

Antitumor effects of telomelysin in combination with paclitaxel or cisplatin on head and neck squamous cell carcinoma

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Abstract. Telomelysin (OBP-301) is a telomerase-specific replication-component adenovirus. Telomelysin has a human telomerase reverse transcriptase (hTERT) promoter element which efficiently kills human cancer cells, but not normal cells. The present study investigated the correlation between the antitumor effect of telomelysin and mRNA expression of hTERT and coxsackievirus and adenovirus receptor (CAR) in head and neck squamous cell carcinoma (HNSCC) *in vitro* and whether telomelysin enhances the antitumor effect of paclitaxel or cisplatin, *in vivo* using a HNSCC xenograft model. We also determined the optimal order for combining telomelysin treatment and chemotherapy as concurrent treatment, telomelysin treatment first and chemotherapy later, chemotherapy first and telomelysin treatment later for achieving the best anticancer effect. The mRNA expression of hTERT and CAR genes was examined by quantitative RT-PCR in 17 HNSCC cell lines. There was no significant correlation between the growth inhibition of telomelysin (ID_{50} for day 3, 5 and 7) *in vitro* and mRNA expression levels of hTERT and CAR. Regarding the correlation between CAR expression and telomelysin ID_{50} for day 3, all cell lines that showed a relative amount of CAR/ β -actin mRNA >0.4 had a low telomelysin ID_{50} . This may indicate that CAR expression contributes to the efficacy of adenovirus infection and the antitumor activity of telomelysin in early stages of treatment. In our *in vivo* study, combining telomelysin and paclitaxel had an additive effect regardless of treatment order. On the other hand, combining telomelysin and cisplatin had additive effect only when cisplatin treatment preceded telomelysin treatment. These results suggest that

paclitaxel is considered innocuous for replication of telomelysin, however cisplatin may influence replication of telomelysin.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer; 500,000-900,000 cases are newly diagnosed annually worldwide (1). The standard treatment for advanced HNSCC includes surgery, radiotherapy and chemotherapy. These treatments modalities prolong the survival of individuals with advanced cases of HNSCC, however the treatment benefit is typically temporary in advanced disease (2). Thus, novel and more effective antitumor agents e.g. targeted molecular therapy, immunotherapy and gene therapy are necessary for treating advanced HNSCC. To improve the therapeutic index, what is needed are agents that target only tumor cells and not normal cells.

Oncolytic adenoviruses have been developed for the treatment of human cancer. These viruses are designed to replicate and selectively kill cancer cells while having a minimum effect on normal cells (3). Such vectors have been approved for clinical trials (4-8). However preclinical and clinical studies have revealed that the clinical application of these agents is hampered by their weak anticancer activity. Therefore, the development of strategies that maximize their anticancer activity is essential to the success of these agents in targeting cancer.

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends (9). Human telomerase reverse transcriptase (hTERT) positively regulates telomerase at the transcriptional level and shows a selectively high activity in growing neoplastic tissues and cells. Many studies have shown that telomerase activity is expressed in $>85\%$ of human cancers, but only in a few normal somatic cell types (10,11). Therefore, telomerase is an attractive target for the treatment of cancer. Telomelysin (OBP-301) is a telomerase-specific replication-component adenovirus that induces selective E1 expression and exclusively kills human cancer cells (12-15).

Adenoviruses initiate both infection and adenovector-mediated gene transfer by attachment of their fiber knobs to a cell surface receptor, the coxsackievirus and adenovirus receptor (CAR) (16). CAR, the primary high-affinity receptor for adenoviruses, is a 46-ku transmembrane glycoprotein and

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belongs to the immunoglobulin superfamily (17,18). Expression of CAR has been studied in numerous cell lines (19-25). In these studies, high expression of CAR correlated with increased adenoviral infection efficacy; cells lacking CAR or expressing low levels of CAR were resistant to adenovirus infection.

