of the worldwide peak use of asbestos in the 20th century [3–6]. Patients with mesothelioma often display increasing pleural effusion containing clusters of tumor cells, and these cells are thought to metastasize and form space-occupying lesions, resulting in breathing difficulty. As mesothelioma remains a lethal and therapy-resistant disease [3–6], the establishment of an effective therapeutic strategy is essential. Although much of the precise mechanism of the pathogenesis of mesothelioma remains unknown, the involvement of chronic inflammation in the pathogenesis has been proposed. After mesothelial cells and macrophages phagocytose asbestos, these cells produce pro-inflammatory cytokines such as interleukin-8 (IL-8), IL-1α, IL-1, IL-6, and tumor necrosis factor-α (TNF-α) [3, 7, 8]. Although the uptake of asbestos often induces cytotoxicity and results in the apoptosis of affected cells, TNF-α produced by macrophages may rescue mesothelial cells from cell death through a nuclear factor-κB (NF-κB)-dependent pathway [8]. In addition, asbestos is thought to stay long in the respiratory system and sustain the chronic inflammation in microenvironmental tissues including adipose tissue, where adipocytes secrete some adipocytokines promoting pro-inflammatory reaction [9]. These global inflammatory environments have been considered to induce the transformation of mesothelial cells and progression of mesothelioma.

NF-κB is a dimeric transcription factor of the Rel family. Generally, NF-κB exists in an inactive form in the cytoplasm by binding to an intrinsic inhibitor, IκB family proteins [10]. Once IκB is phosphorylated, NF-κB translocates from the cytoplasm to the nucleus, after which, NF-κB binds to specific DNA sequences of target genes and induces cell proliferation and various cell functions [10–13]. In previous studies, constitutive activation of NF-κB was observed in some tumor cells [14–17], suggesting that NF-κB is a potential therapeutic target [18, 19]. We previously reported the substantial involvement of constitutive activation of NF-κB in the neoplastic proliferation of mast cells, breast cancer cells, and canine leukemia cells [20–22]. In breast cancer cells in particular, we demonstrated that NF-κB activation was related to the expression of cyclins D1, D2, and D3, which accelerated the effects of cyclin-dependent kinase (CDK) on cell division concurrent with the phosphorylation of retinoblastoma protein. In addition, NF-κB inhibition with a specific inhibitor of IκB kinase β (IKKβ), IMD-0354, suppressed cell cycle progression in breast cancer cells, leading to apoptotic cell death [20]. Study findings revealed the antitumor potential of an NF-κB inhibitor in cancers in which NF-κB substantially contributes to pathogenesis. Exposure to asbestos induces chronic inflammation that is associated with activation of NF-κB signaling and subsequent production of TNF-α and IL-1 [23]. Crucial roles of NF-κB signaling in both malignant transformations have been identified [24]. However, direct evidence regarding NF-κB in triggering mesothelioma formation has been insufficient in clinical cases.

Given the involvement of NF-κB in asbestos-related inflammation and the subsequent tumorigenic transformation of mesothelial cells, drugs that target NF-κB may become promising candidates for the treatment of mesothelioma. Few reports revealed that NF-κB was activated in mesothelioma cells and the use of proteasome inhibitors or antitumor ribonuclease reagents was effective in controlling tumorigenic proliferation and inducing an apoptotic reaction through the prevention of NF-κB activity [21, 22, 25, 26]. However, these reagents may influence the degradation and synthesis of a wide range of functional proteins. Therefore, the development of effective drugs with fewer side effects is necessary. Although previous reports suggested serious roles of NF-κB activation in mesothelioma, only fragmented information has been provided. Thus, more detailed investigation of NF-κB activation relating to tumor progression might be necessary and significant for the establishment of therapy with NF-κB regulation.

In this study, we detected sustained NF-κB activation in three different pathological types of mesothelioma cells. IMD-0354 effectively prevented the activation of NF-κB and the progression of mesothelioma cells. In addition, IMD-0354 successfully suppressed the expression of D-type cyclins, resulting in cell cycle arrest. NF-κB inhibition by IMD-0354 also downregulated sphere formation in mesothelioma cells. Furthermore, NF-κB inhibition effectively suppressed tumor expansion in two transplantation models of mesothelioma. This study revealed that NF-κB may seriously contribute to tumor formation and progression in mesothelioma and that its inhibition will be an effective therapeutic strategy with variations in the cancer hierarchy.

Materials and Methods

Cell culture

Three different human malignant mesothelioma cell lines, MSTO-211H (mixed tissue type), NCI-H2052 (sarcomatoid tissue type), and NCI-H28 (epithelial tissue type), were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.

Reagents

Rabbit anti-phospho-IκBα antibody, rabbit anti-IκBα antibody, and rabbit anti-β-actin antibody were obtained

from Cell Signaling Technology (Beverly, MA). Rabbit anti-NF-κB p65 antibody, anti-Bcl-2 antibody, and anti-Histon H1 antibody were purchased from Santa Cruz Biotechnology (Lake Placid, NY). Rabbit anti-cyclin D1 antibody, anti-cyclin D2 antibody, anti-cyclin D3 antibody, and anti-cyclin E antibody were purchased from PharMingen (San Diego, CA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). IMD-0354, an IKKβ inhibitor [20–22], was kindly provided by the Institute of Medicinal Molecular Design Inc. (Tokyo, Japan). Pemetrexed and cisplatin were purchased from LKT Laboratories, Inc. (St. Paul, MN).

Western blot analysis

After incubation in a serum deprivation condition for 12 h, the medium was changed to serum-containing medium, and simultaneously, cells were exposed to various concentrations of IMD-0354. In total, 2×10^6 cells were collected after 0, 3, 6, 9, and 12 h of treatment. Cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μL of CelLytic-M reagent supplemented with a protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO). Nuclear and cytoplasmic extraction, Western blot analysis, and the calculation of relative intensities were performed as previously described [20].

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

Cells (1×10^5 cells/mL) were applied to each well of 96-well culture plates and incubated in 100 μL of serum-containing RPMI1640 medium in the presence or absence of various concentrations of IMD-0354, cisplatin (Cis, 0.01 μg/mL), and pemetrexed (Pem, 0.1 μg/mL) for 48 h. Beginning at 4 h before the end of culture, 10 μL of 5 mg/mL MTT dissolved in PBS was added to each well. The reaction was stopped by the addition of 100 μL of 10% sodium dodecyl sulfate in 0.01 mol/L HCl. Absorbance was measured at 577 nm using ImmunoMini NJ-2300 (Nalge Nunc International K.K., Tokyo, Japan). The inhibitory rate of proliferation was calculated by the following formula: Inhibitory rate of proliferation = 1 − (average optimal density [OD] in the treatment group/average OD value in the control group).

Cell cycle analysis

After the incubation of cells (2×10^5 cells/mL) in the presence or absence of IMD-0354 (1.25 μmol/L) or pemetrexed (0.1 μg/mL) for 24 h, cells were collected, washed twice with ice-cold PBS, and fixed in 70% ethanol for 30 min with periodic vortexing. Cells were collected and treated with 0.5 μg/mL RNase A for 20 min at 37°C to avoid nonspecific propidium iodide (PI) binding. DNA was stained by incubating cells in 50 μg/mL PI dissolved in 0.1% sodium citrate for 10 min on ice with periodic vortexing. To remove cell clustering, cells were passed through a nylon mesh. DNA contents were analyzed using a flow cytometer (Coulter, Hialeah, FL).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (TUNEL assay)

Cells (1×10^5 cell/mL) were applied to each well of 8-well Permanox slide (Lab-Tek® chamber slideTM System, Thermo Fisher scientific Inc., NY) and incubated in 200 μL of serum-containing RPMI1640 medium in the presence or absence of IMD-0354 (1.25 μmol/L) and pemetrexed (0.1 μg/mL) for 24 h. After the incubation, the cells were fixed immediately in 4% formaldehyde at room temperature for 1 h. Then, TUNEL assay was performed with DeadEndTM Colormetric TUNEL System (Promega, Madison, WI) according to the manufacturer's instruction. The number of positive-labeled cells stained as dark brown was counted in five randomly selected fields under a microscope (200× magnification) and expressed as percentage of total cells counted (at least 500 cells).

Sphere formation assay

Cells (3×10^5 cells/mL) were seeded in 6-well plates and incubated in serum-free medium with or without various concentrations of IMD-0354 for 3 days. To avoid suppression of NF-κB activity, nutrition including AIM V® medium (Life Technologies, Tokyo, Japan) was used as serum-free medium for sphere formation assay. Next, the number of spheres was counted, and their diameters were measured in five visual fields (400× magnification) chosen at random.

Tumor transplantation and measurement of tumor growth

MSTO-211H cells (2×10^6 cells) were incubated with or without 10 μmol/L IMD-0354 overnight and suspended in 200 μL of PBS. Cells incubated with or without IMD-0354 were subcutaneously injected to the right or left side of the shoulders and hips of eight female BALB/c-*nu/nu* mice (purchased from Charles River Japan, Inc., Yokohama, Japan) at the age of 4 weeks. Tumor size was measured twice in a week. The estimated tumor volume was calculated using the following formula: tumor volume = $[(\text{width})^2 \times \text{length}]/2$.

