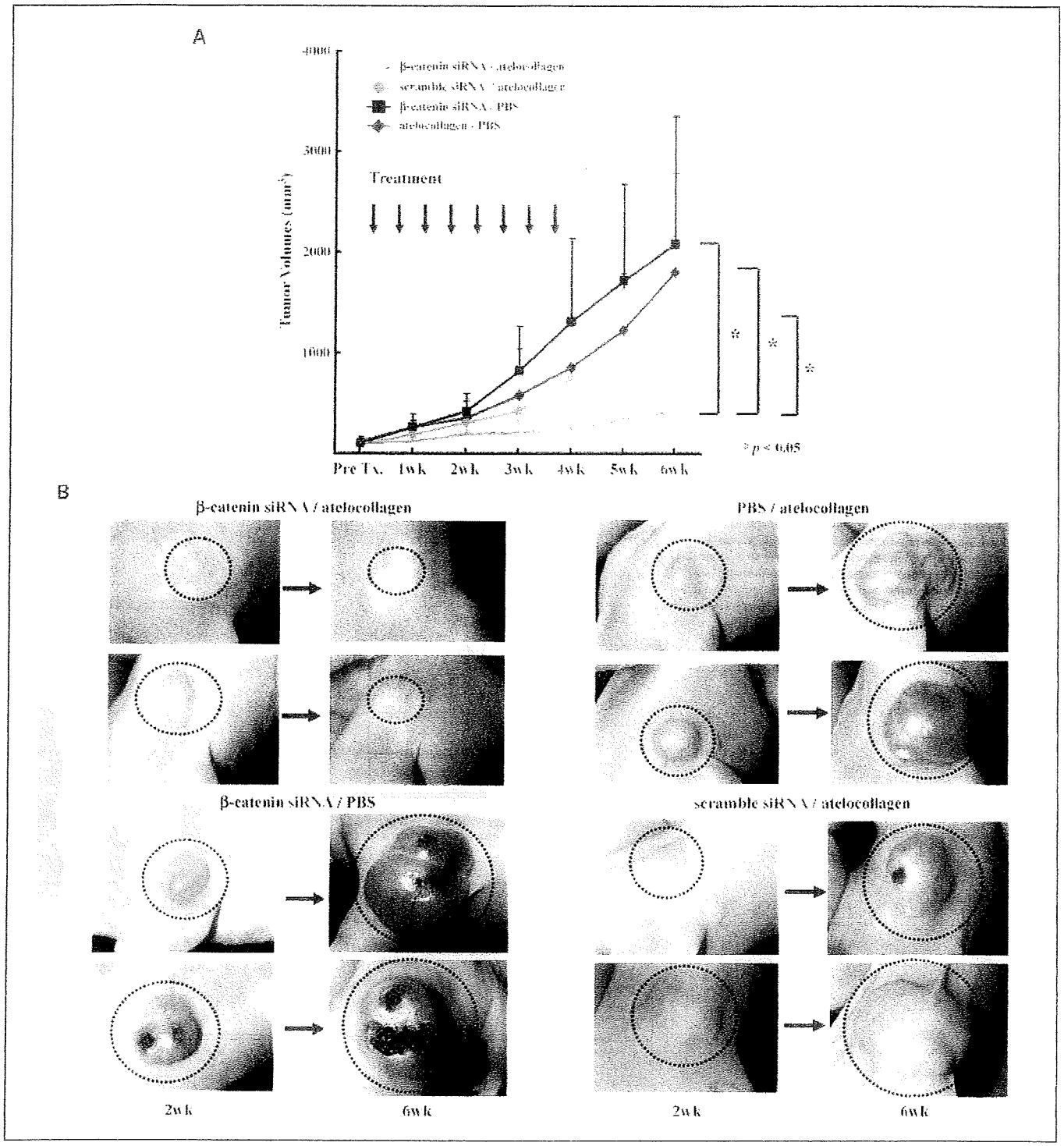

Fig. 5. Antimyeloma effects of β-catenin small interfering RNA in RPMI8226 xenografts. *A*, tumor growth curves. Palpable myeloma tumors (~100 mm^3 in volumes) were treated with s.c. (around the tumors) injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%), scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex, β-catenin small interfering RNA (2.5 μmol/L)/PBS, or PBS/1% atelocollagen twice a week for a total of eight injections (n = 5 per group). Treatment with β-catenin small interfering RNA/atelocollagen complex significantly reduced tumor volumes (P < 0.05). Results are means ± SEs. *B*, representative photos of tumor grafts after 2 and 6 wks of treatment. Dashed circles, myeloma tumors.

and cleaved caspase-3–positive cells. After RPMI8226 tumors had been treated for 1 or 2 weeks with the β-catenin small interfering RNA/atelocollagen complex, a significant decrease in *β-catenin* mRNA was observed (data not shown). After treatment for 4 weeks, β-catenin expression was decreased immunohistologically whereas expression was observed in tumors treated with the scramble small interfering RNA/atelocollagen complex (Fig. 3A, *left*). Because c-myc is a

target of β-catenin, we examined its expression in these 4-week-treated tissues (Fig. 3A, *right*). We found that, like β-catenin, c-myc expression was reduced significantly by treatment with the β-catenin small interfering RNA/atelocollagen complex (Fig. 3B and C). At this time point, we observed that the β-catenin small interfering RNA/atelocollagen complex–treated cells showed a significant increase in apoptotic cells using a TUNEL assay (Fig. 4A, *left*). To clarify the whether caspase was activated by the depletion of β-catenin, we investigated the expression of cleaved caspase-3 in multiple myeloma tumors by β-catenin small interfering RNA/atelocollagen complex treatment. Cleaved capase-3–positive cells were significantly increased in myeloma tumors treated with β-catenin small interfering RNA/atelocollagen complex treatment (Fig. 4A, *right*, and C). In Western analysis, cleaved caspase-3 was overexpressed in multiple myeloma tumors (Fig. 4B). Taken together, our results indicate that treatment with the β-catenin small interfering RNA/atelocollagen complex induced apoptosis of multiple myeloma cells by activating caspase-3.