These findings led us to examine the correlation between the antitumor effect of telomelysin and mRNA expression levels of hTERT and CAR. Antitumor effect of telomelysin for HNSCC was reported previously (26) and we further examined the combination therapy of telomelysin and chemotherapy (cisplatin or paclitaxel) for the purpose of improving the antitumor effect against HNSCC.

Materials and methods

Adenovirus and chemotherapeutic agents. 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 (12,13). Telomelysin was kindly provided by Oncolys Biopharma (Tokyo, Japan). Paclitaxel (Taxol®) and cisplatin (Briplatin®) were purchased from Bristol-Myers Squibb (Park Avenue, NY). Paclitaxel and cisplatin were diluted with saline just before use for *in vivo* studies.

Cell lines and culture conditions. Seventeen human HNSCC cell lines were examined in this study. The origins of these cell lines were the oral floor (YCU-OR891), hypopharynx (YCU-H891), mesopharynx (YCU-M862, KCC-M871 and YCU-M911), larynx (KCC-L871 and YCU-L891), tongue (KCC-T871, KCC-T873, YCU-T891, YCU-T892 and HSC3), and maxillary sinus (KCC-MS871 and YCU-MS861) and metastatic tumors from different tongue carcinomas (KCC-TCM901, KCC-TCM902 and KCC-TCM903). These cell lines were maintained in RPMI-1640 medium (Life Technologies Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. These cells were incubated at 37°C in a moist atmosphere containing 5% CO₂.

Detection of hTERT and CAR mRNAs by quantitative RT-PCR. Cells were grown until nearly confluent in RPMI-1640 supplemented with 10% FBS. Total RNA extracted from 17 human HNSCC cells with phenol solution (Isogen; Nippon Gene Inc., Tokyo, Japan) according to the manufacturer's protocol. One microgram of total RNA was converted into cDNA by using Takara RNA PCR kit (AMV) Ver.3.0 (Takara Bio Inc., Tokyo, Japan). The quantification of relative expression levels of β-actin and hTERT were carried out with Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) using an ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The quantification of relative expression levels of CAR were carried out with TaqMan Gene Expression Master Mix (Applied Biosystems) using an ABI Prism® 7500 Sequence Detection System (Applied Biosystems). PCR was conducted using the following cycle parameters: 50°C for 2 min, 95°C

for 10 min, followed by 60 cycles at 95°C for 15 sec and 60°C 1 min. The primers used to detect each factor were as follows: β-actin: HLUX3000126 (Invitrogen); hTERT: HLUX3011813 (Invitrogen); CAR: Hs00154661 (Applied Biosystems). The results were normalized by the corresponding β-actin levels. Each PCR amplification was performed at least three times and the mean was calculated.

In vivo antitumor activity studies. Female BALB/c nu/nu nude mice, 6-week-old, were obtained from Oriental Yeast (Tokyo, Japan). The mice were maintained in a laminar flow room with a constant temperature and humidity. The animals were maintained and experiments were conducted at the Yokohama City University School of Medicine, Laboratory Animal Facility. The experiments were conducted according to the guidelines for animal experiments set by the Animal Experiment Committee of Yokohama City University School of Medicine. Suspensions of YCU-H891 cells (100 µl) (final concentration, 1x10⁷ cells/100 µl) were injected s.c. into the right flank of the mice. Tumor-bearing mice were randomized (n=6) when the mean tumor volume was 50-100 mm³. Each group was closely matched before treatment, which began one week after cell transplantation. The mice were treated i.t. with telomelysin (5x10⁷ PFU/mouse) or PBS (vehicle) on day 7 or 14. Paclitaxel or saline (vehicle) was administered i.p. (7.5 mg/kg/day 2 times on day 7 and 11 or day 14 and 18). Cisplatin or saline (vehicle) was administered i.p. (2.2 mg/kg/day on day 7 or 14). The control mice were not treated with any material. Treatment group number was 15 and the combination of treatment and administration orders are shown in Fig. 3A and B. Tumor diameters 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; b = width).