Mesothelioma orthotopic tumor model

MSTO-211H cells (1×10^6 cells) in 0.2 mL PBS were injected to the thoracic cavity of 15 severe combined immunodeficiency (SCID) mice. One week after inoculation, mice were randomized to one of the following treatment groups: (1) intraperitoneal (i.p.) PBS, daily administration; (2) i.p. IMD-0354, 10 mg/kg daily administration; or (3) i.p. cisplatin, 2 mg/kg daily administration. Mice were sacrificed when they met the established criteria for minimizing pain and suffering. Survival was calculated from the time of inoculation to the date of death. All experiments with animals were performed in compliance with the standards outlined in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Statistical analysis

A two-tailed Student's *t*-test was performed for comparisons of two groups, and one-way analysis of variance or Bonferroni/Dunnett analysis was performed for comparisons of three or more groups. Modified Wilcoxon test was performed for comparison of survival of two groups. $P < 0.05$ was considered as the level of significance.

Results

Constitutive activation of NF-κB in mesothelioma cells was suppressed by treatment with IMD-0354

To examine the activity of NF-κB, IκBα phosphorylation was assessed in three mesothelioma cell lines: MSTO-211H, NCI-H2052, and NCI-H28. Protein expression was examined by Western blot analysis. IκBα phosphorylation was detected in all cell lines, but there were some differences in the expression profile among the cell lines. In MSTO-211H cells, IκBα phosphorylation was not detected under the serum-deprived condition, but it was induced 3 h after serum stimulation. Conversely, NCI-H2052 cells exhibited IκBα phosphorylation during serum deprivation. Moderate IκBα phosphorylation was detected in NCI-H28 cells under the serum-deprived condition. Phosphorylation was

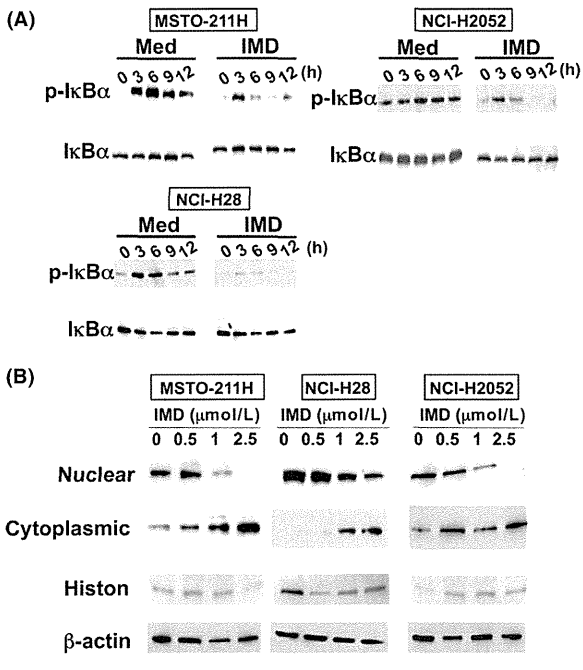


Figure 1. Constitutive activation of NF-κB was suppressed by IKKβ inhibition via a decrease in IκBα phosphorylation. (A) After serum deprivation for 12 h, cells were incubated in flesh medium with 10% fetal bovine serum. At each time point, phosphorylation of IκBα was detected with cells treated with diluent alone (Med) or IMD-0354 (IMD, 2 μmol/L). (B) Cells were incubated with each concentration of IMD-0354 (IMD) for 12 h, and the NF-κB p65 subunit was extracted from the nuclear fraction or cytoplasmic fraction. Representative photos in three individual experiments are shown. NF-κB, nuclear factor-κB.

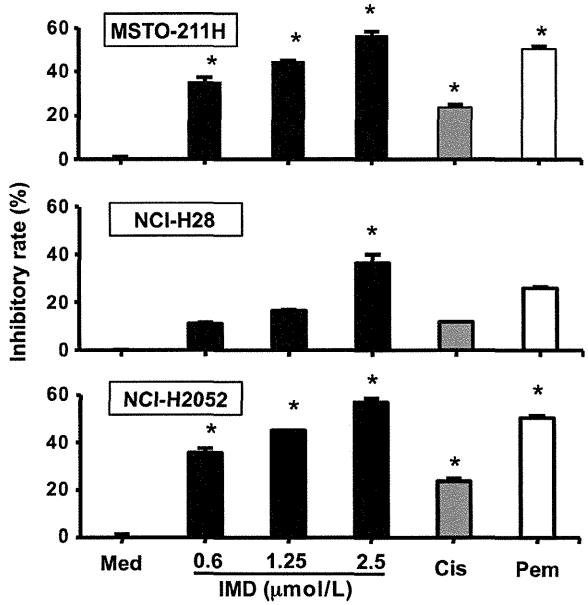


Figure 2. Inhibitory effect of the suppression of NF-κB on the proliferation of mesothelioma cells. MSTO-211H, NCI-H28, and NCI-H2052 cells were incubated in the absense (Med) or the presence of increasing concentrations of IMD-0354 (IMD), cisplatin (Cis, 0.01 μg/mL), and pemetrexed (Pem, 0.1 μg/mL) for 48 h, and the proliferation of cells was determined using the MTT assay. Columns, means of 5–6 different experiments; bars, ±SE. *$P < 0.05$ compared with medium alone. NF-κB, nuclear factor-κB; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

apparently decreased when cells were treated with 2 μM IMD-0354 (Fig. 1A). Next, nuclear translocation of the p65 subunit of NF-κB was assessed. The NF-κB p65 subunit was detected in the nuclear fraction of all untreated cells, but in MSTO-211H and NCI-H2052 cells, the p65 subunit was also present in the cytoplasmic fraction. The nuclear fraction of the NF-κB p65 subunit was decreased in a concentration-dependent manner in all cell lines. In particular, 2.5 μmol/L IMD-0354 completely abolished the nuclear fraction of the NF-κB p65 subunit in MSTO-211H and NCI-H2052 cells (Fig. 1B).

Effect of NF-κB suppression on the proliferation of mesothelioma cells

Next, the effect of NF-κB inhibition on the proliferation of mesothelioma cells was evaluated using an MTT assay after 48 h. As shown in Figure 2, the proliferative activities of both MSTO-211H and NCI-H2052 cells were significantly suppressed by treatment with IMD-0354 as well as chemotherapeutic agents (pemetrexed or cisplatin). Significant suppressive effects on the proliferation of NCI-H28 cells were observed at 2.5 μmol/L IMD-0354. However, no additive effects of IMD-0354 with pemetrexed or cisplatin were identified in any of the cell lines (Fig. S1).

Cell cycle arrest and the downregulation of cell cycle regulatory proteins in mesothelioma cells upon NF-κB inhibition

MTT assay measures cellular activity that reflects proliferation and survival of cells. Therefore, we next checked the effect of NF-κB suppression on cell cycle progression in mesothelioma cells by assessing PI uptake. The cell population at the subG$_1$/G$_1$ phase in all cell lines treated with 1.25 μmol/L IMD-0354 for 24 h was significantly increased (Table 1). In contrast, cells at the S phase were markedly decreased by IMD-0354 treatment in all cell lines (Table 1). Furthermore, to identify whether IMD-0354 induced apoptosis or not, apoptotic cells were detected by TUNEL assay. Unexpectedly,

Table 1. Distribution of cell cycle after treatment.

Cell line	Treatment	Sub G1/G1 (%)	S (%)	G2/M (%)
MST0-211H	Control	39.8 ± 0.7	58.1 ± 0.1	2.1 ± 0.3
	IMD-0354	54.5 ± 0.5*	42.6 ± 0.3*	2.8 ± 0.1
	Pemetrexed	45.4 ± 0.5*	52.5 ± 0.1	2.1 ± 0.5
NCI-H28	Control	65.1 ± 0.6	31.4 ± 0.3	3.6 ± 0.6
	IMD-0354	69.1 ± 0.4*	27.2 ± 0.5*	3.7 ± 0.4
	Pemetrexed	57.2 ± 0.7	38.3 ± 0.6	4.5 ± 0.1
NCI-H2052	Control	49.7 ± 0.5	48.9 ± 0.3	2.8 ± 0.1
experiments	IMD-0354	55.4 ± 0.9*	42.8 ± 0.6*	6.3 ± 0.3
	Pemetrexed	48.7 ± 0.3	48.9 ± 0.3	2.4 ± 0.6

Mean ± SE of three individual experiments are shown.
*$P < 0.05$, when compared to control using a Bonferroni/Dunnett comparison.