Next, we evaluated the size of tumors during the 6 weeks following the treatment (n = 5 per group). At 6 weeks after treatment, the mean tumor volumes were as follows: β-catenin small interfering RNA/atelocollagen complex, 412.2 mm^3; scramble small interfering RNA/atelocollagen complex, 1,317.9 mm^3; β-catenin small interfering RNA/PBS, 2,075.9 mm^3; and PBS/1% atelocollagen, 1,802.3 mm^3. Treatment with the β-catenin small interfering RNA/atelocollagen complex significantly reduced tumor burdens and retarded tumor growth as measured by tumor volumes ($P < 0.05$; Fig. 5A and B). Therefore, these data show that the treatment with the β-catenin small interfering RNA/atelocollagen complex inhibits the proliferation of multiple myeloma tumors.

Discussion

In the present study, we used a mouse xenograft model to show that β-catenin small interfering RNA inhibits growth of multiple myeloma cells. To our knowledge, this is the first report showing that, for hematologic disorders, β-catenin small interfering RNA has growth inhibitory effects *in vivo*. Multiple myeloma cells are maintained and proliferate in the bone marrow through interactions between the bone marrow microenvironment and several cytokine growth factors for multiple myeloma cells, such as Wnt3a, Wnt5a, and Wnt10b (17). Activation of the canonical Wnt signaling pathway stabilizes β-catenin, and its nonphosphorylated form accumulates in the cytoplasm. β-catenin then translocates to the nucleus, where it interacts with T cell factor, driving transcription of target genes such as *c-myc* and *cyclin D1*. The mechanisms underlying aberrant β-catenin expression in multiple myeloma cells remain unclear. However, we have confirmed previous findings that suggested that multiple myeloma cells exhibit higher levels of β-catenin expression than normal hematopoietic cells (7, 9).

In various cancer therapies, RNA interference has been introduced experimentally and numerous methods for small interfering RNA transfection have been developed. At present, methods using viral vectors are the most efficient (18, 19). However, their utility is limited because of their potential to cause mutagenesis and develop cancers (20, 21). Several nonviral carriers have been developed for gene delivery, and atelocollagen is one of the most attractive of these novel carriers. It provides a clinically safe and readily available biomaterial (22).

Because atelocollagen has been developed as an *in vivo* drug delivery system (12, 14, 23, 24), we firstly confirmed the efficacy of three types of β-catenin small interfering RNAs *in vitro* using lipofection reagents as described previously (12, 24). Although small interfering RNAs could not be effectively transfected into myeloma cells *in vitro* (data not shown), our β-catenin small interfering RNAs decreased mRNA and protein levels of β-catenin in A549 and SW480 cells. We confirmed that our β-catenin small interfering RNAs could effectively induced RNA interference against β-catenin. Next, we evaluated the growth inhibition of myeloma cell tumors *in vivo* by β-catenin small interfering RNA/atelocollagen complex. After being administered around the tumors (enveloping the tumors), the β-catenin small interfering RNA/atelocollagen complex releases β-catenin small interfering RNA slowly, allowing it to diffuse into the tumors, where it silences β-catenin expression. We confirmed that treatment with the β-catenin small interfering RNA/atelocollagen complex diminished β-catenin and c-myc expression in immunohistological examinations. In addition, the expression of cleaved capsase-3 in multiple myeloma tumors was increased by the treatment of β-catenin small interfering RNA/atelocollagen complex, and significant increases in apoptotic and cleaved caspase-3–positive cells were observed in multiple myeloma tumors. Taken together, these results indicate that depletion of β-catenin induces apoptosis by activating capsase-3 and inhibit the growth of multiple myeloma cells.

In this study, we showed that the β-catenin small interfering RNA/atelocollagen complex inhibited proliferation of multiple myeloma tumors and that β-catenin might represent a molecular target for therapy against multiple myeloma. Small β-catenin inhibitor molecules have been developed and investigated, and preliminary findings have implicated β-catenin as a novel target for a cancer therapy (3, 9). Our data support these findings. However, because β-catenin is an important molecule for stem cell systems (25–27), systemic administration of β-catenin inhibitors might induce severe adverse effects. Moreover, Wnt/β-catenin signaling is essential for skeletogenesis, and it promotes osteoblast differentiation (28–30). Inhibition of Wnt/β-catenin signaling has been reported to result in the development of multiple myeloma bone disease(31, 32), whereas activation of the Wnt pathway suppresses the disease development (33). Supported by these results, a specific targeting strategy against these cells, such as antibody-combined small interfering RNA, is under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Hiroyuki Yao for his excellent technical assistance and Dainippon Sumitomo Pharmaceutical (Osaka, Japan) and KOKEN Co. Ltd. (Tokyo, Japan) for providing atelocollagen.

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Lung Cancer 66 (2009) 75–79

Contents lists available at ScienceDirect

Lung Cancer

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Monitoring with a non-invasive bioluminescent *in vivo* imaging system of pleural metastasis of lung carcinoma

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ARTICLE INFO

Article history:
Received 16 July 2008
Received in revised form 8 December 2008
Accepted 10 December 2008

Keywords:
Pleural metastasis of lung carcinoma
Malignant pleural effusion
In vivo image system
Bioluminescence imaging
Intrapleural administration
Docetaxel

ABSTRACT

Objectives: The prognosis of patients with malignant pleural effusion (MPE) is poor. An MPE model is necessary for developing new therapeutic strategies. We established a MPE model and investigated the therapeutic effects of the administration of serial doses of docetaxel on intrathoracic disseminated foci using an *in vivo* image system (IVIS).
Methods: First, mice without pleural metastasis of lung carcinoma were given a single injection of docetaxel (Taxotere) in the thoracic cavity. The average weight of each group was evaluated. Next, docetaxel was injected into the thoracic cavity of the mice once a week for 8 weeks. Again, the average weight of each group was evaluated. Finally, A549 lung cancer cells transfected with luciferase gene (A549-Luc) were injected into the intrapleural cavity of the mouse. When pleural metastasis was established, we started repeated intrapleural administration of docetaxel (once a week for 8 weeks). The average weight of each group and the survival period was evaluated. All bioluminescent data were collected and analysed with a Xenogen IVIS® system.
Results: (1) Single administration study: The average weight of mice injected with 50 mg/kg docetaxel dropped 10%. (2) Repeat administration study: In the group of mice injected with 25 mg/kg or 50 mg/kg docetaxel, the average weight dropped by 10%. (3) A549-Luc cells were injected into the thoracic cavity of the mice. Autopsies revealed disseminated foci in thoracic cavity 7 days or more after inoculation. After confirmation of tumor formation, repeated injections of docetaxel were administered. After 21 days, photon counts from the thoracic cavity of untreated mice increased. Repeated intrapleural administration of docetaxel prolonged survival when compared to the control group.
Conclusions: (1) We established a MPE model. (2) IVIS® facilitates faster and more accurate counting of disseminated foci in the pleura (as compared to the previous standard of measuring body weight changes) in a cancerous pleuritis model. (3) Repeated intrapleural administration of docetaxel is effective.

