

Anti-tumor effects of telomelysin for head and neck squamous cell carcinoma

KYOKO FUJITA, MACHIKO KIMURA, NORIO KONDO, ATSUKO SAKAKIBARA,
DAISUKE SANO, YUKARI ISHIGURO and MAMORU TSUKUDA

Department of Biology and Function in Head and Neck, Yokohama City University
Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

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Abstract. Telomelysin is a telomerase-specific replication-competent adenovirus with telomerase reverse transcriptase (hTERT) promoter, which has shown strong anti-tumor effects on a variety of human cancer cells. Human head and neck squamous cell carcinoma (HNSCC) cell lines and a murine HNSCC (NR-S1) model were used to investigate whether telomelysin (OBP-301) had a therapeutic efficacy for HNSCC. We examined the cell killing effects of telomelysin and the induction of tumor cell apoptosis by telomelysin *in vitro*. Based on these data, we examined whether telomelysin therapy produced therapeutic benefits *in vivo*. The results demonstrated that the treatment of telomelysin led to significant tumor regression on the side with subcutaneous NR-S1 tumor. We first confirmed the direct anti-tumor effect of intratumoral telomelysin injections in a murine HNSCC model. Further analyses of the augmented anti-tumor effects revealed that telomelysin increased the source of tumor antigens for immune cells, resulting in the induction of CD4⁺ and CD8⁺ T cells responsible for the *in vivo* tumor regression of treated and untreated tumors. Subsequently, an elevated IFN- γ production of spleen cells was observed in mice treated with telomelysin. These results raise the possibility that telomelysin enhances the immune response in addition to its direct tumor cell killing activity. These findings suggest that telomelysin is a potent agent for the treatment of HNSCC patients with multiple metastases.

Introduction

The incidence of head and neck squamous cell carcinoma (HNSCC) is ~500,000 cases annually worldwide (1).

Combined treatments including surgery, chemotherapy and radiotherapy prolong the survival of advanced cases with HNSCC, but the treatment benefit has been typically temporary in advanced disease (2). Therefore, new approaches are necessary to provide more effective treatment modalities, e.g. molecular-targeted therapy, gene therapy and immunotherapy, against advanced disease.

Telomerase is a rib nucleoprotein complex responsible for the complete replication of chromosomal ends, whose activation is considered to be a crucial step not only in cell proliferation but also in carcinogenesis (3-6). Previous studies have demonstrated a high telomerase activity in >85% of human cancers (4), but in only a few normal somatic cells (5). Human telomerase reverse transcriptase (hTERT) positively regulates telomerase at the transcriptional level and shows a selectively high activity in growing neoplastic tissues and cells. Therefore, it seems reasonable to use hTERT promoter as a cancer-specific promoter. Telomelysin (OBP-301) is a telomerase-specific replication-competent adenovirus that induces selective E1 expression and exclusively kills human cancer cells (7-10).

Deng *et al* reported that the E1A gene showed induction apoptosis (11). It has also been reported that the uptake of antigens from apoptotic cells induces cytotoxic T-lymphocytes (CTLs) which are recognized as critical mediators of anti-tumor immunity (12). Endo *et al* reported that telomelysin-mediated oncolysis stimulated CTL activity (13). These findings led us to examine whether tumor cells killed by telomelysin infection were able to stimulate an immune response *in vivo* by antigens of the apoptotic cells induced by telomelysin. Therefore, we analyzed the anti-tumor activity of telomelysin on human HNSCC cell lines and anti-tumor mechanisms using a murine HNSCC cell line *in vitro* and *in vivo*. The anti-tumor effects of telomelysin on HNSCC have yet to be reported.

