ANTICANCER RESEARCH *36*: 5999-6004 (2016)
doi:10.21873/anticanres.11188

In Vitro and *In Vivo* Anticancer Activity of Human β-Defensin-3 and Its Mouse Homolog

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Abstract. *Background/Aim: Defensins comprise a family of mammalian cationic antimicrobial peptides. We investigated the anticancer effects of human β-defensin-3 (hBD3) and its mouse homolog, Defb14, on lung cancer cells. Materials and Methods: We stained lung cancer cells cultured after treatment with the defensin peptide using propidium iodide and Hoechst 33342. In vivo, Defb14 peptide or vehicle was continuously infused near subcutaneous Lewis lung carcinoma cell tumor in mice. After 9-day infusion, the weights of excised tumors were determined. Results: A 10-min treatment with hBD3 (70 μg/ml) induced propidium iodide uptake in lung cancer cells. The anticancer activity of hBD3 was significantly more potent than the activity of other defensin isoforms. Continuous infusion of Defb14 peptide showed significant tumor-growth suppression in Lewis lung carcinoma cells in mice. Conclusion: Our study demonstrated the suppression of tumor growth by Defb14 peptide in an animal model.*

Antimicrobial peptides have emerged as a part of the host defense mechanism in both animals and plants (1, 2). Specifically, defensins and cathelicidins are antimicrobial peptides found in humans (3). In the defensin family, there are α-defensin and β-defensin subfamilies, which conserve three specific disulfide pairings. These are produced by leukocytes and various types of epithelial cells constitutively, or in response to microbial signals and inflammatory cytokines (4-6).

Human β-defensin-3 (hBD3) was first isolated from human skin (7) and its expression was detected in many tissues, including airway epithelial cells (8, 9). In contrast to other defensin isoforms, the prominent features of hBD3 antimicrobial activity are its salt-independency and its broad antimicrobial spectrum (7, 10, 11).

While antimicrobial peptides have attracted attention as potential molecules to treat cancer (12), previous observations on the anticancer effects of hBD3 have not been consistent. Some studies hypothesized that hBD3 can be oncogenic because hBD3 is frequently overexpressed in oral squamous cell carcinomas (13-15). Winter *et al.* indicated that hBD3 inhibited colon cancer-cell migration, although not cell proliferation (16). Moreover, Phan *et al.* indicated that hBD3 exhibits anticancer effects on various tumor cells through cell permeabilization (17).

Here, we investigated the anticancer activities of various isoforms of β-defensin on lung cancer cells, and confirmed that this anticancer activity is also seen in animal models of cancer.

Materials and Methods

Reagents. Synthetic hBD1, hBD2, hBD3 and human neutrophil peptide-1 (HNP1) peptides were purchased from the PEPTIDE institute (Minoh, Japan), and were dissolved in 0.001% acetic acid to give a final concentration of 2 mg/ml.

We predicted the mature Defb14 peptide spanning 45 COOH-terminal amino acids as a mouse homolog of hBD3, identical to the predicted mature peptide of Defb14 from a previous report (18). At the Peptide Institute (Minoh, Japan), we chemically synthesized mature Defb14 peptide (19, 20). The synthetic peptide was air-oxidized to form three disulfide bonds. The material was eluted as a

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Key Words: Antimicrobial peptide, cytotoxicity, membrane damage.

single peak on reverse-phase high-performance liquid chromatography and confirmed by mass spectroscopy. It was lyophilized and dissolved in 0.001% acetic acid to give a final concentration of 2 mg/ml.

Cell culture. A549 human lung carcinoma cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai-shi, Japan). Lewis lung carcinoma (LLC) cells were obtained from RIKEN BRC (Tsukuba-shi, Japan). The cells were routinely cultured in Dulbecco's modified Eagle's medium, supplemented with 25 U/ml penicillin, 25 μg/ml streptomycin and 10 % (v/v) heat-inactivated fetal calf serum (FCS) at 37˚C in a 5% CO_2-humidified atmosphere. Cell counting was performed using a Coulter Counter (Beckman Coulter, Tokyo, Japan).

Evaluation of propidium iodide (PI) uptake. To evaluate cell membrane damage, we stained the cells cultured in phosphate-buffered saline (PBS) containing 0.9 mM Ca^{2+} and 0.33 mM Mg^{2+} or in Dulbecco's modified Eagle's medium containing 10% (v/v) FCS, using PI and Hoechst 33342. The proportion of damaged cells was determined by dividing the number of PI-positive cells by the number of Hoechst 33342-positive cells in a field of 0.43 mm^2 at the center of the culture dishes.