1. Introduction

Malignant pleural effusion (MPE) is a common and life-threatening problem in patients with advanced malignancies such as primary lung cancer and breast cancer. The prognosis of patients with MPEs remains poor [1], and the mean survival period after confirming the diagnosis has been reported to be approximately 3 months. Patients often experience various manifestations caused by MPE, such as progressive dyspnea, cough, and chest pain, compromising the quality of the remaining short lifespan of these patients. In most cases, MPE is controlled by obliterating the pleural space (pleurodesis) which involves draining the fluid via a chest tube followed by intrapleural instillation of various sclerosing or chemotherapeutic agents [2]. However, pleurodesis sometimes fails to prevent re-occurrence of MPEs. This may result in higher rates of infection, as well as the need for repeated chest tube drainages and longer hospitalizations. Alternatively, prolonged placement of an indwelling pleural drainage catheter has been proposed for controlling MPEs [3,4]. Putnam et al. conducted a randomized study comparing placement of an indwelling pleural catheter with pleurodesis using doxycycline in the treatment of patients with MPE. They demonstrated that placement of an indwelling pleural catheter resulted in a shorter length of hospitalization for the patient.

Implantable access systems can be used for controlling malignant effusion. Such systems had been initially used for intraperitoneal administration of chemotherapeutic agents to control peritoneal effusion caused by malignant tumors such as ovarian

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0169-5002/$ – see front matter © 2009 Published by Elsevier Ireland Ltd.
doi:10.1016/j.lungcan.2008.12.010

cancer [5–7]. Driesen et al. used an implantable access system for repeated intrapleural immunotherapy in patients with malignant mesothelioma [8].

Docetaxel, a taxane, is a newer chemotherapeutic agent that is widely used for the treatment of malignant tumors such as non-small cell lung cancer (NSCLC) and breast cancer. Docetaxel promotes microtubule polymerization and inhibits depolymerization, which results in a mitotic arrest in the G_2M phase of the cell cycle [9]. Docetaxel also induces apoptotic cell death by stimulating the phosphorylation of bcl-2 [10]. Many experimental studies showed that intravenous administration of docetaxel was effective in the treatment of mice bearing human cancer subcutaneous xenografts. In addition, one experimental study showed that intraperitoneal administration of docetaxel was safe and effective in the treatment of mice bearing ovarian carcinoma xenografts implanted intraperitoneally [11]. These results suggest that intrapleural docetaxel administration may be safe and effective for treating patients with MPE; however, safety and efficacy should be examined in animal models prior to clinical use.

In mice subcutaneous tumor models, tumor growth can be easily monitored by caliper measurements. However, in pleural metastasis models, it is difficult to measure tumor growth continuously and to evaluate response to a treatment. Surrogate markers such as weight loss may be employed to monitor toxicity, but the exact treatment efficacy is usually evaluated only after mice are sacrificed. In addition to computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET), an *in vivo* non-invasive bioluminescent imaging (BLI) system has recently been developed using the adenosine triphosphate (ATP)-dependent light-emitting reaction of the firefly (*Photinus pyralis*) luciferase and its substrate D-luciferin [D-(−)-2-(6′-hydroxy-2′-benzothiazolyl) thiazone-4-carboxylic acid] [12,13]. Using this system, temporal and spatial monitoring of pathophysiologic processes *in vivo* can be performed, therefore reducing the number of animals needed to achieve statistical power [12–19]. Thus, in this study, we first established pleural metastasis of lung carcinoma model, and then demonstrated the efficacy of intrapleural administration of docetaxel via continuous monitoring of pleural metastasis during the treatment using a non-invasive bioluminescent *in vivo* imaging system (IVIS).

2. Materials and methods

2.1. Cell line and animals

The human lung cancer cell line A549 (adenocarcinoma) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained at 37 C in RPMI-1640 medium (GibcoBRL, Oaisley, Scotland) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT).

Female BALB/c *nu/nu* mice were purchased from SLC Japan (Kyoto, Japan), and were maintained in a pathogen-free environment. All animal studies were performed in accordance with guidelines established by the Animal Welfare Committee of Kyoto University.

2.2. Evaluating the safety of intrapleural docetaxel administration

Docetaxel was a kind gift from Aventis Pharma Japan (Tokyo, Japan). A total of 42 five-week-old female BALB/c *nu/nu* mice were used in this study. There were 7 mice in each of the 6 treatment groups: 100 μl or 200 μl of saline, 5 mg/kg docetaxel in 100 μl or 200 μl of saline, or 50 mg/kg docetaxel in 100 μl or 200 μl of saline. To evaluate the safety of a single intrapleural administration of docetaxel, animals (bearing no tumor) were administered a single dose in the left pleural cavity and then maintained with no additional treatment. Body weights were measured twice a week to monitor toxicity.

2.3. Determining the optimal docetaxel dose in repeated intrapleural administration

Five-week-old female BALB/c *nu/nu* mice, bearing no tumor, were given weekly injections in the left thoracic cavity. Animals were treated at doses of 0 mg/kg, 1.0 mg/kg, 2.5 mg/kg, 5.0 mg/kg, 7.5 mg/kg, 10.0 mg/kg, 12.5 mg/kg, 25.0 mg/kg, or 50.0 mg/kg of docetaxel for 8 weeks (4 animals per treatment group). Each dose of docetaxel was dissolved in 100 μl of physiological saline. The optimal dose was defined as the maximum dose that did not cause treatment-related death or body weight loss of greater than 10% throughout the 8-week docetaxel treatment as well as the 8 weeks of follow-up.