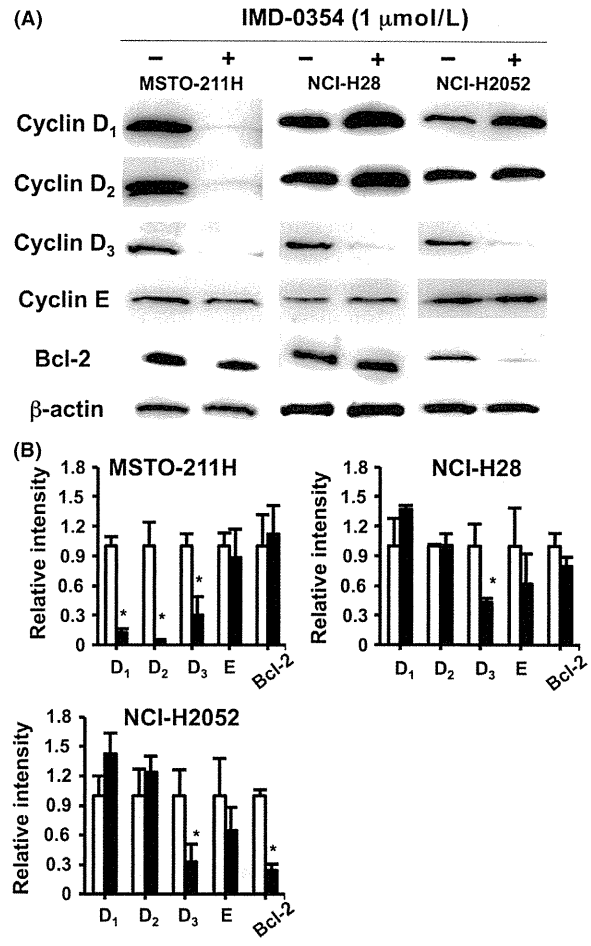


Figure 3. Downregulation of cell cycle regulatory and antiapoptotic proteins in mesothelioma cells upon NF-κB inhibition. (A) The expression of cyclins D1, D2, and D3 was suppressed in MSTO-211H cells and that of cyclin D3 was decreased in NCI-H28 and NCI-H2052 cells by treatment with IMD-0354 (1 μmol/L) for 24 h. In NCI-H2052 cells, Bcl-2 expression was suppressed by IMD-0354 treatment (representative of three different experiments). (B) The relative intensity of each factor was normalized to the protein expression of β-actin as an endogenous control. The intensity in the absence of IMD-0354 was scored as 1. D1, cyclin D1; D2, cyclin D2; D3, cyclin D3; E, cyclin E. Columns, means of five independent experiments; bars, ±SE. *$P < 0.05$ compared with medium alone. NF-κB, nuclear factor-κB.

percentages of TUNEL-positive cells were not increased markedly by treatment with IMD-0354 within 24 h (Fig. S2).

The expression of cyclins and their contribution to cell proliferation were previously demonstrated in various cell types [21, 27]. Since NF-κB inhibition resulted in the increase in cell numbers at the subG$_1$/G$_1$ phase, we examined the expression of cyclins related to the S phase entry in these mesothelioma cell lines. As shown in Figure 3A, cyclins D1, D2, D3, and E were detected in all mesothelioma cell lines, and cyclin D3 expression was significantly decreased by NF-κB suppression in all cell lines. Cyclins D1 and D2 were also significantly downregulated in MSTO-211H cells upon NF-κB inhibition. In contrast, in NCI-H2052 cells, cyclin D1 and D2 expression was increased by NF-κB suppression. Similarly, cyclin D1 was upregulated in NCI-H28 cells upon NF-κB inhibition. At the same time, we assessed the expression of the antiapoptotic protein Bcl-2 in these samples. The expression of Bcl-2 was significantly decreased upon NF-κB inhibition only in NCI-H2052 (Fig. 3).

Sphere formation was inhibited upon NF-κB suppression

Recently, the tumor sphere-culture system has been discussed because of its microenvironmental characteristics that are disadvantageous for the survival and proliferation of cancer cells [28]. In addition, patients with mesothelioma often exhibit pleural effusion containing clusters of tumor cells in sphere-like clusters, and these clusters are believed to relate to metastasis to the thoracic cavity in patients with mesothelioma [1]. Therefore, we examined the capability of three mesothelioma cell lines to form spheres under serum deprivation. Moreover, we assessed the influence of NF-κB inhibition on sphere formation, especially in MSTO-211H cells, which were most susceptible to NF-κB inhibition. As shown in Figure 4A, only MSTO-211H cells could form spheres and survive under serum deprivation. Therefore, we assessed the influence of NF-κB suppression on sphere formation by measuring the diameter and number of spheres. Sphere formation was suppressed by NF-κB inhibition in an IMD-0354 concentration-dependent manner (Fig. 4A and B).

Tumor growth was suppressed by NF-κB inhibition

Next, we examined the influence of NF-κB inhibition on tumor growth by in vivo experiments using eight BALB/c nude mice. We used MSTO-211H cells because of their susceptibility to NF-κB suppression as shown in our experiments. In the same mice, we observed different rates of tumor growth between the right and left sides (Fig. 5A). The growth of tumors derived from cells

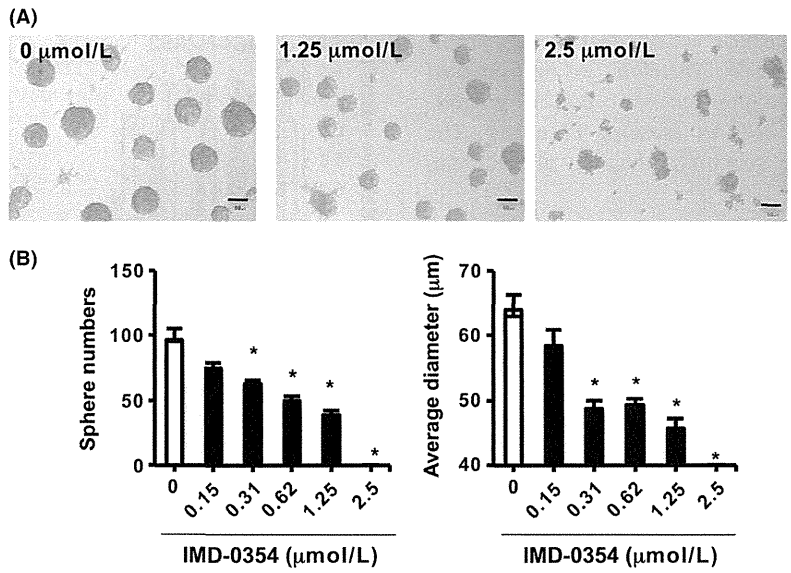
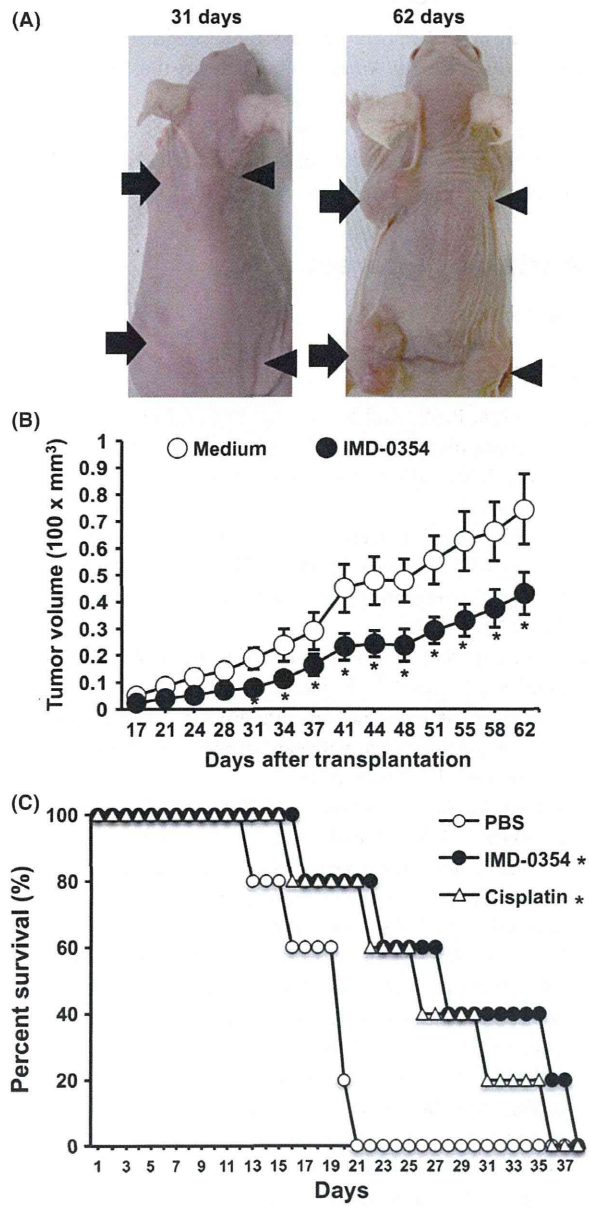


Figure 4. Inhibitiory effect of NF-κB suppression on sphere formation in mesothelioma cells. (A) MSTO-211H cells could form spheres in a serum-deprivation condition. Numbers and diameter of spheres were reduced by treatment of IMD-0354. The bar in the photo indicates 50 μm. Representative photos of 5–6 individual experiments were provided. (B) NF-κB suppression inhibited the growth and survival of sphere-forming cells. Columns, means of 5–6 different experiments; bars, ±SE. *$P < 0.05$ compared with medium alone. NF-κB, nuclear factor-κB.

treated overnight with 10 μmol/L IMD-0354 was suppressed compared to the findings in the absence of treatment (Fig. 5B). In addition, we injected MSTO-211H cells into the thoracic cavity of SCID mice to examine the effects of IMD-0354 treatment on tumor growth. Mice treated daily with IMD-0354 and cisplatin survived significantly longer than PBS-treated mice (Fig. 5C). On the other hand, no significant difference in survival was observed between the IMD-0354 and cisplatin treatment groups.