Evaluation of apoptosis. Terminal deoxynucleotidyl transferase-mediated cUDP nick end-labeling (TUNEL) was done for the evaluation of apoptosis. This was evaluated using an Apoptosis In Situ Detection Kit (Wako Chemical, Osaka, Japan). The apoptotic index was calculated as the percentage of positive cell nuclei stained with peroxidase relative to the total number of cells in the fields at x400 magnification. Six fields per histological section were included in the analysis.

Evaluation of vessel density. In order to estimate the vessel density, the vessels in the tumor tissues were stained with an anti-CD31 antibody (1:200; Dako, Denmark). The tumors were excised and blocked with 2% goat serum and 1% bovine serum albumin in PBS and stained with the antibody. Slides were developed using 3,3'-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories, Inc., Burlingame, CA). Vessel density was determined by counting the stained vascular endothelial cells in the fields at x100 magnification. Six fields per histological section were included in the analysis.

Statistical analysis. For statistical analyses of the correlation between ID₅₀ of telomelysin and mRNA expression of hTERT or CAR, we used Spearman's correlation coefficient or by the rank test. For statistical analyses of *in vivo* antitumor activity,

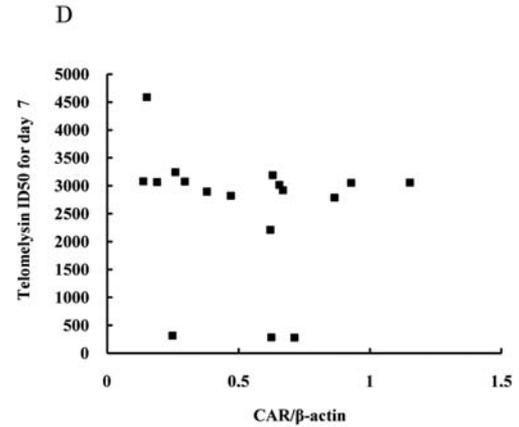
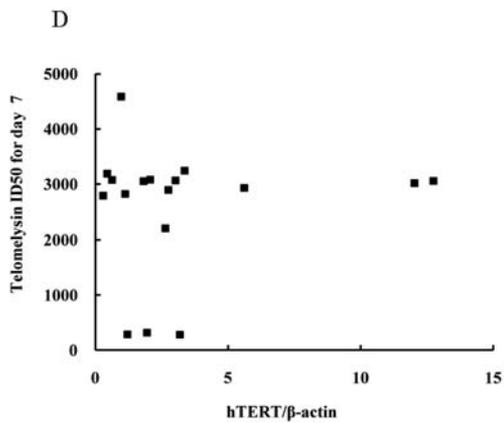
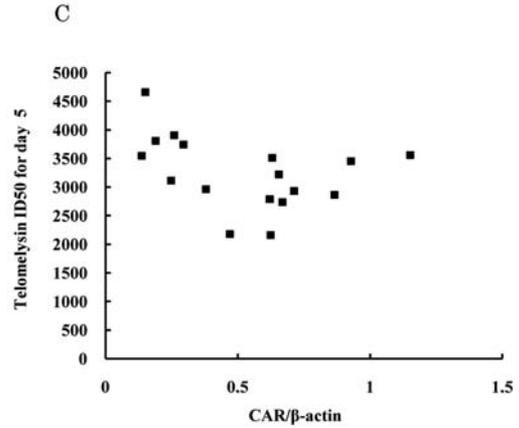
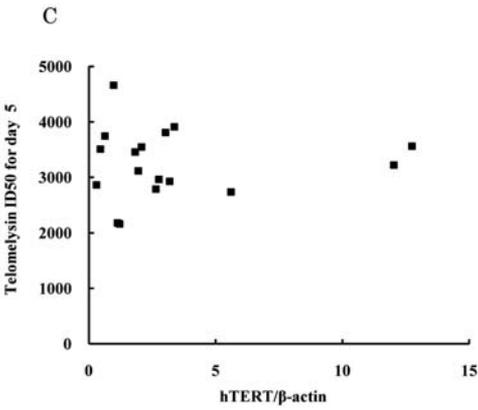
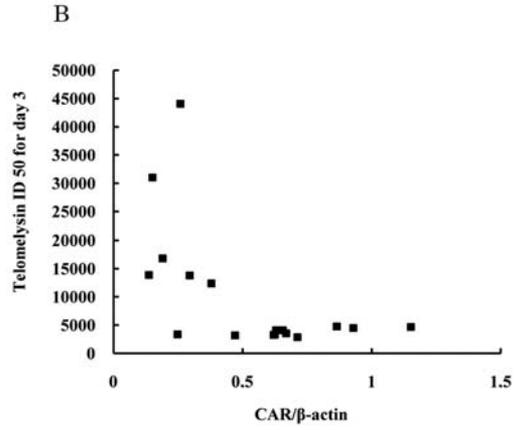
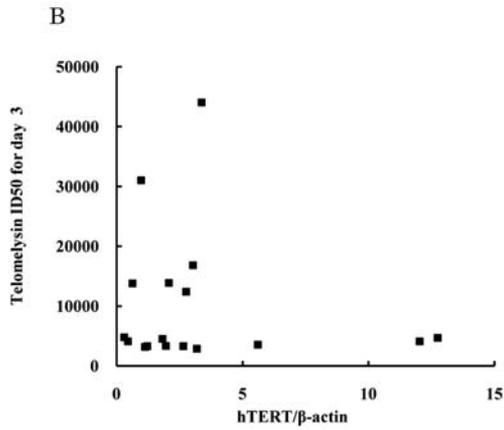
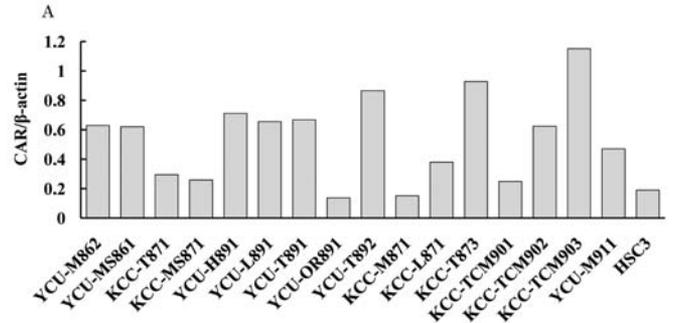
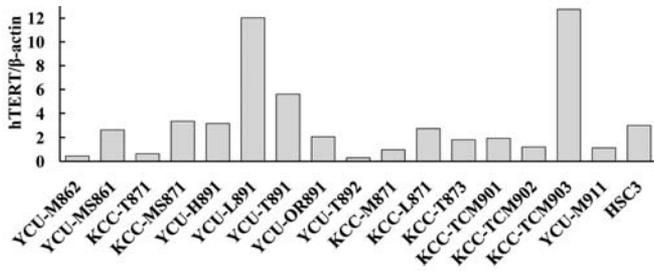


Figure 1. (A) Expression of hTERT mRNAs in HNSCC cell lines. Expression of mRNA was determined by real-time RT-PCR in 17 HNSCC cell lines. The level of each mRNA was normalized relative to β -actin mRNA. (B) Correlation between hTERT expression and telomelysin ID₅₀ for day 3. (C) Correlation between hTERT expression and telomelysin ID₅₀ for day 5. (D) Correlation between hTERT expression and telomelysin ID₅₀ for day 7. Correlation was not observed in all groups ($P < 0.05$). Statistical correlation was analyzed with Spearman's correlation coefficient or by the rank test.