2.4. Establishing A549 cell lines that express the firefly luciferase

A549 cells were co-transfected with the firefly luciferase gene (pGL3 Basic plasmid; Promega, Madison, WI) in combination with a vector for neomycin resistance, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were selected with G418 (Geneticin; Invitrogen). The surviving colonies were harvested and cultured independently. Cell clones were screened using a bioluminescence IVIS® (Xenogen). The A549 clone showing the highest luciferase activity (A549-Luc) was used in the present *in vivo* study. To evaluate how the number of cells effects the photon counts, A549-Luc cells were diluted to 1.0×10^5 cells per well, 5.0×10^4 cells per well, 2.5×10^4 cells per well, or 1.25×10^4 cells per well. Bioluminescence imaging with a charge-coupled device (CCD) camera (IVIS, Xenogen) was initiated 1–2 min after 150 μg/ml of D-luciferin (Xenogen) was added each well.

2.5. Treatment of pleural metastasis of lung carcinoma with intrapleural docetaxel injection

A549-Luc cells (5.0×10^6) were injected directly into the left pleural cavity of five-week-old female BALB/c *nu/nu* mice. IVIS was used to monitor the pleural metastasis of lung carcinoma. When establishment of pleural metastasis was ensured, mice were randomized into the control or docetaxel group (10 per group).

Mice in the docetaxel group received 8 weekly intrapleural injections of docetaxel at the previously determined optimal dose (10 mg/kg). Control mice were injected with the same volume (100 μl) of physiological saline on the same schedule as the docetaxel group. Pleural metastasis of lung carcinoma was monitored with IVIS.

2.6. Bioluminescence imaging with IVIS

Mice were anesthetized with isoflurane inhalation, and were subsequently intraperitoneally (i.p.) injected with 100 μl of 7.5 mg/ml D-luciferin (Xenogen). Bioluminescence imaging with a CCD camera (IVIS, Xenogen) was initiated 10 min after injection; imaging times ranged from 1 to 60 s, depending on the amount of luciferase activity. Bioluminescence from the region of interest (ROI) was defined manually, and the data were expressed as photon-flux (photons/s/cm^2/steradian). Background photon-flux was defined using an ROI from a mouse that was not given an i.p. injection of D-luciferin. All bioluminescent data were collected and analyzed using IVIS.

2.7. Statistics

The Stat View 5.0 statistical software package (SAS Institute Inc., Cary, NC) was used for all statistical analyses. The chi-squared test was used to compare photon counts. Continuous data between two groups were compared using: (1) Student's *t*-test, if the distribution of samples was normal, or (2) Mann–Whitney *U*-test, if the sample distribution was asymmetrical. Continuous data among three or more groups were compared using: (1) a one-way analysis of variance (ANOVA), if the distribution of samples was normal, or (2) the Kruskal–Wallis *H*-test, if the sample distribution was asymmetrical. The postoperative survival rate was analyzed by the Kaplan–Meier method, and the differences in survival rates were assessed by the log-rank test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Safety of single intrapleural injection of docetaxel

Female BALB/c *nu/nu* mice, five weeks in age and bearing no tumor, were administered a single injection of docetaxel (5 mg/kg or 50 mg/kg) in the left pleural cavity. Each dose was prepared in two dilutions using 100 μl or 200 μl of saline. Control mice were given 100 μl or 200 μl of saline alone. No deaths occurred in any of the 6 treatment groups. Although, no significant weight loss was observed after a single dose of 5 mg/kg docetaxel, a 10% decrease in weight was documented at 2 days following the injection of 50 mg/kg docetaxel (Fig. 1). The volume of saline used to prepare the docetaxel did not affect the weights of the mice; therefore, docetaxel was dissolved in 100 μl of saline in subsequent studies.

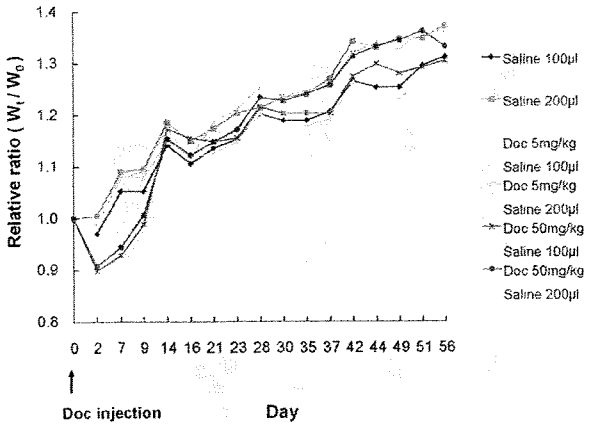

Fig. 1. Body weight changes after a single intrapleural injection of docetaxel Docetaxel was injected into the pleural cavity of non-tumor-bearing mice on Day 0. Docetaxel (Doc) was dissolved in 100 μl or 200 μl of physiological saline and administered at 0 mg/kg, 5 mg/kg, or 50 mg/kg. There were 7 mice in each group.

3.2. Determination of optimal dose for repeated intrapleural administration of docetaxel

To determine the optimal dose in repeated intrapleural administration of docetaxel for the treatment of pleural metastasis of lung carcinoma, mice were injected with docetaxel (0 mg/kg, 1.0 mg/kg, 2.5 mg/kg, 5.0 mg/kg, 7.5 mg/kg, 10.0 mg/kg, 12.5 mg/kg, 25.0 mg/kg, and 50.0 mg/kg) once per week for 8 weeks. Marked weight loss was observed in all mice administered 25 mg/kg or 50 mg/kg docetaxel. In addition, all mice in these treatment groups died before the completion of the 8 weekly injections (49 and 21 days respectively) (Fig. 2).

In contrast, no deaths or weight loss over 10% was documented in any group given a dose of 7.5 mg/kg docetaxel or less. At doses of 10–12.5 mg/kg of docetaxel, marginal weight loss was occasionally observed, but no deaths. Based on these results, we used 10 mg/kg of docetaxel in further repeated intrapleural treatment experiments.