XTT assay. We performed an XTT assay using a Cell Proliferation Kit (Roche Diagnostics, Minato-ku, Tokyo, Japan) to measure the number of viable cells. In accordance with the protocol, 100 μl of A549 cell suspension (10^5 cells/ml) was seeded in a 96-well plate, and cultured for 48 h with 20 μg/ml hBD3 or with 0.001% acetic acid. The absorbance was measured at 492 nm.

5'-Bromo-2' deoxyuridine (BrdU) assay. We performed a BrdU assay using 5'-Bromo-2' deoxyuridine Labeling & Detection Kit (Roche Diagnostics). A549 cell suspension (100 μl of 10^5 cells/ml) was seeded in a 96-well plate and cultured for 24 h. Subsequently, BrdU was added with 20 μg/ml hBD3 or with 0.001% acetic acid, and the cells were incubated for an additional 12 h before the measurement of BrdU incorporation.

Implantation of cancer cells and subcutaneous infusion of the DefB14 peptide. All the following procedures were approved by the Animal Care and Use Committee of University of Tokyo (P08-095). After anesthetizing 3-month-old C57BL/6J male mice (CLEA Japan, Meguro-ku, Tokyo, Japan) with ketamine and xylazine, we subcutaneously injected 100 μl of LLC cell suspension (1×107 cells/ml PBS) to form tumors on the back of mice. Eight days after LLC implantation, we selected the mice that had palpable tumors in the back, and grouped them randomly into a Defb14-treated group (n=8) and a vehicle-treated group (n=8). In the Defb14-treated group, an ALZET MINI-Osmotic Pump (model 2002; DURECT, Cupertino, CA, USA) containing 4.17 mg/ml of Defb14 peptide in PBS was implanted subcutaneously, near the tumor. This pump infused the contained liquid at a rate of 0.5 μl/h, which corresponds to 50 μg/day of Defb14 peptide. In the vehicle-treated group, an ALZET MINI-Osmotic Pump containing 0.01% ascetic acid in PBS was implanted subcutaneously, near the tumor. At the start of the infusion, the palpable tumor size was measured. After 9 days of infusion, the weights of the incised tumors was determined.

Statistics. The variables were compared by Student's *t*-test. Statistical significance was defined as a *p*-value of less than 0.05.

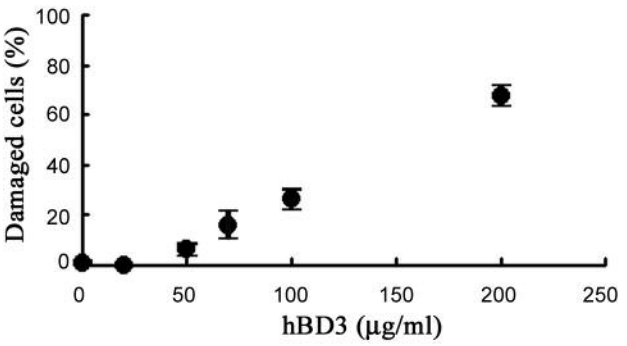

Figure 1. *Propidium iodide (PI) uptake after a 10 min treatment with human β-defensin-3 (hBD3). The percentage of damaged cells was determined by dividing the number of PI-positive cells by the number of all the nuclei after 10 min treatment with hBD3. Data are the mean and the standard error (n= 9, 7, 8, 4, 8 and 5 at 0, 20, 50, 70, 100 and 200 μg/ml hBD3, respectively). The percentage of damaged cells increased linearly with increasing concentration of hBD3. Treatment with 70 μg/ml or higher concentrations of hBD3 induced a significantly higher proportion of damaged cells compared to no treatment with hBD3 ($p<0.01$).*

Results

Treatment with hBD3 induced immediate cell membrane damage. A 10-min treatment with hBD3 induced PI uptake in numerous A549 cells, indicating immediate cell-membrane damage. The proportion of damaged cells increased linearly with increasing concentration of hBD3. Treatment with a concentration of hBD3 of 70 μg/ml or higher induced significantly higher proportion of damaged cells ($p<0.01$) (Figure 1).