Figure 2. (A) Expression of CAR mRNAs in HNSCC cell lines. Expression of mRNA was determined by real-time RT-PCR in 17 HNSCC cell lines. The level of each mRNA was normalized relative to β -actin mRNA. (B) Correlation between CAR expression and telomelysin ID₅₀ for day 3. (C) Correlation between CAR expression and telomelysin ID₅₀ for day 5. (D) Correlation between CAR expression and telomelysin ID₅₀ for day 7. Correlation was not observed in all groups ($P < 0.05$). Statistical correlation was analyzed with Spearman's correlation coefficient or by the rank test.

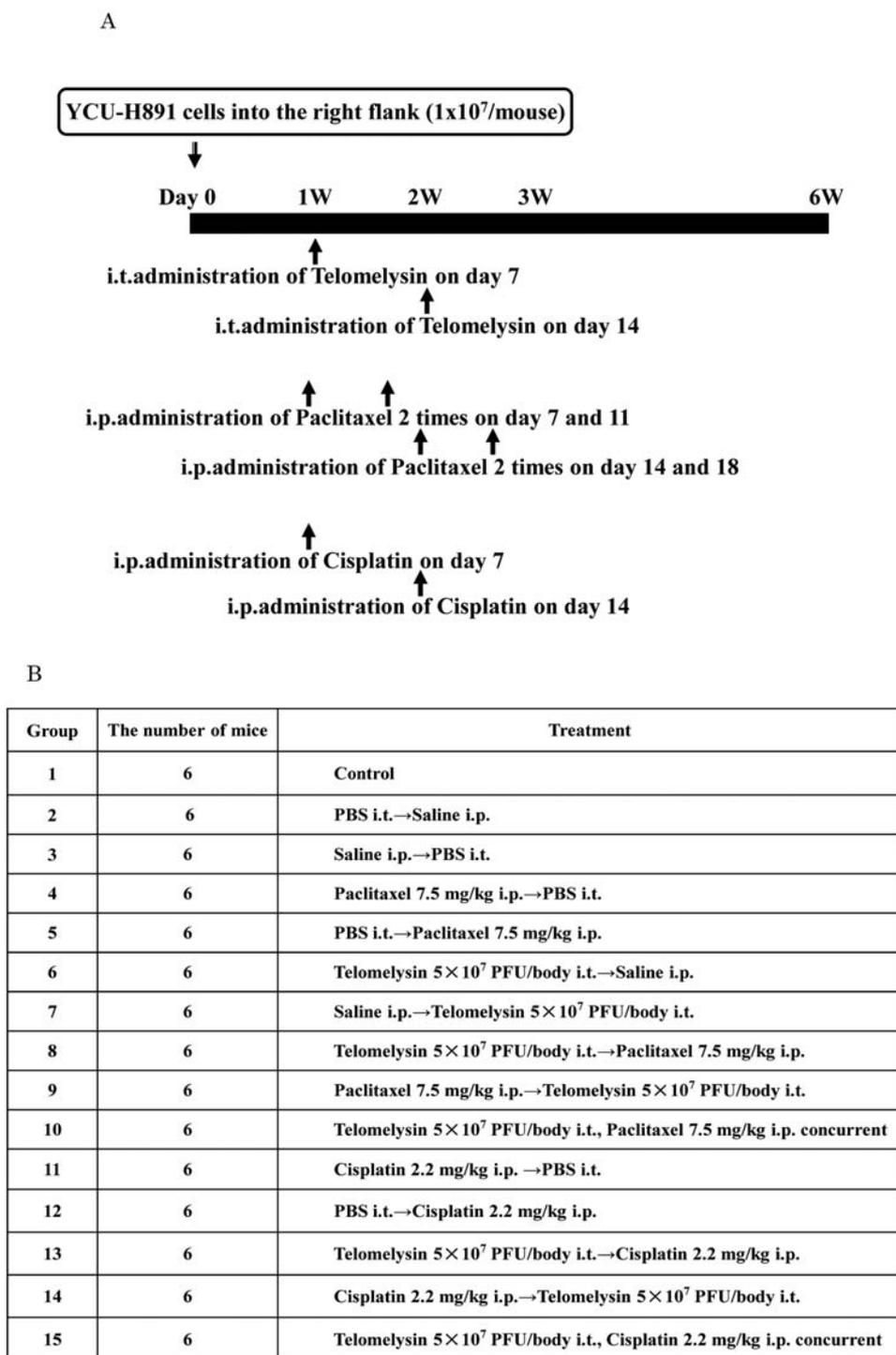


Figure 3. Animal experiment regimens. (A) Administration schedule of telomelysin and chemotherapeutic agents. Mice were inoculated with 1×10^7 YCU-H891 cells on day 0. Telomelysin (5×10^7 PFU/mouse) was administered i.t. on day 8 or 14. Paclitaxel (7.5 mg/kg) was administered i.p. 2 times on day 7 and 11, or day 14 and 18. Cisplatin (2.2 mg/kg) was administered i.p. on day 7 or 14. (B) The group number was 15 and 6 mice were used in each group. Telomelysin treatment alone and three orders of combination treatment of telomelysin and chemotherapy - telomelysin and chemotherapy concurrently, telomelysin and then chemotherapy, chemotherapy and then telomelysin were examined.

apoptotic index, vessel density, we used Student's paired t-test. A value of $P < 0.05$ was considered significant.

Results

Effect of expression of hTERT and CAR mRNAs on 17 HNSCC cell lines and correlation with telomelysin ID₅₀. To determine the correlation between the telomelysin ID₅₀ and expression

of hTERT and CAR, we analyzed levels of mRNA for hTERT and CAR in 17 HNSCC cell lines by quantitative RT-PCR. Although expression levels of hTERT mRNA, which plays a key role in telomerase activation (27), varied widely, all cell lines expressed mRNA encoding hTERT (Fig. 1A). In an earlier study, we reestablished the telomelysin ID₅₀ for of day 3, 5 and 7 (26). The correlation between expression levels of hTERT mRNA and telomelysin ID₅₀ was not