Discussion

In this study, we revealed for the first time that NF-κB was constitutively activated in three human mesothelioma cell lines, and this activation was related to the proliferation and sphere formation of mesothelioma cells and tumor development in vivo. NF-κB is already known to have several important cell functions in various cell types [20–22]. However, in mesothelioma cells, the true roles of NF-κB and the underlying factors are not fully understood. The biggest issues faced by patients with mesothelioma are the symptoms of lethal cachexia and difficulty in breathing caused by aggressive tumor progression and the subsequent development of space-occupying lesions in the thoracic cavity [2, 3]. Therefore, the control of proliferative and metastatic functions is especially important in mesothelioma cells.

We have presented the different profiles of IκBα phosphorylation following serum stimulation in each cell line. However, when NF-κB was suppressed by IMD-0354 treatment, cell cycle in these cells was equally suppressed, accompanied by inhibition of the S phase entry. These results suggest that IMD-0354 exhibited suppressive effects on the tumorigenic proliferation of mesothelioma cells, regardless of these different pathologic characters, by blocking cell cycle. NF-κB inhibition rarely involved in strong induction of apoptosis of mesothelioma cells might reveal clinical benefit of the inhibitor in terms of avoidance of serious adverse effects [20].

Figure 5. Inhibitory effects of NF-κB inhibition on the in vivo progression of mesothelioma. (A) After incubating MSTO-211H cells with or without IMD-0354 (1 μmol/L) overnight, cells were subcutaneously injected into female BALB/c-*nu/nu* mice as described in the Materials and Methods section. Cells incubated with IMD-0354 were injected into two sites in the right side (arrowheads), and cells incubated without IMD-0354 were injected into two sites in the left side (arrow) of mice. Left, 31 days after injection; right, 62 days after injection. (B) The growth of tumors derived from IMD-0354-treated cells was delayed compared to the findings in the absence of treatment. Symbols, means of 16 sites in eight mice; bars, ±SE. $*P < 0.05$ compared with mice transplanted cells that were incubated without IMD-0354. (C) Therapeutic potency of NF-κB inhibition in an orthotopic model of mesothelioma. MSTO-211H cells were injected into the thoracic cavity of 15 mice. One week after inoculation, mice were treated with PBS (five mice), 10 mg/kg IMD-0354 (five mice), or 2 mg/kg cisplatin (five mice) daily. Survival was calculated from the time of inoculation to death. Modified Wilcoxon test was performed for comparison of survival of each group, and statistical significances were obtained between the IMD-0354-treated group and the PBS-treated group, as well as between the Cisplatin-treated group and the PBS-treated group. No statistical significance was identified between the IMD-0354 treated group and the Cisplatin-treated group. $*P < 0.05$ compared with mice treated PBS alone. PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB.

Previous studies that assessed the relationships between tumorigenic proliferation and cell cycle regulation in mesothelioma cells only focused on cyclin D1 [29–31]. In this study, we showed that NF-κB suppression decreased the expression of cyclins D1, D2, and D3 in MSTO-211H cells and also suppressed the expression of cyclin D3 in NCI-H28 and NCI-H2052 cells. This remarkable downregulation of cyclin D1, D2, and D3 expression in MSTO-211H cells may be associated with the inhibitory effect of NF-κB suppression on cell proliferation and cell cycle progression. Conversely, cyclin D1 and D2 expression in NCI-H2052 cells and cyclin D1 expression in NCI-H28 cells were not suppressed, indicating that a compensatory reaction exists for cyclin D3 suppression. As the cell cycles of both NCI-H2052 and NCI-H28 cells were arrested, NF-κB may regulate cell proliferation and the cell cycle primarily through cyclin D3. The expression of Bcl-2 was decreased by NF-κB suppression in NCI-H2052 cells of 3 cell lines used. NF-κB may also involve in an antiapoptotic reaction, as observed in other cancer cells such as breast cancer cells, in a certain type of mesotheliomas [20].

We performed sphere-culture experiments under serum deprivation as a model for tumor cell clusters in pleural effusions. MSTO-211H cells formed spheres in the serum-deprived condition, and NF-κB suppression inhibited sphere growth. Previous study showed that NF-κB pathway affected cell functions relating metabolic adaptation and cell transformation [32, 33]. Therefore, NF-κB may have roles in the formation and maintenance of spheres in nutrient-starved circumstances, such as the tumor relating microenvironment of the thoracic cavity. In addition, NF-κB activation may protect mesothelioma cells under disadvantageous conditions and induce the first step of pleural dissemination.

Finally, we evaluated the suppressive effects of IMD-0354 on the in vivo progression of mesothelioma using two xenograft models. In the subcutaneous implanted model, the pretreatment of cells with an NF-κB inhibitor delayed tumor growth. As mesothelioma grows in the thoracic cavity, we injected MSTO-211H cells into the thoracic cavity of SCID mice as an orthotopic model for therapeutic evaluation. IMD-0354 treatment significantly extended the survival of mice compared to PBS injection. In addition, IMD-0354 displayed similar efficacy as cisplatin, a major chemotherapeutic agent for mesothelioma. These in vivo experiments showed that IMD-0354 is a potential therapeutic agent for mesothelioma. According to the result obtained in in vitro experiments, no additive effect on combination of IMD-0354 with cisplatin was shown.

We confirmed that IMD-0354 was not associated with any serious side effects and toxicity in a previous study [20]. Direct contribution of NF-κB to tumorigenesis of mesothelial cells has been undefined in clinical cases; however, asbestos-induced chronic inflammation in which NF-κB plays a crucial role has been implicated as the main cause of malignant transformation of mesothelial cells. Therefore, the NF-κB pathway must be an important molecular target in the progression of mesothelioma, and IMD-0354 has a potential role in anti-mesothelioma therapy as well as prevention of mesothelioma formation in patients seriously exposed to asbestos.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inhibitory effect of IMD-0354 combined with other chemotherapeutic reagents on the proliferation of mesothelioma cells. MSTO-211H cells, NCI-H28 cells, and NCI-H2052 cells were incubated with IMD-0354 (IMD, 0.3 μmol/L), cisplatin (Cis, 0.01 μg/mL), and pemetrexed (Pem, 0.1 μg/mL) as indicated for 48 h, and the proliferation of cells were determined by MTT assay. Columns, means of five to six different experiments; bars, mean ± SE.

Figure S2. Effect of IMD-0354 on the apoptosis of mesothelioma cells. MSTO-211H, NCI-H28, and NCI-H2052 cells were incubated in the absence (Med) or presence of IMD-0354 (IMD, 1.25 μmol/L) and pemetrexed (Pem, 0.1 μg/mL) for 24 h. Apoptosis was detected by the TUNEL assay as described in Materials and Methods. Numerals in each photo represents mean ± SE of TUNEL-positive cells in each cell line of five different experiments. $*P < 0.05$ compared with cells treated diluent alone.

Figure S3. Photos of other three of eight mice subjected to the expariment performed in Figure 5. Cells incubated with IMD-0354 were injected into two sites in the right side (arrowheads), and cells incubated without IMD-0354 were injected into two sites in the left side (arrows) of mice. Upper, 31 days after injection; lower, 62 days after injection.

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Production of stem cell factor in canine mast cell tumors

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ABSTRACT

Mast cell tumor (MCT) is the most common cutaneous tumor in dogs. We recently revealed that production of stem cell factor (SCF) contributes to the proliferation of neoplastic mast cells in an autocrine/paracrine manner. The aim of the present study was to determine the contribution of the mechanism in clinical MCTs. In consequence, high SCF expression (>10 times compared to HRMC cells) was observed in 5 of 7 MCT samples used in the study regardless of KIT mutation, which was confirmed in immunohistochemical analysis. In addition, production of SCF was observed in Ki-67-positive cells in the MCT xenograft. These results indicate the broad contribution of SCF autocrine/paracrine mechanism on clinical MCTs, providing the rationale for the clinical use of KIT inhibitors regardless of KIT mutation.