Materials and methods

Human cell lines and culture conditions. Human HNSCC cell lines were examined in this study. The primary sites of these cell lines were the oral floor (YCU-OR891), hypopharynx (YCU-H891), mesopharynx (YCU-M862, YCU-M911 and KCC-M871), larynx (KCC-L871 and YCU-L891), tongue (YCU-T891, YCU-T892, KCC-T871, KCC-T873 and HSC-3),

Correspondence to: Dr Kyoko Fujita, Department of Biology and Function in Head and Neck, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
E-mail: t056043b@yokohama-cu.ac.jp

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Table I. Antiproliferative activity of telomelysin.^a

HNSCC cell line	Primary sites	Telomelysin ID ₅₀ (MOI)		
		Day 3	Day 5	Day 7
YCU-OR891	Oral floor	13875	3549	3077
YCU-H891	Hypopharynx	2886	2931	279
YCU-M862	Mesopharynx	4120	3510	3186
YCU-M911	Mesopharynx	3189	2179	2819
KCC-M871	Mesopharynx	31041	4661	4585
KCC-L871	Larynx	12389	2962	2894
KCC-L891	Larynx	4099	3223	3015
YCU-T891	Tongue	3570	2736	2918
YCU-T892	Tongue	4776	2865	2788
KCC-T871	Tongue	13787	3744	3075
KCC-T873	Tongue	4523	3455	3050
HSC-3	Tongue	16814	3811	3065
KCC-TCM901	Lung metastasis (tongue)	3365	3115	314
KCC-TCM902	Lung metastasis (tongue)	3322	2161	282
KCC-TCM903	Lung metastasis (tongue)	4684	3560	3055
KCC-MS871	Maxillary sinus	44057	3909	3243
KCC-MS861	Maxillary sinus	3311	2791	2208

^aIn the proliferation assay described in Materials and methods, tumor cells were exposed to telomelysin for 24 h. Data are expressed as ID₅₀, the telomelysin quantity (MOI) that causes 50% inhibition of cell proliferation.

lung metastasis of tongue cancer (KCC-TCM901, KCC-TCM902 and KCC-TCM902) and maxillary sinus (KCC-MS871 and YCU-MS861). These cell lines were maintained in RPMI-1640 medium (Life Technologies Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37°C in an atmosphere containing 5% CO₂.

Mice and cell line. Four-week-old female C3H (H-2k^k) mice were purchased from Oriental Yeast, Tokyo, Japan. They were maintained in the specified pathogen-free conditions and used after approval by the Animal Care and Use Committee of Yokohama City University School of Medicine. A murine squamous cell carcinoma cell line (NR-S1) originating from the bucal mucosa was cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Adenovirus. We used the recombinant replication-selective, tumor-specific adenovirus vector telomelysin (OBP-301), in which the hTERT promoter element drives the expression of E1A and E1B genes linked with an internal ribosome entry site (7,8).

In vitro proliferation assays. MTT assay was performed to measure cell viability using human HNSCC cell lines and a mouse HNSCC cell line. These cells were plated in 96-well U-bottomed plates (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ, USA) at a concentration of 1x10³ cells/

well. Plates were allowed to incubate for 24 h prior to drug treatment. After a 3-, 5- and 7-day exposure to drugs, the MTT assay was carried out with Tetra Color One (Seigaku Co., Ltd., Tokyo, Japan). Relative growth inhibition was calculated compared to vehicle-treated control cells, and ID₅₀ values acted as the drug concentration showing 50% survival.

Annexin V binding assay. To evaluate apoptosis in NR-S1 cells, an Annexin V binding assay was performed. Tumor cells were double stained with FITC-conjugated Annexin V and propidium iodide (Roche). Cells undergoing early apoptosis were determined as the percentage of Annexin V-positive propidium iodide-negative cells by FACScan with a Cell Quest 1.0 software package (Becton-Dickinson).

In vivo anti-tumor activity studies. Female C3H/HeJ mice, 4 weeks old, were obtained from Oriental Yeast. The mice were maintained in a laminar flow room with constant temperature and humidity. Suspensions of 100 µl of NR-S1 cells (final concentration: 5x10⁶ cells/ml) were injected subcutaneously into the two sides of the mice. Tumor-bearing mice were randomized (n=7) when the mean tumor volume was ~100 mm³. Each group was closely matched before treatment, which began 10 days after cell transplantation. The mice were randomly assigned to the treatment groups with an intratumoral injection of PBS (control) or telomelysin in 100 µl buffer. As for the telomelysin administration, the left-side tumors were intratumorally injected with either doses of a single administration or three separate administrations of 10⁸ plaque-forming units (PFU) per tumor. Tumor diameters