3.3. Bioluminescent monitoring of pleural metastasis of lung carcinoma during treatment

A549 cells were transfected with the firefly luciferase gene, and plated at 1.0×10^5 cells per well, 5.0×10^4 cells per well, 2.5×10^4 cells per well, or 1.25×10^4 cells per well. Photon counts, as detected by IVIS, highly correlated to the number of cells in each well ($r^2 = 0.99$) (Fig. 3).

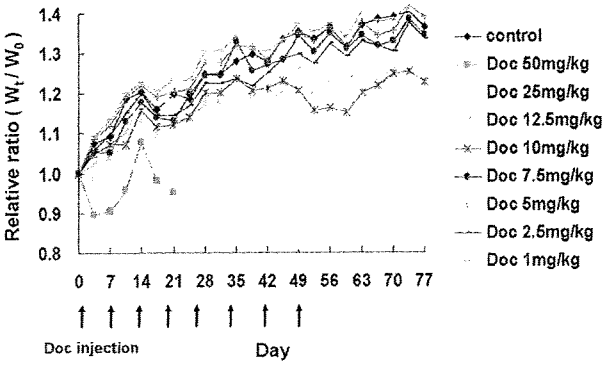

Fig. 2. Repeated intrapleural injections of docetaxel in non-tumor-bearing mice Docetaxel was administered weekly (Days 0, 7, 14, 21, 28, 35, 42, and 49) into the left pleural cavity of the mice. Docetaxel was dissolved in 100 μl of physiological saline and the mice were given a 0 mg/kg, 1 mg/kg, 2.5 mg/kg, 5 mg/kg, 7.5 mg/kg, 10 mg/kg, 12.5 mg/kg, 25 mg/kg, or 50 mg/kg dose. The day of first docetaxel injection is shown as Day 0. There were 4 mice in each group.

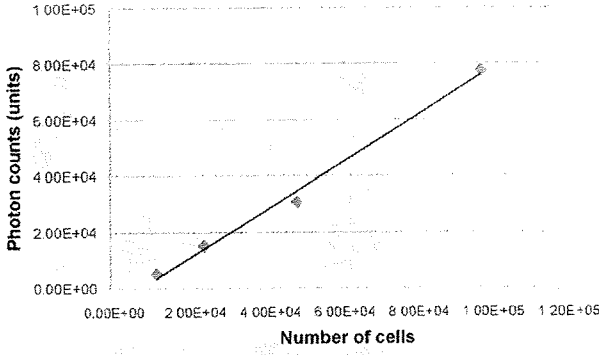

Fig. 3. Correlation of cell number and photon counts A549-Luc cells were diluted to 1.0×105 cells per well, 5.0×104 cells per well, 2.5×104 cells per well, and 1.25×104 cells per well and the photon counts were measured using a bioluminescent *in vivo* imaging system (IVIS) ($r2 = 0.99$).

A549-Luc cells (5×10^6) were injected into the left thoracic cavity of each mouse. Seven days after the cell implantation, establishment of pleural metastasis of lung carcinoma were confirmed using IVIS (Fig. 4(A)). Mice were then randomly divided into 2 treatment groups (control or 10 mg/kg docetaxel).

After treatment, pleural metastasis was monitored using IVIS (Fig. 4(A)); the photon intensity, a measurement of viable tumor volume, was also determined by the system (Fig. 4(B)). The average photon intensity did not increase following treatment with docetaxel; whereas, a significant increase in the photon intensity was observed in the control group (Fig. 4(A) and (B)).

Although 8 weekly intrapleural injections were initially planned, all mice in the control group died before "Day 49," the day of the 8th intrapleural injection. Autopsies revealed that all of the mice died due to enlargement of the pleural metastasis. All 10 mice in the docetaxel group survived until "Day 42" when the 7th intrapleural injection was scheduled. However, 2 of the 10 mice died just after the intrapleural injection was performed. Autopsies revealed that these mice had drug-induced interpleural adhesions and subsequently died of unintentional injuries to the lung during the intrapleural. Thus, we decided to not administer the 8th dose. The remaining 8 mice in the docetaxel group were sacrificed on "Day 53" (3 weeks after the final (7th) intrapleural injection of docetaxel). Autopsies revealed that 4 of these mice possessed drug-induced intrapleural adhesions; yet, virtually no disseminated tumor was observed in any mouse.

There was a significant survival advantage in the docetaxel group as compared with the control group ($p < 0.01$) (Fig. 5(A)). A slight increase in the average body weight was seen in the docetaxel group during repeated injections, but a significant (more than 10%) decrease was observed in the control group ($p < 0.01$) (Fig. 5(B)).

4. Discussion

In this study, we first established a pleural metastasis of lung carcinoma model. We then assessed the efficacy of repeated intrapleural injections of docetaxel by monitoring the tumors using the non-invasive IVIS.

In previous pleural metastasis models, tumor cells were injected into the pleural space of the mouse after skin and intercostal muscle incisions. This ensured that the tumor cells were implanted in the pleural space. In this study, however, intrapleural injections were performed without incisions. Our method was simple