Mast cell tumors (MCTs), characterized by the aberrant expansion of neoplastic mast cells, account for approximately 20% of all cutaneous tumors in dogs (Withrow et al., 2013). MCTs, especially high-grade ones, often relapse or behave in an aggressive manner, metastasizing to local lymph nodes, the liver, and the spleen (Withrow et al., 2013). In the first decade of the 21st century, two tyrosine kinase inhibitors, toceranib phosphate and masitinib mesylate, were approved by the Food and Drug Administration and the European Medicines Agency for the treatment of canine MCTs (London et al., 2009; Hahn et al., 2010). In clinical trials, each agent achieved clinical responses in approximately 50% of the MCT patients, including those with MCTs that expressed wild-type KIT. Because both agents exert their activity by abrogating adenosine 5′-triphosphate (ATP) binding to the ATP-binding pocket of KIT, ATP-dependent KIT activation should be necessary in tumors that are sensitive to the agents. However, KIT mutations have been reported in approximately 20% of MCTs, including those in the clinical trials of the two agents (London et al., 2009; Hahn et al., 2010). Thus, there must be mutation-independent KIT activation in at least some MCTs.

SCF is a cytokine that bind the KIT and promotes mast cell migration, proliferation and survival (Tsai et al., 1991). Recently, we discovered an autocrine/paracrine mechanism of stem cell factor (SCF) as a novel mechanism of mast cell tumorigenesis (Amagai et al., 2013a). If the mechanism exists in clinical MCTs, it will explain the clinical benefits of KIT inhibitors in KIT mutation-negative MCTs. However, no studies have examined SCF expression in clinical MCTs. Thus, we examined SCF expression in clinical MCTs to determine the impact of the mechanism on mast cell tumorigenesis in clinical settings. All experiments using clinical cases complied with the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

First, real-time RT-PCR analysis was carried out to detect SCF expression in each sample. Tissue samples were obtained in the Animal Medical Center of Tokyo University of Agriculture & Technology. They were diagnosed as MCTs and pathologically classified according to the Patnaik grading (Fig. 1A). From these, complementary DNA (cDNA) was obtained and SCF was quantified as described previously (Amagai et al., 2013b). Because mast cells contain large amounts of proteases such as tryptase (Pejler et al., 2007), some samples were too damaged for proper extraction of RNA (cycle threshold [*Ct*] values of β-actin > 25). Therefore, damaged samples were excluded from the analysis. Based on our observation that SCF expression level was extremely higher in HRMC mast cell line than the ones in other mast cell lines (Amagai et al., 2013a), we compare the SCF expression level in all the samples with the one in HRMC cells.

Interestingly, the expression of SCF in clinical samples was much higher than that in HRMC cells (Fig. 1B). Compared to HRMC cells, more than 10 times higher SCF expression was observed 5 in 7 clinical samples. According to the sequence analysis of the c-*kit* gene, MCT-6 and MCT-132 expressed internal tandem duplica-

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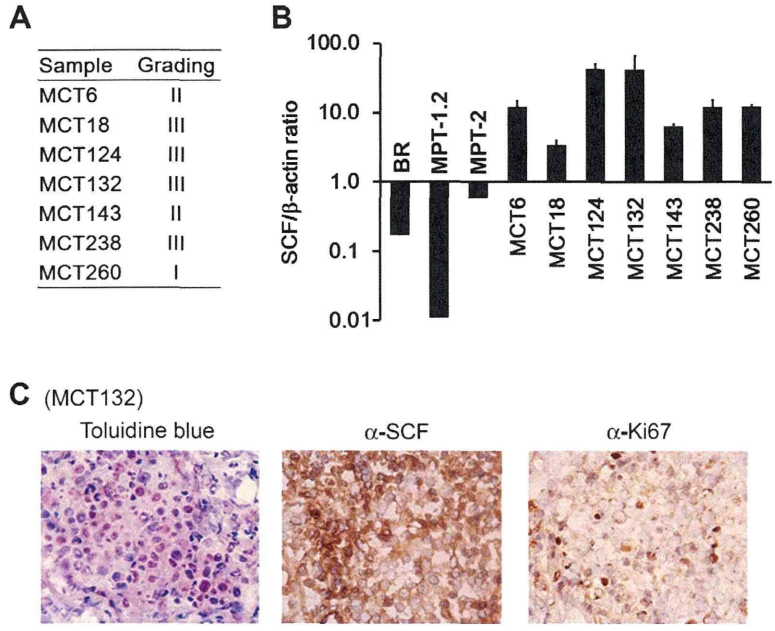

Fig. 1. SCF expression in clinical MCT tissues. (A) Patnaik grading of each clinical samples. (B) SCF mRNA expression in each clinical tissue. Real-time RT-PCR was conducted 3 times. The expression levels (based on the relative *Ct* values of SCF and GAPDH) ± SD were calculated. In the graph, the expression of SCF in each sample is shown relative to that in HRMC cells. BR, MPT-1.2, and MPT-2 cells are all mast cell lines. Others are the clinical MCT samples. (C) Representative data from immunohistochemical analyses (MCT132). Samples were stained with toluidine blue, anti-SCF (α-SCF), and anti-Ki67 (α-Ki67). Original magnification of all tissues was 200×.

tions, which were 1727-1783 ITD and 1769-1827 ITD with 1 base insertion respectively (the numbers correspond to the GenBank Accession No. AF044249), but not in other MCTs (Amagai et al., 2013b). SCF expression was seen in both mutation-positive and -negative tumors. In contrast, SCF expression in other mast cell lines, BR, MPT-1.2, and MPT-2 cells (Amagai et al., 2013c) were much lower than the ones in HRMC cells and the clinical samples (Fig. 1B). Next, SCF expression at the protein level was confirmed with paraffin section immunohistochemistry, using anti-SCF antibody (polyclonal, Abcam, Cambridge, MA) as a primary antibody (1:1000 dilution) and the methods described previously (Okamoto et al., 2011). To analyze the cells in the growth phase, we also conducted paraffin section immunohistochemistry using Ki-67 antibody (clone SP6, Abcam), a marker of cell proliferation, as a primary antibody (1:2000 dilution). As shown in Fig. 1C, the tumor tissue was SCF-positive, and part of the tumor was also positive for Ki-67. Similar staining patterns were obtained in all other samples examined. It suggests that SCF production contributes significantly to tumor growth.

We maintain an *in vivo*, wild-type KIT-expressing MCT xenograft (MCT-TP) derived from the MCT of a 12-year-old female pointer, which has been passaged in BALB/c-*nu/nu* mice more than 20 times. To validate the results in Fig. 1, immunohistochemical analysis was carried out using MCT-TP tissue. Part of the tumor, especially cells in the peripheral region, was positive for SCF and Ki-67, though most Ki-67-negative tumor cells and murine stromal cells did not express SCF (Fig. 2A). Using the MCT-TP isolated from BALB/c-*nu/nu* mice, flow cytometry analysis was also conducted to detect the SCF expression in both the tumor and stromal cells. The tissue was digested with Hank's balanced salt solution containing FBS, collagenase, and DNase I. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI), anti-mouse $H2K_d$-FITC, and anti-KIT-APC antibody, then fixed, permeabilized, and stained with anti-SCF antibody and anti-rabbit-Alexa594 antibody (Life Technologies). Main live cell population was selected based on the incorporation of DAPI with forward and side scatters (FSC/SSC, Fig. 2B). Then either $H2K_d$- or KIT-positive cells were gated, which represent murine stromal cells and MCT-TP-derived tumor cells, respectively (Fig. 2B). Approximately 40% of MCT-TP cells expressed SCF, whereas murine stromal cells were negative for SCF (Fig. 2B).

These results indicate that tumor cells express SCF especially in the growth phase. It also provides a rationale for administering KIT inhibitors to patients with KIT mutation-negative MCTs. Because the number of cases we studied was limited, further investigation must take place to support the findings. In addition, further investigation will be necessary to determine the mechanism of tumor resistance toward these agents, which has been observed in about 50% of MCTs (London et al., 2009; Hahn et al., 2010). In cases when the growth of tumor cells is independent of SCF, unknown mutations or autocrine/paracrine actions of other cytokines, such as interleukin 3 (IL-3) or IL-4 (Ihle et al., 1983), may be necessary for the growth or survival of mast cells. Alternatively, the concentration of KIT inhibitors at the tumor site may be low. In fact, wild-type KIT is reportedly less sensitive to agents than mutant KIT (Ma et al., 2002). Moreover, the overexpression of KIT protein may compensate for the inhibitory effect of the agents, as reported in other tyrosine kinases such as HER2 (Dowsett, 2001).