SPANDIDOS PUBLICATIONS by Spearman's correlation coefficient or by the (Fig. 1B-D). These results suggest that the hTERT promoter element is an important target in human cancer therapy, but its expression does not correlate with the therapeutic efficacy of telomelysin. Although all cell lines expressed CAR mRNA (Fig. 2A), correlation was not observed between CAR mRNA and the telomelysin ID₅₀ (Fig. 2B-D). The cell lines that showed a relative mRNA value >0.4 for the ratio CAR/ β -actin, had a low telomelysin ID₅₀.

In vivo studies of the antitumor effects of telomelysin in combination with cisplatin and paclitaxel on HNSCC xenografts. We reported an inhibitory effect of telomelysin on tumor growth in the HNSCC xenograft model (26). Here we investigated the *in vivo* antitumor activity of combination therapy of telomelysin and paclitaxel or cisplatin in nude mice bearing YCU-H891 squamous cell carcinoma xenografts. To investigate the influence of chemotherapeutic agents on infection efficacy and antitumor activity combined with telomelysin, we administered combination therapy of telomelysin and chemotherapy in three orders: telomelysin treatment and chemotherapy concurrently, telomelysin treatment and then chemotherapy, chemotherapy and then telomelysin. As shown in Fig. 4A, paclitaxel treatment alone (7.5 mg/kg/day, 2 times) or telomelysin alone (5×10^7 PFU/mouse) did not cause a complete regression of tumors, however combination therapy of the two reduced the tumor volume compared to telomelysin or paclitaxel alone (Fig. 4A). There was a marked reduction in tumor volume after a regimen of concurrent telomelysin and paclitaxel and after paclitaxel then telomelysin compared to telomelysin alone ($P < 0.05$; Fig. 4A).