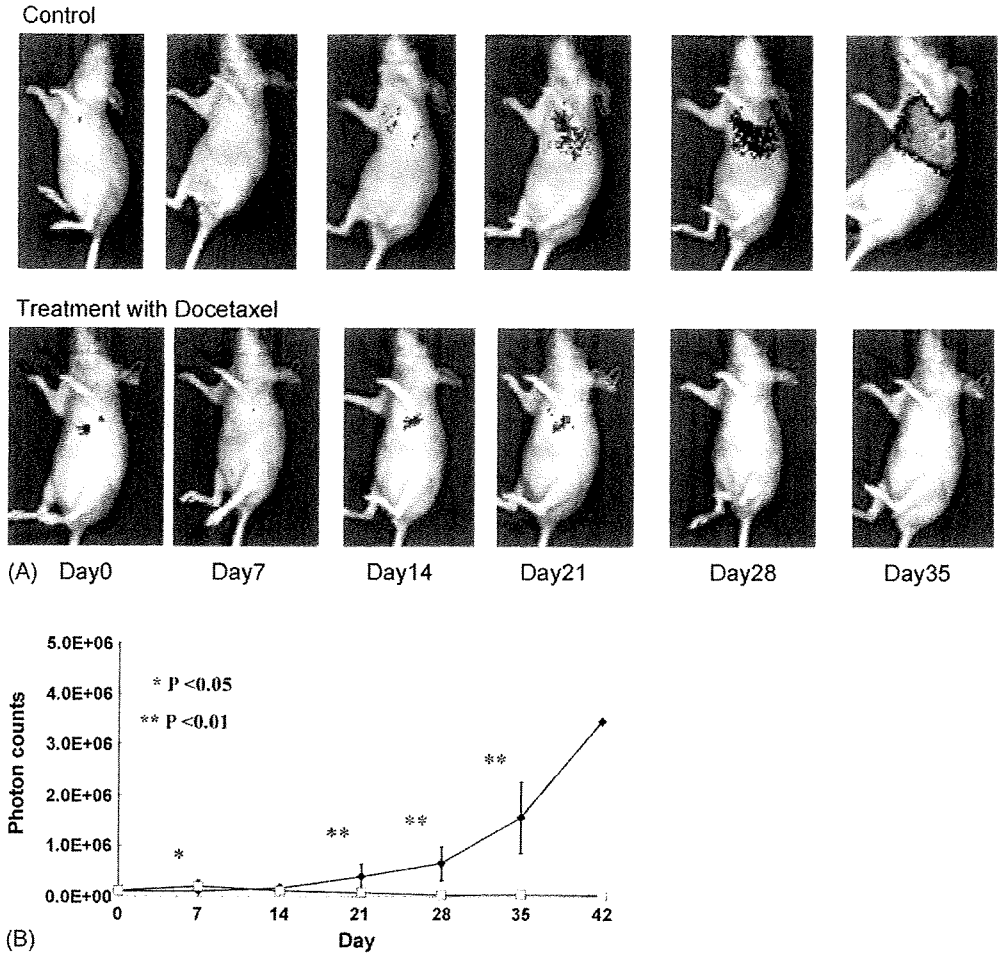

Fig. 4. Treatment of tumor-bearing mice with repeated intrapleural docetaxel injections A549-Luc cells (5 × 106) were injected into the left thoracic cavity of the mice. Seven days later, establishment of pleural metastasis of lung carcinoma was confirmed using a bioluminescent *in vivo* imaging system (IVIS). Mice were then randomized into a group with repeated intrapleural docetaxel treatment (10 mg/kg of docetaxel dissolved in 100 μl physiological saline) or a control group (the same volume of physiological saline). Pleural metastasis was monitored with the bioluminescent *in vivo* imaging system (A). The day of first docetaxel (or physiological saline) injection is defined as "Day 0." The bioluminescence intensity, a measurement of viable tumor volume, was also quantified by the system, and is indicated as photon-flux (photons/s/cm^2/steradian) (B).

as well as less invasive than the previous techniques. When evaluating the efficacy of treatment, it is essential that the tumor cells are consistently implanted in the intrapleural cavity prior to treatment. To address the issue, we used IVIS to ensure the establishment of pleural metastasis of lung carcinoma, as well as to monitor the efficacy of the treatments. In preliminary experiments, we attempted to establish pleural metastasis mouse models using the human lung cancer cell lines SBC-3, SBC-5, A549, and H1299 transfected with the firefly luciferase gene. Autopsies revealed that only the A549-Luc cell line consistently established disseminated tumors in all mice. Thus, we used the A549-Luc cell line to inoculate the thoracic cavity of the nude mice used in this study.

In a clinical trial, Shoji et al. performed repeated intrapleural administration of 5-FU and CDDP via a catheter in patients with MPE, and reported a favorable median survival of more than 1 year [20]. However, 5-FU was not active in lung cancer patients. More potent chemotherapeutic agents, such as docetaxel, have been developed in the 1990s. Therefore, we conducted an *in vivo* study to assess the safety and efficacy of intrapleural docetaxel treatment. First, we demonstrated that single or repeated intrapleural injections of docetaxel can be safely performed without inducing significant toxicities, especially acute hyperreaction. Next, we demonstrated a significantly prolonged survival in the docetaxel group as compared to the control animals. In addition, when autopsied, almost no pleural metastasis of lung carcinoma was observed in any mouse treated with docetaxel, whereas all mice in the control group died due to enlarged pleural metastasis. In our experiments, we clearly demonstrated that repeated intrapleural injections of docetaxel are safe and effective in our mouse model.

We repeatedly monitored the efficacy of docetaxel treatment using IVIS. A significant decrease in the average photon-flux count was observed during docetaxel treatment. Luminescence of luciferase requires both ATP and O_2; in addition, luminescence is not seen in necrotic sites. Therefore, photon-flux counts represent the volume of viable tumors. In our study, IVIS revealed a significant increase in the average photon-flux in the control mice on "Day 21," before any mice exhibited significant weight loss or died ($p < 0.01$). These findings indicate that IVIS is superior in speed and provides a more accurate representation of pleural metastasis. In conclusion, we established a pleural metastasis of lung carcinoma model using A549-Luc cells and demonstrated that the intrapleural administration of docetaxel may be a safe and effective treatment modality for MPE. Application of this treatment technique should be examined in clinical trials.

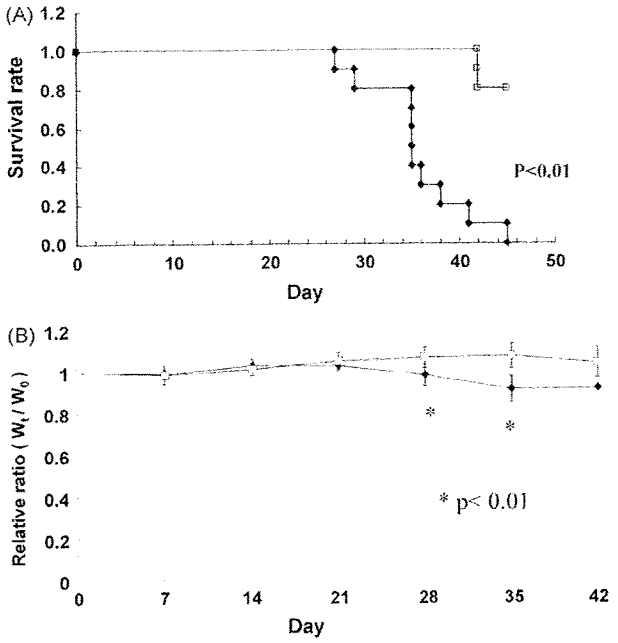

Fig. 5. Survival curves and relative body weight changes of the docetaxel treatment group and the control group are shown in (A) and (B), respectively. Repeated intrapleural docetaxel treatment significantly improved survival without inducing significant toxicities.