Although there are many single mutations that drive the aberrant expansion of tumor cells, SCF expression is unlikely to be regulated by a single mutation, considering the strong correlation with the cell cycle (Fig. 2). Because the blood supplies most nutrition required for tumor growth, some humoral factors, such as cytokines or chemokines, may trigger SCF production by tumor cells. It should be noted that SCF expression in mast cell lines used was much lower than the clinical samples (Fig. 1B). For this, two possibilities can be assumed; each cell line lost its ability to produce SCF production in the process of the establishment, or was originally derived from SCF production-free/low tumor cells, because original tumors might be heterogenic in terms of SCF

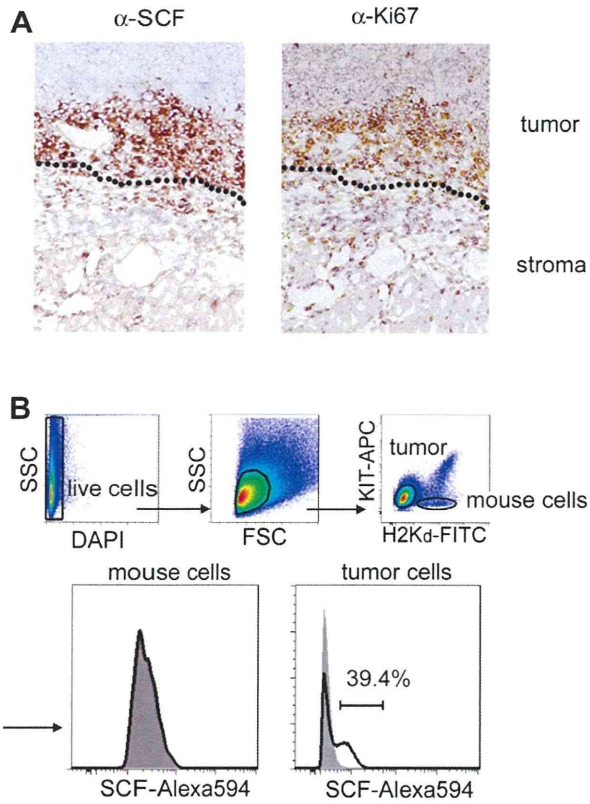

Fig. 2. SCF production in the MCT xenograft. (A) Representative data from immunohistochemical analyses. Samples were stained with anti-SCF (α-SCF) and anti-Ki67 (α-Ki67). The dotted line indicates the border between tumor and stroma. Original magnification of all tissues was 200×. (B) Flow cytometry analysis of SCF protein production. Plots in the upper row are density plots. Main live cell population was selected based on DAPI incorporation with FSC and SSC, then either $H2K_d$- or KIT- positive cells were gated. Both $H2K_d$ and KIT double positive cells were not gated because they are considered to be the doublet formed by dog and mouse cells according to their density. Histograms in the lower row indicate the expression level of SCF in each fraction. Around 40% of tumor cells were positive for SCF, though mouse cells were negative. Gray area, 2nd Ab control; black lines, anti-canine SCF antibody and 2nd Ab staining.

production (Fig. 1C, 2). In either case, the tumor microenvironment must be taken into account to understand the role of SCF production in clinical MCTs.

In conclusion, the present study indicates the involvement of the SCF autocrine/paracrine mechanism in the survival and/or expansion of canine MCTs.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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CASE REPORT

Heterogeneity of internal tandem duplications in the c-*kit* of dogs with multiple mast cell tumours

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Mast cell tumours are one of the most common neoplasms in dogs. Mutations in the proto-oncogene c-*kit*, especially internal tandem duplications of exon 11, are considered to play a crucial role in mast cell tumourigenesis. In this report, two cases that suffered from multiple mast cell tumours containing an internal tandem duplication in the primary lesion but not in the secondary lesions are described. This finding indicates the existence of heterogenous c-*kit* gene mutations in each site of multiple mast cell tumours. Additionally, these results raise the possibility that the contribution of internal tandem duplications in the malignant transformation of mast cells is quite limited. It is proposed that, for clinicians, genetic analysis of several regions of multiple mast cell tumours is necessary for predicting prognosis and tumour response to KIT inhibitors.

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INTRODUCTION

Canine mast cell tumours (MCTs) account for 16 to 21% of all cutaneous tumours (Welle *et al.* 2008). They often relapse or behave in an aggressive manner, metastasizing to local lymph nodes, liver and spleen (Welle *et al.* 2008). KIT tyrosine kinase, which is a receptor for stem cell factor encoded by the proto-oncogene c-*kit*, is necessary for the growth and differentiation of mast cells (Tsai *et al.* 1991, Irani *et al.* 1992). Mutations in the c-*kit* gene, especially point mutations in either exon 11 or exon 17, have been reported to cause neoplastic proliferation of mast cells in humans and rodents (Furitsu *et al.* 1993, Tsujimura *et al.* 1994). As internal tandem duplications (ITDs) in exons 11 and 12 of the c-*kit* gene, which encodes the juxtamembrane domain of KIT, have been identified in approximately 15% of MCTs, the involvement of ITDs has been given serious consideration in the tumourigenesis of canine mast cells (London *et al.* 1999, Zemke *et al.* 2002, Letard *et al.* 2008). ITD mutations have also been shown to induce KIT autophosphorylation and cytokine-independent growth of Ba/F3 cells in the absence of stem cell factor (Letard *et al.* 2008). ITDs were often found in MCTs classified as grade II or III using the Patnaik grading (Patnaik *et al.* 1984); therefore, ITDs have been regarded as highly relevant to the tumourigenesis and the progression of malignant MCTs. To confirm the previous reports, ITDs in 83 MCT specimens obtained from dogs presented to the Animal Medical Center at Tokyo University of Agriculture and Technology were detected. Amongst them, two cases that expressed heterogenous c-*kit* gene mutations in each site of multiple MCTs were identified.

CASE HISTORIES

All dogs within the study were referred to the Animal Medical Center at Tokyo University of Agriculture and Technology for MCTs. After appropriate surgical removal or fine needle aspiration, polymerase chain reaction (PCR) analysis was carried out on samples obtained from MCTs to detect KIT ITDs in 56 dogs with a solitary MCT and 12 dogs with multiple MCTs (Table 1), for evaluation of the sensitivity to chemotherapeutic treatment

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Table 1. Tumour grading of the analysed specimens

Breed	Grading													
	Solitary tumour							Multiple tumour						
	I	I-II	II	II-III	III	N.D.	Total	I	I-II	II	II-III	III	N.D.	Total
American cocker spaniel			1	1			2				1	1 (1)		2
Boxer		1					1							0
Bulldog			1 (1)				1							0
Flat-coated retriever				1			1					1 (1)		1
Golden retriever						2	2					1		1
Jack Russel terrier	1			1			2							0
Labrador retriever		2	8	1	1 (1)		12					1		1
Maltese			1				1							0
Miniature dachshund						1	1			1				1
Pug						1	1					1		1
Pyrenean mountain dog			1				1							0
Setter			1				1							0
Shetland sheepdog			1				1							0
Shiba Inu		2	2 (1)				2			1				1
Shih-tzu							0		1					1
Mongrel/unknown	1	2	7	3	6	6	25			1		2 (2)		3
Total	2	7	23	7	7	10	56	0	1	3	1	7	0	12

MCT Mast cell tumour, ITD Internal tandem duplication
Actual number of MCT cases analysed is indicated. Each tissue was pathologically classified by Patnaik grading. Figures in parentheses are the number of cases in which ITDs were detected

with a small-molecule tyrosine kinase inhibitor. Tissue samples were pathologically classified according to the Patnaik grading (Table 1). All experiments using clinical cases complied with the standards specified in the guidelines of the University Animal Care and Use Committee and performed with written informed owner consent. From these samples, genomic DNA was extracted using the Maxwell® 16 kit (Promega) and the sequence of c-*kit* exon 11 and intron 11 was amplified using Platinum Taq DNA polymerase (Invitrogen) and the following primer sets: forward (5′-CCC ATG TAT GAA GTA CAG TGG AAG-3′) and reverse (5′-GTT CCC TAA AGT CAT TGT TAC ACG-3′). Subsequently, PCR products were amplified using BigDye® Terminator ver.3.1 (Life Technologies) and sequenced using an ABI3100 (Life Technologies). In dogs with multiple MCTs, PCR analysis was performed on each lesion. In cases where more than two bands were amplified, the larger variant (>190 bp) was cut from the agarose gel and sequenced.

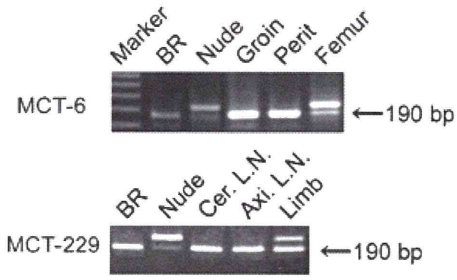
Amongst dogs with multiple MCTs, there were two cases in which ITDs were detected. The first case was a 12-year-old male crossbreed dog with MCTs in the femur, inguinal lymph node and peritoneal cavity (MCT-6). The second case was a 14-year-old female crossbreed dog with tumours in the forelimb, cervical lymph node and axillary lymph node (MCT-229). The primary lesions were located in the femur and forelimb. Tyrosine kinase inhibitors had not been administered to either dog but the first case was treated with prednisone as a neoadjuvant/adjuvant chemotherapy. Interestingly, ITDs were only identified in the primary lesions but not in the metastatic lesions (Fig 1A). Sequencing data of each sample obtained from the two cases are shown in Fig 1B. The underlined sequence and capital letters corresponded to the tandem repeat in each specimen.