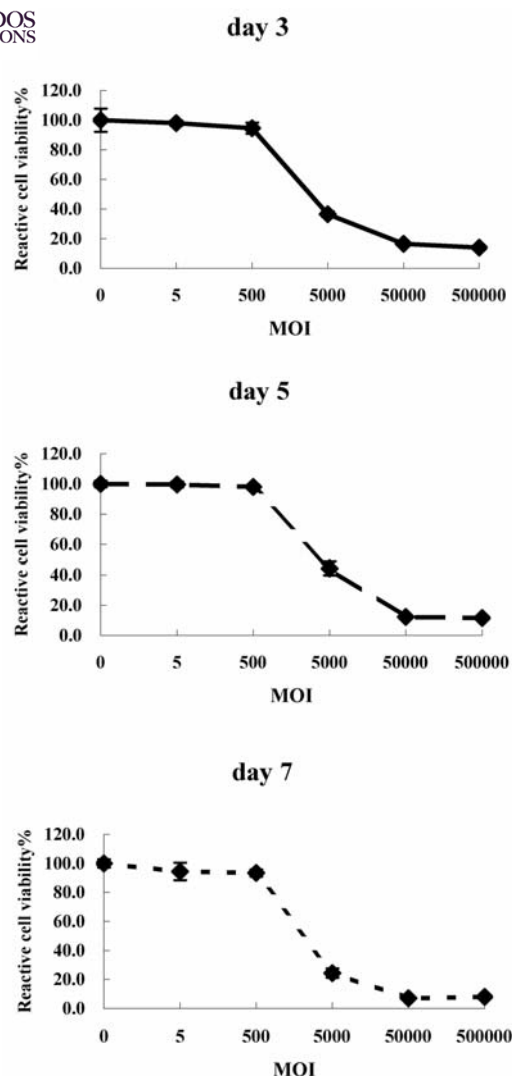


Figure 1. *In vitro* cell killing effect of telomelysin on NR-S1 murine SCC cells. NR-S1 cells were infected with telomelysin at the indicated multiplicity of infection (MOI) values, and surviving cells were quantitated over 3, 5 and 7 days after infection by MTT assay. Error bars indicate SE.

in the control and treated groups were measured weekly with a Vernier caliper. Tumor volume (V) was determined by the equation: $V = ab^2/2$ (a, length and b, width).

IFN- γ assay. Spleen cells ($1 \times 10^6/\text{ml}$) isolated from telomelysin-treated (triple administration) and control mice were added to 6-well plates and stimulated with mitomycin-treated NR-S1 HNSCC cells ($1 \times 10^5/\text{ml}$). Supernatants were collected 5 days later, centrifuged ($1500 \times g$ for 15 min), and stored at -80°C . Levels of IFN- γ were assessed by ELISA (Bioscience).

Immunohistochemical assay. The mice were sacrificed on day 7 after being treated three times with PBS or telomelysin. Tumors were resected and stored in Tissue-Tek OCT compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) at -80°C until immunohistochemical analysis. We immunohistochemically evaluated the explants of tumor-bearing mice for the presence of CD4 (Santa Cruz Biotechnology, Inc., CA, USA) or CD8 (Chemicon International, Inc., CA, USA) T cells

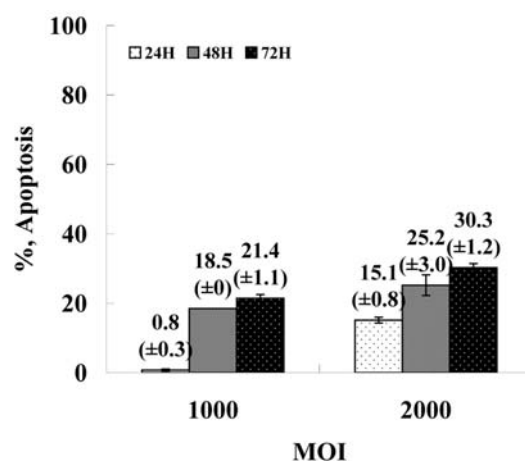


Figure 2. Telomelysin-induced apoptosis of NR-S1 cells. NR-S1 cells were treated with various concentrations of telomelysin *in vitro*. Tumor cell apoptosis harvested after a 24 and 48 h coincubation with telomelysin was assessed by Annexin V/propidium iodide staining and FACScan analysis. Error bars indicate SE.

and CD56 (Santa Cruz Biotechnology, Inc.) after streptavidin peroxidase immunization.