Conflict of Interest

None declared.

Acknowledgments

We gratefully acknowledge the contribution of Masaki Nogawa and the helpful comments of Philip C. Mack and Jamie McCall on several points in this paper.

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Bone Marrow Angiotensin AT_1 Receptor Regulates Differentiation of Monocyte Lineage Progenitors From Hematopoietic Stem Cells

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Background—The angiotensin II (Ang II) type 1 (AT_1) receptor is expressed in bone marrow (BM) cells, whereas it remains poorly defined how Ang II regulates differentiation/proliferation of monocyte-lineage cells to exert proatherogenic actions.

Methods and Results—We generated BM chimeric apoE$^{-/-}$ mice repopulated with AT_1-deficient (Agtr1$^{-/-}$) or wild-type (Agtr1$^{+/+}$) BM cells. The atherosclerotic development was significantly reduced in apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice compared with apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice, accompanied by decreased numbers of BM granulocyte/macrophage progenitors (GMP:c-Kit$^+$Sca-1$^-$Lin$^-$CD34$^+$CD16/32$^+$) and peripheral blood monocytes. Macrophage-colony-stimulating factor (M-CSF)–induced differentiation from hematopoietic stem cells (HSCs:c-Kit$^+$Sca-1$^+$Lin$^-$) to promonocytes (CD11b^{high}Ly-6G^{low}) was markedly reduced in HSCs from Agtr1$^{-/-}$ mice. The expression of M-CSF receptor c-Fms was decreased in HSCs/promonocytes from Agtr1$^{-/-}$ mice, accompanied by a marked inhibition in M-CSF–induced phosphorylation of PKC-δ and JAK2. c-Fms expression in HSCs/promonocytes was mainly regulated by TNF-α derived from BM CD45$^-$CD34$^-$ stromal cells, and Ang II specifically regulated the TNF-α synthesis and release from BM stromal cells.

Conclusions—Ang II regulates the expression of c-Fms in HSCs and monocyte-lineage cells through BM stromal cell–derived TNF-α to promote M-CSF–induced differentiation/proliferation of monocyte-lineage cells and contributes to the proatherogenic action. (***Arterioscler Thromb Vasc Biol.* 2009;29:1529-1536.)**

Key Words: bone marrow progenitors ■ angiotensin ■ monocyte ■ atherosclerosis ■ M-CSF

The angiotensin II (Ang II) type 1 (AT_1) receptor exerts proatherogenic actions.[1] AT_1 receptor–deficient (Agtr1$^{-/-}$) mice showed a significant reduction of atherosclerotic development,[2,3] and treatment with AT_1 receptor blocker (ARB) reduced the size of atherosclerotic lesions both in experimental animals and humans.[4] AT_1 receptors are present in a variety of cells, including endothelial cells, vascular smooth muscle cells, and bone marrow (BM) stem cells and progenitors.[5,6] Recently, Cassis et al demonstrated that Ang II–induced atherosclerosis was significantly attenuated in LDL receptor–deficient (LDLr$^{-/-}$) mice whose BM cells were repopulated with Agtr1$^{-/-}$ cells.[7] Fukuda et al also reported that atherosclerotic lesion development was significantly reduced in apoE-deficient (apoE$^{-/-}$) mice with Agtr1$^{-/-}$ marrow.[8] However, no information regarding the role of the BM-AT_1 receptor on the differentiation/proliferation and properties of BM stem cells and progenitors has been reported in these previous studies.

Monocytes and macrophages play a crucial role in the pathogenesis of atherosclerosis, which is characterized by plaque progression, destabilization, and subsequent plaque rupture, through foam cell formation, migration/proliferation of resident vascular smooth muscle cells, and degradation of extracellular matrix.[9] Along with the previous studies showing the effect of diet-induced hypercholesterolemia on BM and leukocyte,[10] Swirski et al reported that hypercholesterolemia induced a surprisingly profound expansion of blood monocytes as well as BM monocyte-lineage cells.[11] However, the relative contribution of the BM renin–angiotensin system to hypercholesterolemia-associated monocytosis has not been fully investigated.[12]

In the present study, we focused on the action of the AT_1 receptor expressed in BM cells and studied whether (1) Ang II affects the differentiation/proliferation from BM stem cells into monocyte-lineage cells, and (2) hypercholesterolemia-associated monocytosis contributes to the development of AT_1-mediated atherosclerosis. Our results demonstrated for the first time that (1) Ang II promotes M-CSF–induced differentiation from hematopoietic stem cells (HSCs; c-Kit$^+$Sca-1$^+$Lin$^-$) into monocyte-lineage cells through up-regulation of the M-CSF receptor c-Fms, and that (2) TNF-α

Received September 28, 2008; revision accepted July 14, 2009.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.187732