The cytotoxic effects of hBD3 were significantly more prominent than HNP-1 ($p<0.01$). Neither hBD1 nor hBD2 induced PI uptake into A549 cells, even at a concentration of 200 μg/ml, for 10 min. Interestingly, Defb14 also showed potent cytotoxicity against A549 cells (Figure 2).

Time-course of cell damage induced by hBD3. Evaluating the time-dependency of the hBD3 cytotoxic effect, we found that the proportion of PI-positive cells increased sharply after 10 min. However, between 10 and 20 min, the increase of PI-positive cells was relatively lower. A slight increase was also seen 40 min after co-incubation with hBD3 (Figure 3A). In prolonged incubation with hBD3 in culture medium containing 10% (v/v) FCS, hBD3 also induced PI uptake into A549 cells, and the proportion of damaged cells increased almost linearly until 540 min after co-incubation (Figure 3B).

Because of the time-dependent increase in cytotoxicity induced by hBD3, we evaluated the cytotoxic effects of prolonged incubation with the hBD3 peptide at a lower concentration. After a 24-h co-incubation with 20 μg/ml of hBD3 in culture medium containing 10% (v/v) FCS, a

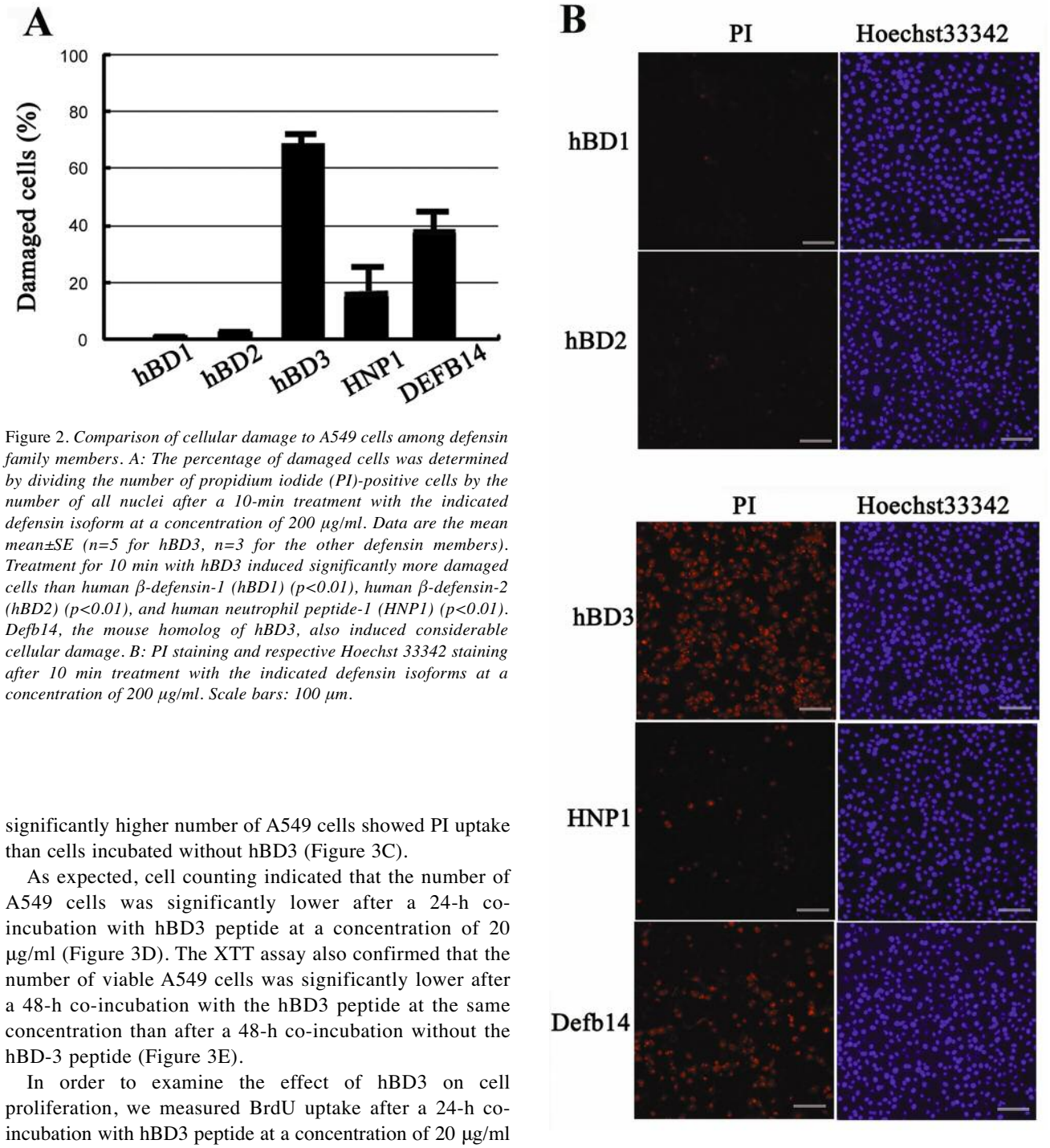

Figure 2. *Comparison of cellular damage to A549 cells among defensin family members. A: The percentage of damaged cells was determined by dividing the number of propidium iodide (PI)-positive cells by the number of all nuclei after a 10-min treatment with the indicated defensin isoform at a concentration of 200 μg/ml. Data are the mean mean±SE (n=5 for hBD3, n=3 for the other defensin members). Treatment for 10 min with hBD3 induced significantly more damaged cells than human β-defensin-1 (hBD1) (p<0.01), human