Statistical analysis. For statistical analyses of *in vivo* anti-tumor activity, IFN- γ assay and immunohistological analysis, we used Student's paired t-test. $P < 0.01$ was considered significant.

Results

Antiproliferative effects of telomelysin on human HNSCC lines. We initially determined the antiproliferative effects of telomelysin on HNSCC lines. Cells were cultured with media alone (control) or media containing telomelysin (0-5000 multiplicity of infection, MOI). As shown in Table I, HNSCC lines were sensitive to telomelysin. The ID_{50} of telomelysin on day 7 ranged between 279 and 4585 MOI.

Antiproliferative effects of telomelysin on NR-S1 cells. The cell killing effect of different concentrations of telomelysin on the NR-S1 cell line was determined. As illustrated in Fig. 1, significant growth inhibitory effects were observed on days 3, 5 and 7 following telomelysin treatment at >500 MOI.

Analysis of apoptosis in NR-S1 cells induced by telomelysin *in vitro*. To determine the apoptotic rates in NR-S1 cells treated with telomelysin, we subtracted the percentage of spontaneous apoptosis in untreated tumor cell cultures from telomelysin-induced apoptosis values. In the Annexin V binding assay the apoptotic rate determined as the percentage of Annexin V-positive propidium iodide-negative cells treated with telomelysin for 72 h was 21.4 ± 1.1 and $30.3 \pm 1.2\%$ at the concentrations of 1000 and 2000 MOI, respectively (Fig. 2).

***In vivo* study of the anti-tumor effects of telomelysin on NR-S1 xenografts.** We investigated the *in vivo* anti-tumor activity of telomelysin in the NR-S1 xenografts. Tumor growth was significantly inhibited by treatment with telomelysin on the

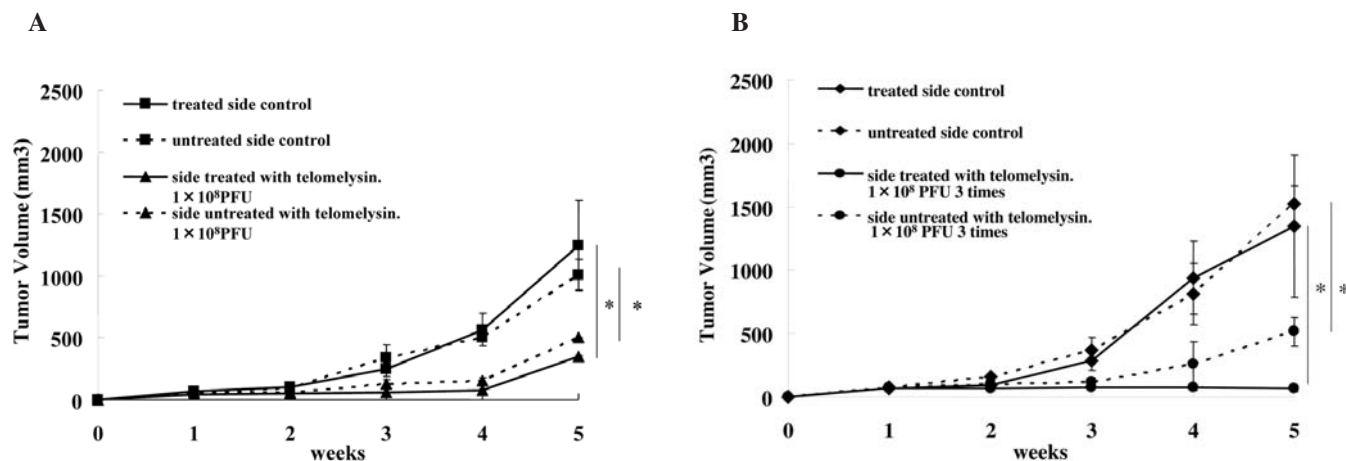


Figure 3. (A and B) Effects of telomelysin on tumor growth on the two sides of the HNSCC xenograft model. (A) One- and (B) three-time telomelysin administration of 1×10^8 PFU. Antitumor potential of telomelysin was significantly stronger than the control in the murine SCC model. Error bar, SE (n=7 mice per group) and * $P < 0.01$ versus control.

