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 ribonucleoprotein 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),
lung metastasis of tongue cancer (KCC-TCM901, KCC-TCM902 and KCC-TCM903) and maxillary sinus (KCC-MS871 and KCC-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) 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 1x10^3 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: 5x10^6 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 10^6 plaque-forming units (PFU) per tumor. Tumor diameters

<table>
<thead>
<tr>
<th>HNSCC cell line</th>
<th>Primary sites</th>
<th>Telomelysin ID50 (MOI)</th>
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<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
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<tr>
<td>YCU-OR891</td>
<td>Oral floor</td>
<td>13875</td>
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<tr>
<td>YCU-H891</td>
<td>Hypopharynx</td>
<td>2886</td>
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<td>12389</td>
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<td>KCC-L891</td>
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<td>YCU-T891</td>
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<tr>
<td>KCC-MS861</td>
<td>Maxillary sinus</td>
<td>3311</td>
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</table>

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

**IFN-γ assay.** Spleen cells (1x10^6/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 (1x10^5/ml). Supernatants were collected 5 days later, centrifuged (1500 x 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 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 ID50 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±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
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 10⁸ 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 (10⁶ 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
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.
tumor cells and induced CD4+ and CD8+ T cells responsible for 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 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. 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 apoptotic bodies from 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 splintered within the xenograft tumor in nude mice (8,14,17). In this study, we analyzed an immunocomplete 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.

References