β-defensin-2 (hBD2) (p<0.01), and human neutrophil peptide-1 (HNP1) (p<0.01). Defb14, the mouse homolog of hBD3, also induced considerable cellular damage. B: PI staining and respective Hoechst 33342 staining after 10 min treatment with the indicated defensin isoforms at a concentration of 200 μg/ml. Scale bars: 100 μm.*

significantly higher number of A549 cells showed PI uptake than cells incubated without hBD3 (Figure 3C).

As expected, cell counting indicated that the number of A549 cells was significantly lower after a 24-h co-incubation with hBD3 peptide at a concentration of 20 μg/ml (Figure 3D). The XTT assay also confirmed that the number of viable A549 cells was significantly lower after a 48-h co-incubation with the hBD3 peptide at the same concentration than after a 48-h co-incubation without the hBD-3 peptide (Figure 3E).

In order to examine the effect of hBD3 on cell proliferation, we measured BrdU uptake after a 24-h co-incubation with hBD3 peptide at a concentration of 20 μg/ml (Figure 3F). There was no significant difference in BrdU uptake between cells co-incubated with and without hBD3.

Effect of Defb14 infusion on tumor growth in mice. Subsequently, we evaluated the anticancer activity of Defb14 *in vivo*. Before beginning the Defb14 peptide infusion, there were no differences in tumor size between the Defb14-treated group and the vehicle-treated group (248.06±9.80 mm^3 and 245.06±22.66 mm^3, respectively, p=0.45). After a 9-day infusion of Defb14 or vehicle, the tumor weights were significantly lower in the Defb14-treated group than in the control group (p=0.015). We observed no apparent damage to normal tissues around the site of Defb14 infusion (Figure 4).