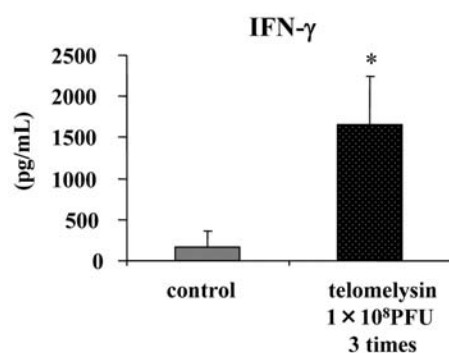


Figure 4. Effects of telomelysin on IFN- γ secretion by spleen cells. The cells were stimulated with mitomycin-treated NR-S1 cells and incubated for 5 days. The supernatants were collected and evaluated by ELISA for the production of IFN- γ . Columns, mean of a triplicate sample; error bars indicate SE and * $P < 0.01$ versus control.

treated side, and tumor reduction was observed in the untreated side of the group receiving telomelysin injection compared to the control group ($P < 0.01$; Fig. 3A and B). The telomelysin treatment courses of 10^8 PFU/day, i.e., once or three times, revealed a dose-dependent inhibition of the tumor growth on the treated side of mice with tumor xenograft.

IFN- γ production in spleen cells of mice treated with telomelysin. To evaluate the immunological response to the telomelysin treatment, IFN- γ production was assessed in cultures of spleen cells isolated from the control group, and in telomelysin-treated cultures. IFN- γ was measured in the supernatants of spleen cells which were stimulated with mitomycin-treated NR-S1 (Fig. 4). Spleen cells isolated from mice treated with telomelysin released a significantly higher level of IFN- γ compared to that of spleen cells from the control group.

Immunohistochemical analysis. The infiltration of CD4⁺ or CD8⁺ T-lymphocytes in tumors receiving telomelysin

injections on the treated and untreated sides was significantly enhanced compared with that observed in the control group. There was almost no infiltration of CD56⁺ cells in either the treated or control groups (Fig. 5A and B).