Besides the specimens from multiple MCT cases, the analysis was conducted with 56 specimens from solitary MCT cases. Although ITDs were observed in five cases, no identical sequence was detected amongst these (Fig 1C). The absolute ITDs/case proportion was 8·4% in total (7 in 83 specimens). With regard to grading, ITDs were observed in MCTs with high pathological grades (grades II and III).

DISCUSSION

To the authors' knowledge, this is the first report to identify heterogeneity in the existence of ITD in KIT of multiple MCT cases. On the basis of the observation in these two cases, subclones with different KIT phenotypes or non-clonogenic tumour cell expansions in the neoplastic mast cells of one dog are possible. To date, the neoplastic growth of canine mast cells has been thought to result from either KIT mutations in the juxtamembrane or extracellular domain (Letard *et al.* 2008), production of growth factors, including autocrine/paracrine mechanism of stem cell factor (Amagai *et al.* 2013) or other unknown mechanisms. Compared with other gain-of-function mutations such as point mutations, which have been reported in aggressive mastocytosis (Longley *et al.* 1999), ITDs are unlikely to develop via a DNA replication error. In humans, ITDs are frequently observed in the Flt3 tyrosine kinase gene in patients with haematological disorders, such as acute myeloid leukaemia (AML) (Meshinchi *et al.* 2001, Shin *et al.* 2002, Gale *et al.* 2008). Indeed, Flt3 ITDs have been detected in 15 to 25% of AML patients (Meshinchi *et al.* 2001, Shin *et al.* 2002, Gale *et al.* 2008). Interestingly, this is similar to the proportion of KIT ITDs detected in canine MCTs. In addition, Shin *et al.* (2002) reported that approximately half of affected patients acquired ITDs during relapse. Assuming that the functional role of ITDs correlates between human Flt3 and canine KIT, ITDs could potentially contribute to malignancy; however, the formation of the tumour or tumour metastasis may not depend on ITDs. The concept that ITDs contribute to

A



B

MCT-6 Femur

1721 1731 1741 1751 1761 1771
aacaattatg tttacataga cccaacacag cttccttacg atcacaaatg ggagtttccc
1781
agaTATGTTT ACATAGACCC AACACAGCTT CCTTACGATC
ACAAATGGGA GTTTCCCAGA aacaggc
1790

MCT-229 Forelimb

1721 1731 1741 1751 1761 1771
aacaattatg tttacataga cccaacacag cttccttacg atcacaaatg ggagtttccG
ACCCAACACA GCTTCCTTAC GATCACAAAT GGGAGTTTCCc
1781 1790
cagaaacagg c

MCT-6, 229 Other sites of tumor (wild-type)

1721 1731 1741 1751 1761 1771
aacaattatg tttacataga cccaacacag cttccttacg atcacaaatg ggagtttccc
1781 1790
agaaacaggc

C

Specimens	Mutations	Patnaik grading
MCT-0	1768-1807 ITD, ins 17 bp	II
MCT-6	1727-1783 ITD	II
MCT-13	1733-1789 ITD	III
MCT-132	1769-1827 ITD, ins 1 bp	III
MCT-205	1740-1795 ITD	III
MCT-221	1749-1790 ITD	II
MCT-229	1738-1779 ITD	III

FIG 1. Screening analysis of c-*kit* internal tandem duplications (ITDs). Analyses of three different parts of mast cell tumour (MCT) from the same dog (A). Polymerase chain reaction (PCR) for exon 11 and intron 11 was performed on the genomic DNA derived from 83 canine MCT specimens. They were analysed using 1·5% agarose gel electrophoresis. BR cells (Caughey *et al*. 1988) expressed point mutations in c-*kit*, although the expected length of the PCR products was identical to the one obtained from wild-type c-*kit* gene. "Nude" is derived from the MCT possessing ITD mutation in the juxtamembrane domain, which was subcultured in BALB/c Slc-*nu/nu* mice (Kobie *et al*. 2007). ITDs were found only in one site of each specimen. Perit, peritoneal cavity; Cer L.N., superficial cervical lymph node; Axi L.N., axillary lymph nodes; Limb, forelimb. Sequence of KIT ITDs in MCT-6 and MCT-229 (B). PCR products were sequenced and the summary of mutations was indicated. The figure shows the base number of c-*kit* mRNA corresponding to the reference of GenBank accession no. AF044249. The risk of nucleotide misincorporation because of the lack of proofreading activity in Taq polymerase (Tindall & Kunkel 1988, Lawyer *et al*. 1993) was compensated by repeating the analysis in each sample thrice. The underlined sequence and the one written in capital letters correspond to the tandem repeat in each specimen. Sequences of ITDs (C). The figure indicates the base number of c-*kit* mRNA corresponding to the reference of GenBank accession no. AF044249

tumour malignancy is supported by the results of this study and a previous report (Zemke *et al*. 2002) where ITDs were identified only in malignant MCTs of higher pathological grades.

As reported previously, several KIT mutations other than ITD have been demonstrated in MCTs (Letard *et al*. 2008). Different types of mutations might exist in the two dogs of this report. However, each lesion of multiple MCTs may express ITDs or other gain-of-function mutations, as well as the wild-type c-*kit*. Therefore, simply determining the presence or absence of KIT mutations by analysing a single tumour region is likely to be insufficient. MCTs that contain ITDs have been reported to be more sensitive to small-molecular inhibitors targeting KIT, such as masitinib mesylate and toceranib phosphate (Hahn *et al*. 2008, 2010, London *et al*. 2009). Therefore, in the case of multiple MCTs, genetic analysis of several parts of the tumour may be preferable to predict tumour response to KIT inhibitors and tumour prognosis accurately.

Regarding the proportion of ITD, it was much less than shown in previously published reports (London *et al*. 1999, Zemke *et al*. 2002, Letard *et al*. 2008) despite the large number of malignant tumours examined in this study (Table 1). This notion also indicates the importance of analysing c-*kit* gene to select the appropriate treatment in each case.

In conclusion, the current results not only confirmed the small population of ITDs in MCT specimens but also provide the possibility that MCTs are derived from multiple clones or exhibit non-clonogenic expansions in the same dog. These data may lead to a better understanding of MCTs and potentially enhance the course of therapeutic intervention against MCTs.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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NOTE *Internal Medicine*

Increased Expression of the Antiapoptotic Protein MCL1 in Canine Mast Cell Tumors

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ABSTRACT. Myeloid cell leukemia sequence 1 (MCL1) is a potent antiapoptotic protein that plays a critical role in cell survival and drug resistance in various cancers. However, to the best of our knowledge, the role of MCL1 in mast cell tumors (MCTs) has not been investigated in dogs. Here, we detected increased MCL1 expression in MCT cell lines, regardless of the presence of a c-*kit* mutation. MCL1 expression increased when the cells were exposed to specific inhibitors of mitogen-activated protein kinase or Janus kinase-signaling pathways, thus protecting the cells from apoptosis, but not when KIT or phosphatidylinositol-3 kinase signaling cascades were inhibited. These results indicate that MCL1 expression may contribute to MCT survival and confer drug resistance.
KEY WORDS: mast cell tumor, MCL1.

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Myeloid cell leukemia sequence 1 (MCL1) is a member of the Bcl-2 protein family, which plays an antiapoptotic role in various kinds of cells [2, 20]. It was originally identified in a human leukemia cell line, ML-1, and is currently known as an essential factor responsible for the long lifespan of mature B and T cells [11, 17]. MCL1 is also critical in the malignancy and drug resistance of cancer cells [10, 15, 18, 19, 23]. Upregulation of MCL1 is reported to associate with tumor malignancy in breast cancer, ovarian cancer and non-Hodgkin lymphomas [10, 15, 18]. In addition, Wertz *et al.* [23] have shown that MCL1 downregulation increases the sensitivity of ovarian and breast adenocarcinomas, non-small-cell lung cancer, leukemia and lymphoma to chemotherapy.

As MCL1 contributes to the maintenance of hematopoietic cells, involvement of MCL1 in the survival of malignant hematopoietic cells has been suggested [1, 24, 25]. Aichberger *et al.* [1] showed that MCL1 is essential for the survival of neoplastic human mast cells, suggesting that it may act as a novel therapeutic target. In dogs, mast cell malignances are known as mast cell tumors (MCTs), which are one of the most commonly encountered neoplasms in veterinary medicine [5]. However, little information is available about the role of MCL1 in canine MCTs. Therefore, we examined the expression and regulatory pathway of MCL1 in canine MCT cell lines.