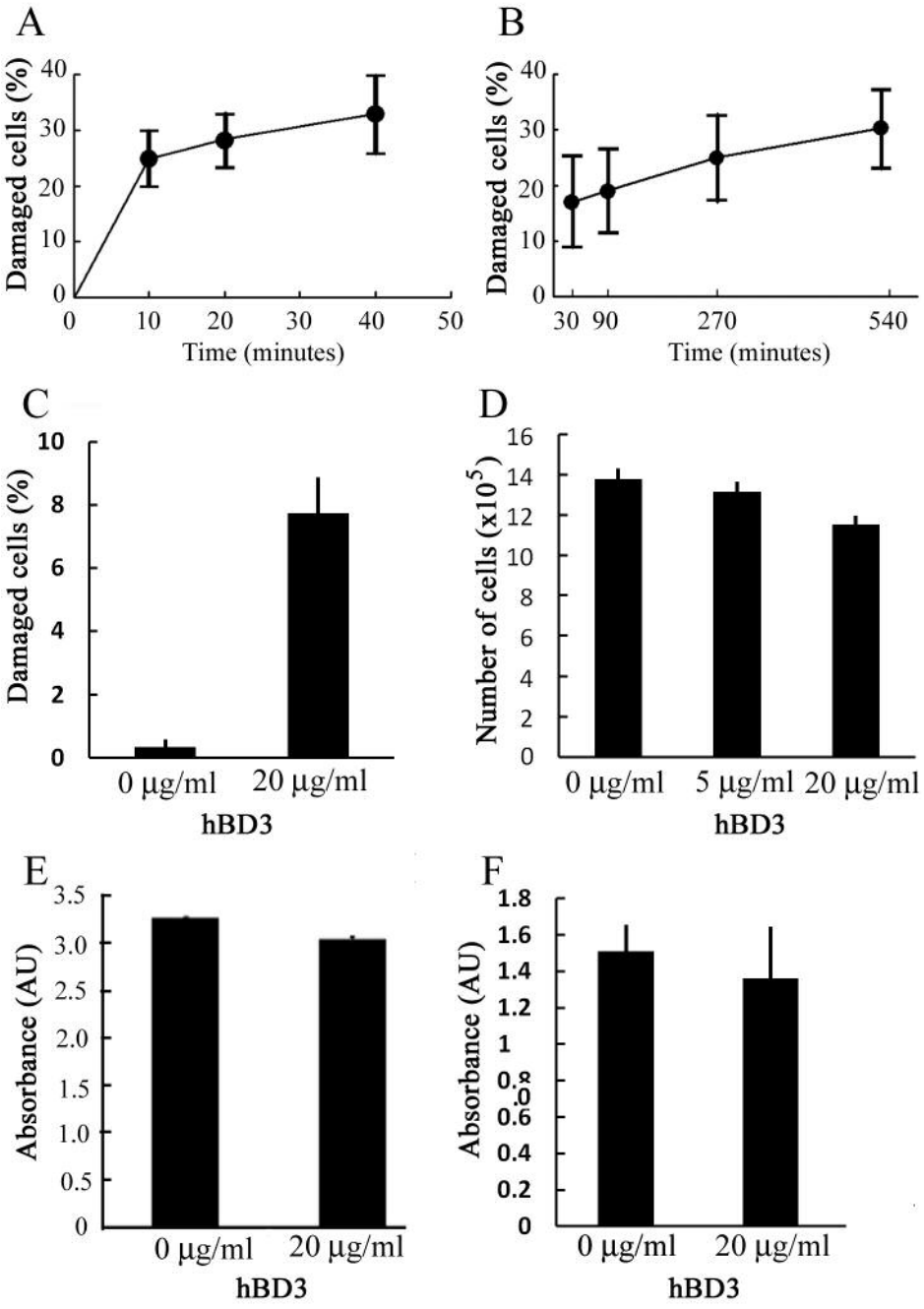

Figure 3. *Time dependency of cellular damage induced by human β-defensin-3 (hBD3). A: Propidium iodide (PI) uptake by A549 cells was observed after 10-, 20- and 40-min incubation with 70 µg/ml of hBD3 in phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.33 mM Mg^{2+}. The proportion of damaged cells rapidly increased in 10 min. Data are mean±SE (n=5 for each incubation time). B: Prolonged incubation with 100 µg/ml of hBD3 in culture medium containing 10% (v/v) fetal calf serum (FCS) led to nearly 20% of the A549 cells taking-up PI after 30-min incubation and the proportion of damaged cells continued to increase gradually after 90-, 270- and 540-min incubation. Data are the mean±SE (n=3 for each incubation time). C: The cytotoxic effects of prolonged incubation with hBD3 peptide were evaluated at a lower concentration. After 24-h co-incubation with 20 µg/ml of hBD3 in culture medium containing 10% (v/v) FCS, a significantly higher number of A549 cells showed PI uptake than without hBD-3 ($p<0.01$). Data are the mean±SE (n=4 in each group). D: Cell counting indicated that the number of A549 cells was significantly lower after 24-h co-incubation with 20 µg/ml hBD3 peptide than without hBD3 ($p<0.01$). Data are the mean±SE (n=4 in each group). E: The XTT assay showed that the number of A549 cells was significantly lower after 24-h co-incubation with 20 µg/ml hBD3 peptide than without hBD-3 ($p<0.01$). Data are the mean±SE (n=5 in each group). F: There was no significant difference in 5'-bromo-2' deoxyuridine (BrdU) uptake between cells co-incubated with 20 µg/ml hBD3 peptide for 24 h and cells incubated without hBD3 peptide. Data are the mean±SE (n=4 in each group).*