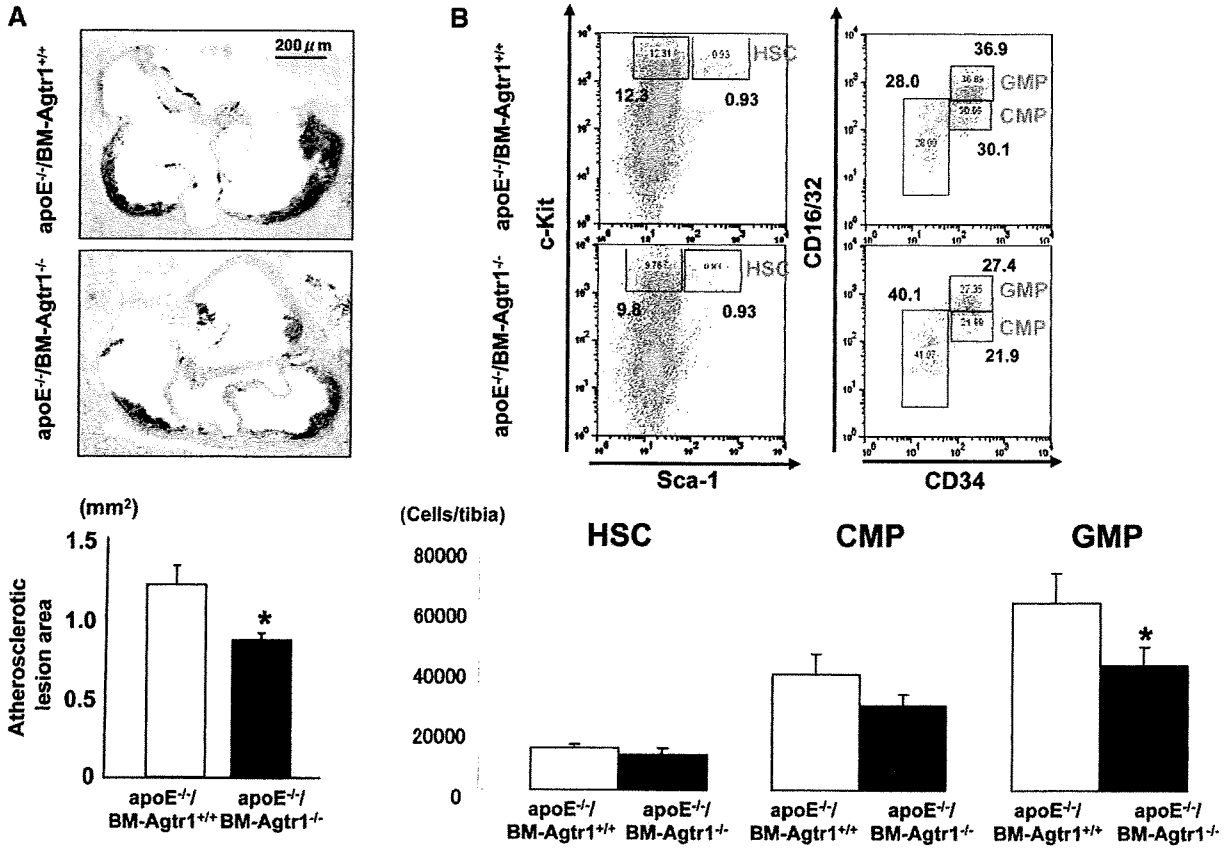

Figure 1. Attenuated atherosclerosis accompanied by the reduction of monocyte-lineage cells on ablation of marrow AT_1. A, Two-month-old apoE$^{-/-}$ mice whose BM was repopulated with Agtr1$^{-/-}$ or Agtr1$^{+/+}$ cells. Quantitative analysis showing the lower area of atherosclerotic lesions in apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice than apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice. Values are the mean±SE for at least 8 mice in each group. *P<0.05 vs apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice. B, Flow cytometry of c-Kit and Sca-1 expression in lineage-negative BM populations and CD16/32 and CD34 expression in c-Kit^{+}Sca-1^{-}lineage-negative BM populations of apoE$^{-/-}$/BM-Agtr1$^{+/+}$ and apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice fed a Western diet for 2 months. Quantitative analysis showing a decrease in GMP number in apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice. HSCs indicates hematopoietic stem cells; CMP, common myeloid progenitors; GMP, granulocyte and macrophage progenitors. Values are the mean±SE for at least 5 mice in each group. *P<0.05 vs apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice.

derived from BM CD45^{-}CD34^{-} stromal cells growth-controlled by Ang II specifically regulates the c-Fms expression in promonocytes (CD11b^{high}Ly-6G^{low}), thus leading to the increased numbers of circulating monocytes that modulate AT_1-mediated proatherogenic activities.

Methods

A full description of all methods can be found in the Data Supplement (available online at http://atvb.ahajournals.org).

Animal Preparation

ApoE$^{-/-}$ mice (C57BL/6) and AT1a receptor-deficient (Agtr1$^{-/-}$) mice (C57BL/6) were obtained from Taconic Co Ltd (Germantown, NY) and Tanabe Seiyaku Co Ltd (Osaka, Japan), respectively. BM cells of 2-month-old female apoE$^{-/-}$ recipient mice were repopulated with male Agtr1$^{-/-}$ or Agtr1$^{+/+}$ cells. The percentage chimerism determined by transplanting GFP-overexpressing BM cells was 96±2% of peripheral blood mononuclear cells.[13] Furthermore, BM CD45^{-}CD34^{-} stromal cells, HSCs, and myeloid progenitors (MP:c-Kit^{+}Sca-1^{-}Lin^{-}) were almost completely (more than 99%) replaced by GFP-positive cells (supplemental Figure I). All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto Prefectural University School of Medicine.

Statistical Analysis

All data are expressed as the mean±SE. Mean values were compared using ANOVA. If a statistical significant effect was found, Fisher test was performed to detect the difference between the groups. P<0.05 was considered statistically significant.

Results

BM-AT_1 Deficiency Attenuates Atherosclerosis Concomitant With the Reduction of BM Monocyte-Lineage Cells

Consistent with the previous reports,[8] apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice showed a significant reduction of atherosclerotic lesions compared with apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice (31%, P<0.05; Figure 1A). At 3 months after BMT, the numbers of white blood cells and monocytes were similar between the 2 groups (supplemental Table I). However, after 2 months of a Western diet feeding, white blood cells and monocytes were significantly less abundant in apoE$^{-/-}$/BM-Agtr1$^{-/-}$ mice than apoE$^{-/-}$/BM-Agtr1$^{+/+}$ mice by 40% and 39%, respectively (P<0.05; supplemental Table II).

HSCs (c-Kit^{+}Sca-1^{+}Lin^{-}) have been shown to differentiate into common myeloid progenitors (CMP:c-Kit^{+}Sca-1^{-}Lin^{-}CD34^{+}CD16/32^{-}) and then granulocyte/macrophage progenitors (GMP:c-Kit^{+}Sca-1^{-}Lin^{-}CD34^{+}CD16/32^{+}), followed by the terminal differentiation into BM promonocytes (CD11b^{high}Ly-6G^{low}).[14,15] We examined BM-AT_1–me-