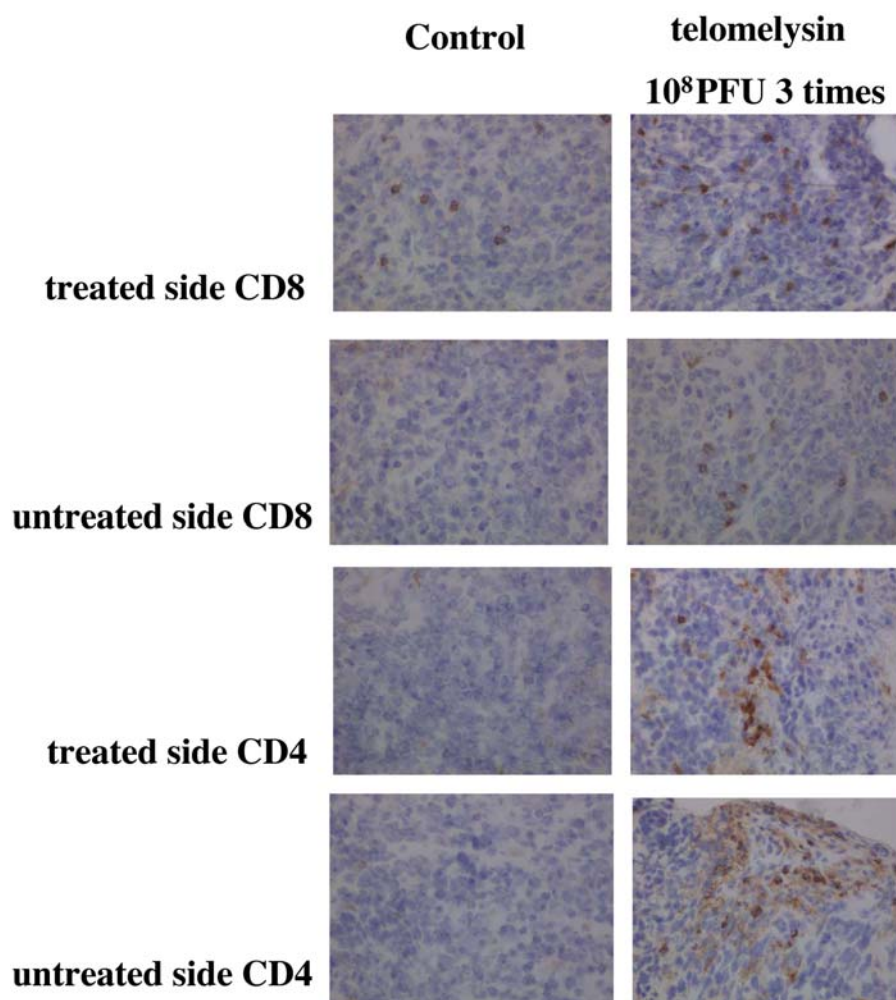
Discussion

The successful multidisciplinary treatment of advanced head and neck cancer is a significant issue. Neither cytotoxic therapy nor immunotherapy alone or in combination with definitive treatments, i.e., surgery and/or radiotherapy, can successfully resolve this issue. Different treatment modalities may provide an improvement in the outcome of advanced cases. In the present study, we evaluated the potential utility of the intratumoral administration of telomelysin, an anticancer virus.

Telomelysin has been shown to be effective against human cancers (7,8,10). This virus was genetically designed to replicate under the control of the hTERT promoter specifically in tumor cells causing specific 'oncolysis'. Therefore, telomelysin does not cause significant toxicity in normal human cells and tissues (7,8,10).

This is the first study on the anti-tumor effects of telomelysin on HNSCC. The present results demonstrated that telomelysin has a strong anti-tumor effect against human HNSCC cells *in vitro* and against a murine HNSCC cell line *in vitro* and *in vivo*. Kishimoto *et al* reported that the reactive hTERT mRNA expression in HNSCC cell lines was detected by real-time RT-PCR analysis (14). Therefore, telomelysin showed killing activity against HNSCC in *in vitro* and *in vivo* studies. The cell growth inhibition *in vitro* and the mouse model with tumor xenografts treated with telomelysin (10^8 PFU/day, once or three times) showed a dose-dependent inhibition of tumor growth on the treated side. The intratumoral administration of telomelysin is a rational therapy for HNSCC patients because most patients have visible tumors in the head and neck regions.

Endo *et al* (13) reported that telomelysin replication produced the endogenous danger-signaling molecule, uric