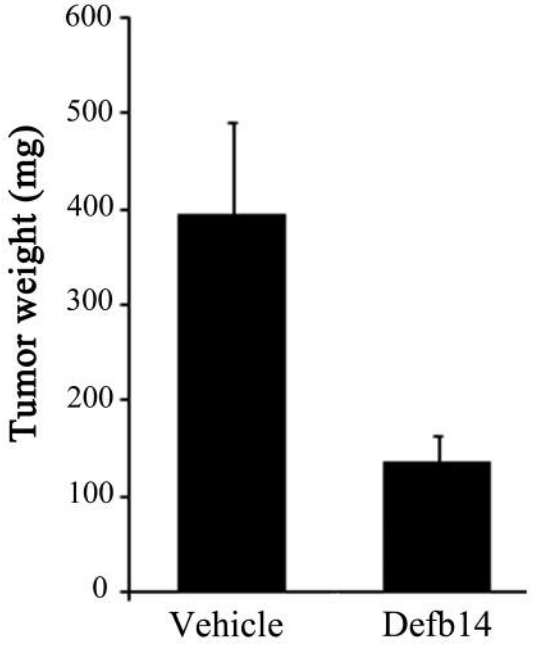

Figure 4. *Anticancer activity of Defb14 peptide in mice. Eight days after subcutaneous implantation of Lewis lung carcinoma cells into the back of C57BL/6J mice, the mice in which the tumors were palpable were grouped randomly into a Defb14-treated group (n=8) and a vehicle-treated group (n=8). In the Defb14-treated group, 50 μg/day of Defb14 peptide was subcutaneously infused near the tumor. After 9-day infusion of Defb14 peptide, the excised tumor weights were significantly lower in the Defb14-treated group than in the control group (p=0.015). Data are mean±SE (n=8 in each group).*

Discussion

In this study, we investigated the anticancer activities of hBD3 and Defb14. As far as we are aware of, this is the first study to confirm the anticancer activity of defensin in an animal model.

Although many studies have evaluated the association between carcinogenesis and antimicrobial peptides (12), the results have been inconsistent. Some studies indicated that defensin promoted cancer growth (21, 22), whereas other studies showed that defensin is cytotoxic (16, 17, 23-26). Our results suggest that the cytotoxic effect is specific to hBD3 and its mouse homolog, Defb14, among members of the defensin family. Although many studies indicated the cytotoxic effects of HNP1 (23-25), our study showed that the anticancer activity of hBD3 was markedly more potent than that of HNP1. In addition, the presence of serum did not abolish the cellular damage induced by hBD3, whereas a previous report indicated that membrane permeabilization by HNP1 did not occur in the presence of 5% serum (25).

The inconsistency between our findings compared to those from previous investigations reporting that tumors overexpressing hBD3 or Defb14 showed enhanced solid-tumor growth remains unclear (14, 15, 27). This may have occurred because of differences in the concentration of hBD3 and Defb14 used, although the concentration of 20 μg/ml defensin used in our study was not excessively higher than that at which antimicrobial activity was observed (10). Additionally, different pathways may function, if the tumors themselves overexpress defensin (15, 27).

Our results for the anticancer activity of hBD3 in lung cancer cells were consistent with those in a recent report by Phan *et al*. (17). In addition to the *in vitro* anticancer activity, we first demonstrated the suppression of tumor growth by the Defb14 peptide in mice. Although high concentrations of Defb14 can have harmful effects on normal cells, we observed no damage to normal tissues around the site of Defb14 infusion. Because hBD3 can induce cytokine production, and also be chemotactic for immune cells (8, 18, 28-31), we were unable to determine whether the suppression of tumor growth in our animal model was because of direct cytotoxic effects of Defb14 or immunoregulatory activities of Defb14. Defb14 may also negatively affect angiogenesis in tumors, although a previous investigation indicated that Defb14 promoted angiogenesis (27). It is difficult to clarify the contribution of immune reactions because cell damage can induce secondary inflammatory responses. Although the precise mechanism remains to be clarified, our observations represent a crucial first step in the application of hBD3 peptide in anticancer therapy.

In conclusion, our study clarified the characteristics of hBD3 anticancer activity on lung cancer cells, and demonstrated for the first time, the suppression of tumor growth by the Defb14 peptide in an animal model.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (24591154, 15K08899).

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Received July 23, 2016
Revised August 15, 2016
Accepted August 18, 2016