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We used 4 MCT cell lines that express different types of wild-type or mutant KIT proteins: HRMC cells expressing wild-type KIT [16], BR cells possessing KIT with a point mutation in its juxtamembrane domain [7], MPT-2 cells expressing KIT with an internal tandem duplication in its juxtamembrane domain [12] and MPT-1.2 cells possessing KIT with an N508I point mutation in its extracellular domain [4]. BR cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 10% FBS and antibiotics, while other cell lines were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Hyclone, Logan, UT, U.S.A.) and antibiotics. To investigate whether these cell lines express MCL1, western blot analysis was performed. The protocol was performed as described previously [21]. Both first and second antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Because

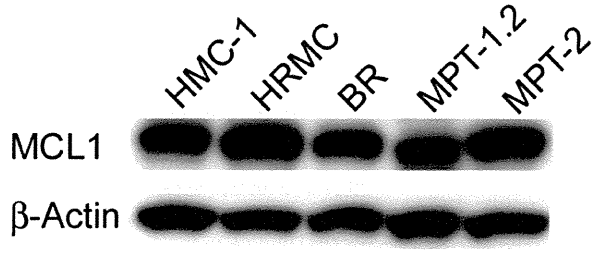

Fig. 1. Detection of MCL1 expression in canine MCT cell lines. The total protein extracted from 10^5 cells was loaded in the lanes of a 12.5% gel for SDS-PAGE. Human-derived HMC-1 cells were used as the positive control.

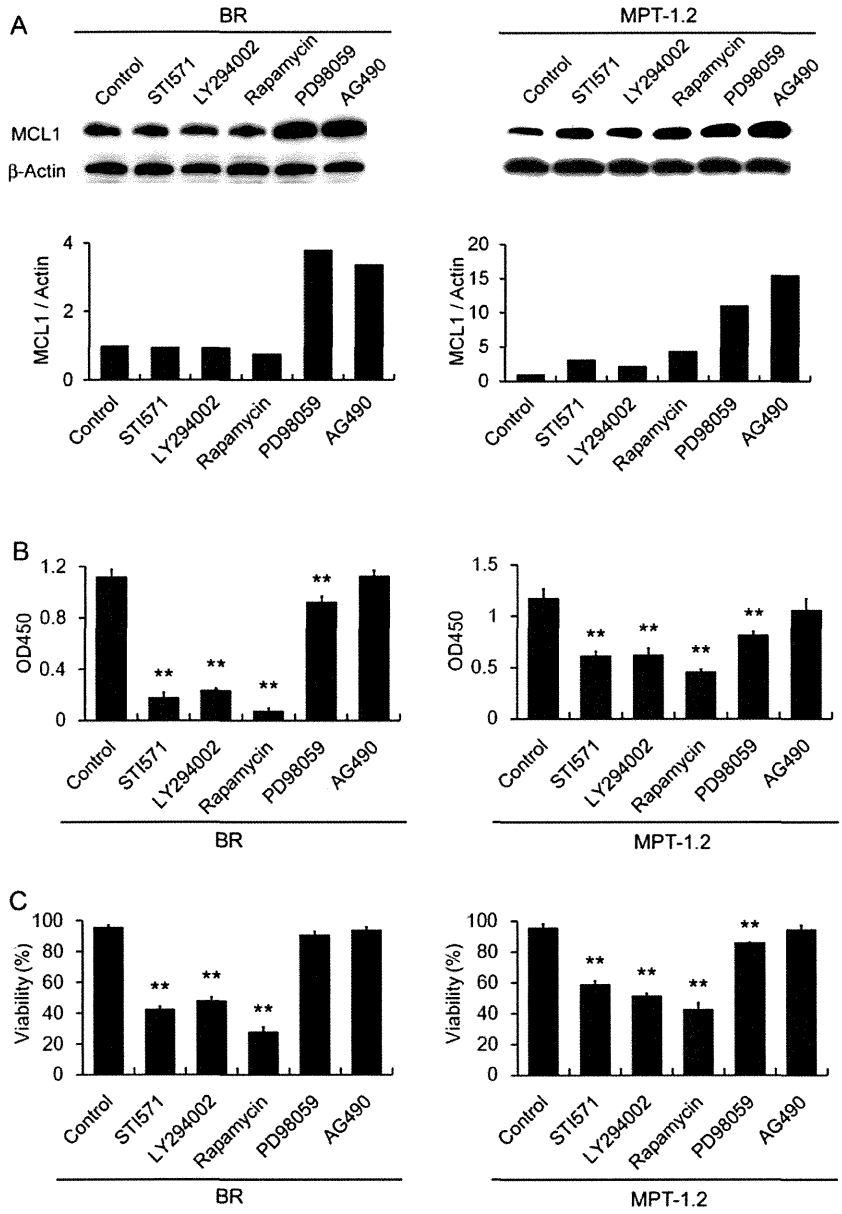

Fig. 2. Effect of each inhibitor on MCL1 expression in neoplastic mast cells.
Changes in MCL1 expression levels for each molecule inhibitor (A). The cells were treated with each inhibitor for 12 hr. They were then lysed, and the total protein extracted from 10^5 cells was loaded in the lanes of a 12.5% gel for SDS-PAGE. The graphs below indicate the relative intensity of the MCL1/β-actin ratio. Absorbances of each cell line that was incubated with each inhibitor for 72 hr (B). The experiment was conducted 3 times, and the mean ± SD values are shown. **, $P<0.01$ as compared to the control by Dunnett's test. Viabilities of each cell line that was incubated with each inhibitor for 72 hr (C). The experiment was conducted 3 times, and the mean ± SD values are shown. **, $P<0.01$ as compared to the control by Dunnett's test. The concentrations of the reagents in each experiment are as follows: STI571, 100 nM; LY294002, 5 μM; rapamycin, 100 nM; PD98059, 10 μM; and AG490, 10 μM.

human-derived HMC-1 mast cells have been reported to express MCL1 [1], they were used as a positive control. As shown in Fig. 1, MCL1 was detected in all the MCT lines that were examined, with expression levels that were as high as those of HMC-1 cells.

We next examined the changes in levels of MCL1 expres-

sion in all the MCT cell lines under treatment with several molecular inhibitors in order to determine the signaling pathway that mainly regulates MCL1 expression. In addition to the KIT inhibitor STI571 (also known as imatinib mesylate) [9], we evaluated the effect of selective inhibitors that target either phosphoinositide 3-kinase (PI3K) (LY294002) [22], mammalian target of rapamycin (rapamycin) [6], Janus kinase (JAK) 2 (AG490) [13] or mitogen-activated protein kinase (MAPK) (PD98059) [3]. Cell proliferation was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and a trypan blue dye exclusion test, according to previously described methods [21]. Cells were incubated with fixed concentrations of each reagent for 12 hr, and the MCL1 expression levels were determined by western blot analysis. The concentrations were determined according to the IC_{50} values of each reagent as reported previously [4]. Although STI571, LY294002 and rapamycin suppressed cell proliferation, MCL1 expression in BR cells remained unchanged (Fig. 2A–C). In contrast, both PD98059 and AG490, which did not suppress the proliferation of the cells, dramatically enhanced MCL1 expression in BR cells (Fig. 2A–C). Similar results were seen in MPT-1.2 cells (Fig. 2A–C), MPT-2 cells and HRMC cells (data not shown).

The PI3K pathway has been reported to play a critical role in the regulation of MCL1 expression in humans [13]. Interestingly, the expression levels of MCL1 in the MCT cells were not altered by the PI3K-signaling inhibitors, LY294002 and rapamycin. Therefore, it is likely that the regulatory pathway of MCL1 is different in humans and dogs. Additionally, MCL1 expression increased after suppression of both MAPK and JAK/STAT signaling pathways. The results suggest that MCL1 expression is upregulated by the inactivation of MAPK and/or JAK/STAT signaling pathways in order to rescue the cells from apoptosis. KIT-PI3K pathways directly promote cell proliferation in MCTs independently of anti-apoptotic signals through MCL1. Proliferation and survival of cells are reciprocally regulated [8]. KIT-PI3K pathways promote signals associating with cell proliferation as well as cell survival, which may be suppressed by KIT-MAPK or KIT-JAK/STAT pathways. Therefore, resistance to apoptosis may be evoked when MAPK or JAK/STAT signals are inhibited, resulting in MCL1 upregulation.

Focusing on the relationship between chemosensitivity and MCL1 expression, MCL1 expression was enhanced by the reagents that did not suppress the growth of each cell line, suggesting that MCL1 contributes to the circumvention of apoptosis against certain cytotoxic agents. In fact, Moulding *et al.* [14] have reported the importance of MCL1 in circumventing apoptosis in malignant hematopoietic cells through the downregulation of a proapoptotic protein Bak. This finding suggests that MCL1 inhibitors can overcome chemoresistance in MCTs in certain settings.

In conclusion, we detected increased expression of MCL1 in canine MCT cells, raising the possibility of their contribution to the cell survival and drug resistance. Further investigation of the functional role of MCL1 in MCTs may lead to a better understanding of the MCT biology and provide a novel therapeutic approach against canine MCTs.

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