B

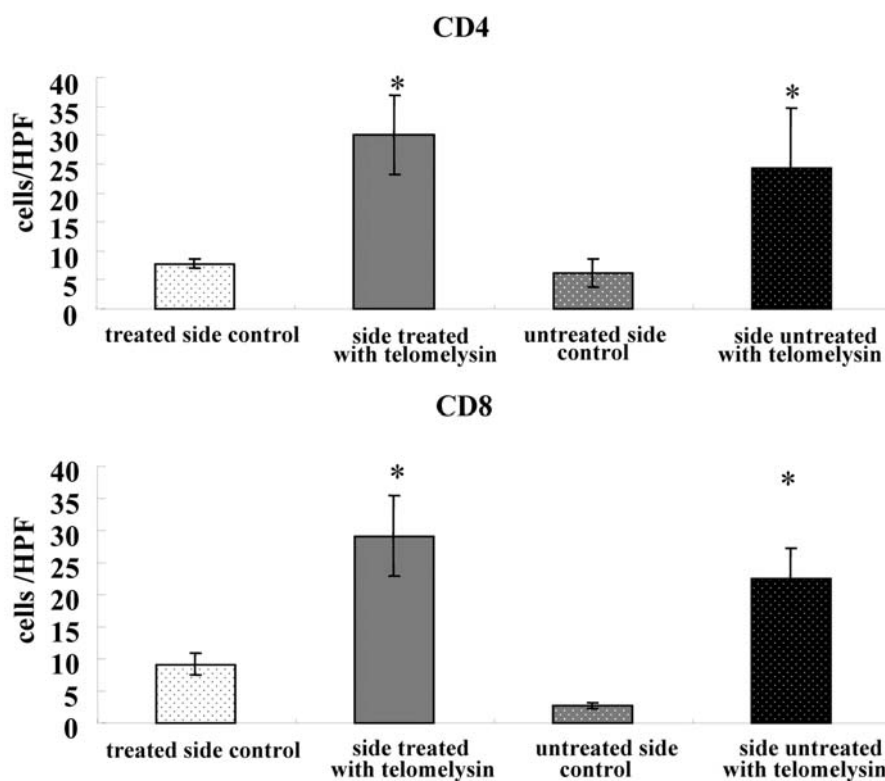


Figure 5. (A and B) Seven days after telomelysin treatment, control and treated mice were sacrificed. Tumors from the treated and untreated sides were sectioned and stained for CD4 and CD8 T-cell infiltration. Magnification, x400. Five fields per histological section were included in the analysis. Error bars indicate SE and *P<0.01 versus control.

acid, in infected human tumor cells, which in turn stimulated dendritic cells (DCs) to produce IFN- γ and IL-12 *in vitro*. We demonstrated that telomelysin induced the apoptotic cell death of NR-S1 cells and that treatment with telomelysin led to tumor regression in treated and untreated tumors in an immunocompetent murine SCC model. In tumors on the untreated side, we found that telomelysin increased apoptotic tumor cells and induced CD4⁺ and CD8⁺ T cells responsible for the *in vivo* tumor regression of treated and untreated tumors. Subsequently, elevated IFN- γ production of spleen cells in mice treated with telomelysin was observed.

Recent studies have shown that telomelysin is effective not only as a direct cytotoxic agent but also as an immunostimulatory agent which can induce specific CTL for the remaining antigen-bearing tumor cells (13). Nicolas *et al* have debated on whether necrotic or apoptotic cells can stimulate DCs to cross-present cell-derived peptides (15). Loading of DCs with tumor antigens can be facilitated by the selective use of a stress regimen that promotes apoptosis, which raises the possibility of a receptor-mediated uptake of apoptotic materials (12,15,16). Therefore, telomelysin may increase the source of tumor antigens for immune cells, and accelerate or sustain the anti-tumor immunity, resulting in therapeutic effects. Our data support this hypothesis.

We investigated the anti-tumor effect of telomelysin combined with DCs (data not shown). The results demonstrated that treatment with telomelysin combined with the injection of DCs led to complete tumor regression, in contrast to only partial eradication of the tumors with DCs alone. This approach is based on previous findings that DCs are able to take up apoptosis of tumor cells and present antigens associated with tumor cells (12,16). Although DCs alone did not induce reproducible anti-tumor effects on the untreated tumor side, the combination of telomelysin treatment and administration of intratumoral DCs produced stronger anti-tumor effects on remote tumors. We hypothesize that the increased apoptotic tumor cells, following treatment with telomelysin, release tumor antigens that are taken up by DCs.

In contrast, Huang *et al* and other researchers reported that telomelysin was transported to the untreated tumor through the bloodstream and then sprinted within the xenograft tumor in nude mice (8,14,17). In this study, we analyzed an immunocompetent mouse model. Therefore, the anti-tumor activity of telomelysin on the untreated side may not only be a direct effect of the bloodstream but also an immunological activity induced by increasing the source of tumor antigens.

In the present study, we successfully demonstrated the anti-tumor effects of telomelysin *in vitro* and *in vivo*. Telomelysin may be applied for the therapy of advanced HNSCC patients with multiple tumors, especially multiple metastases.

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