Regarding cisplatin, cisplatin then telomelysin caused marked reduction in tumor volume compared to telomelysin alone ($P < 0.05$; Fig. 4B). Combining these two drugs did not show a combination effect on tumor volume for telomelysin then cisplatin or for concurrent telomelysin and cisplatin.

Effect of combination treatment on apoptosis in the xenograft model. Histopathological analysis of the xenograft samples was done to examine apoptotic events, one of antitumor mechanisms (Fig. 5A). The all combination protocols of telomelysin and paclitaxel showed an increased number of TUNEL-positive cells (Fig. 5B). The apoptotic index was significantly higher in tumors from mice treated with telomelysin and paclitaxel concurrently, or with telomelysin then paclitaxel compared to paclitaxel alone ($P < 0.05$; Fig. 5B).

Telomelysin in combination with cisplatin, for all combination protocols show a higher apoptotic index, but statistical significance was not reached (Fig. 5C).

Effect of combination treatment on microvascular content in the xenograft model. To determine the effects of the combination treatment of telomelysin and paclitaxel or cisplatin on tumor neovascularization, the vessel density of the xenograft tumors was examined to clarify antitumor mechanisms (Fig. 6A). The all combination protocols of telomelysin and paclitaxel showed a lower number of microvessels positive for CD31 staining compared to telomelysin or paclitaxel alone, but there was no statistical significance (Fig. 6B). For the combination of telomelysin and cisplatin, all the protocols

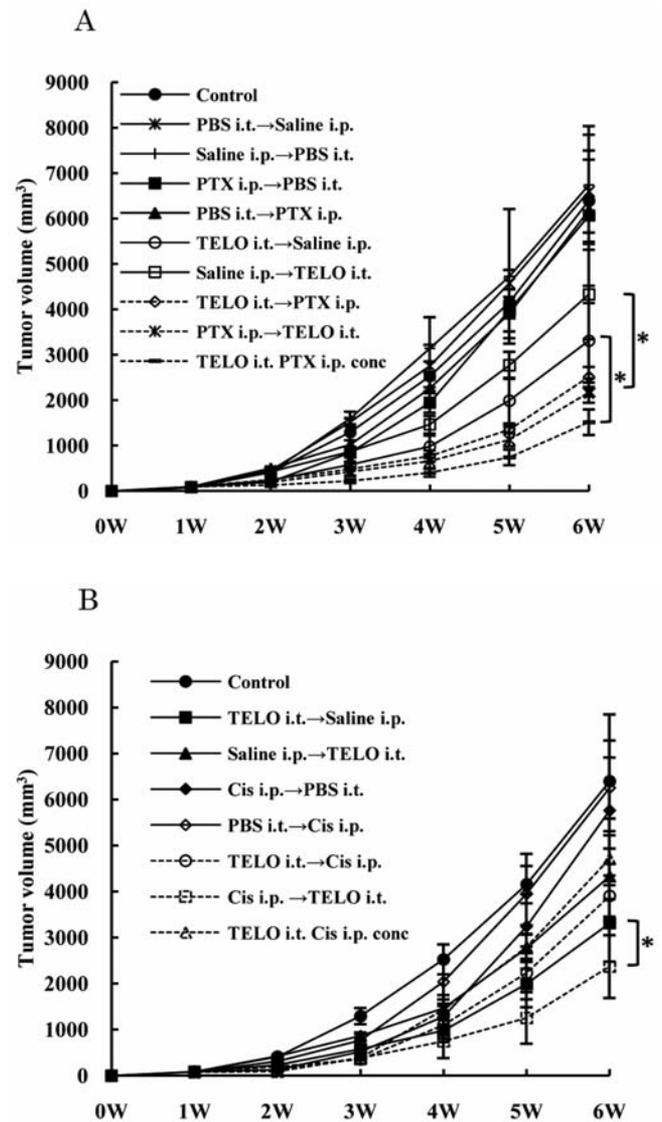


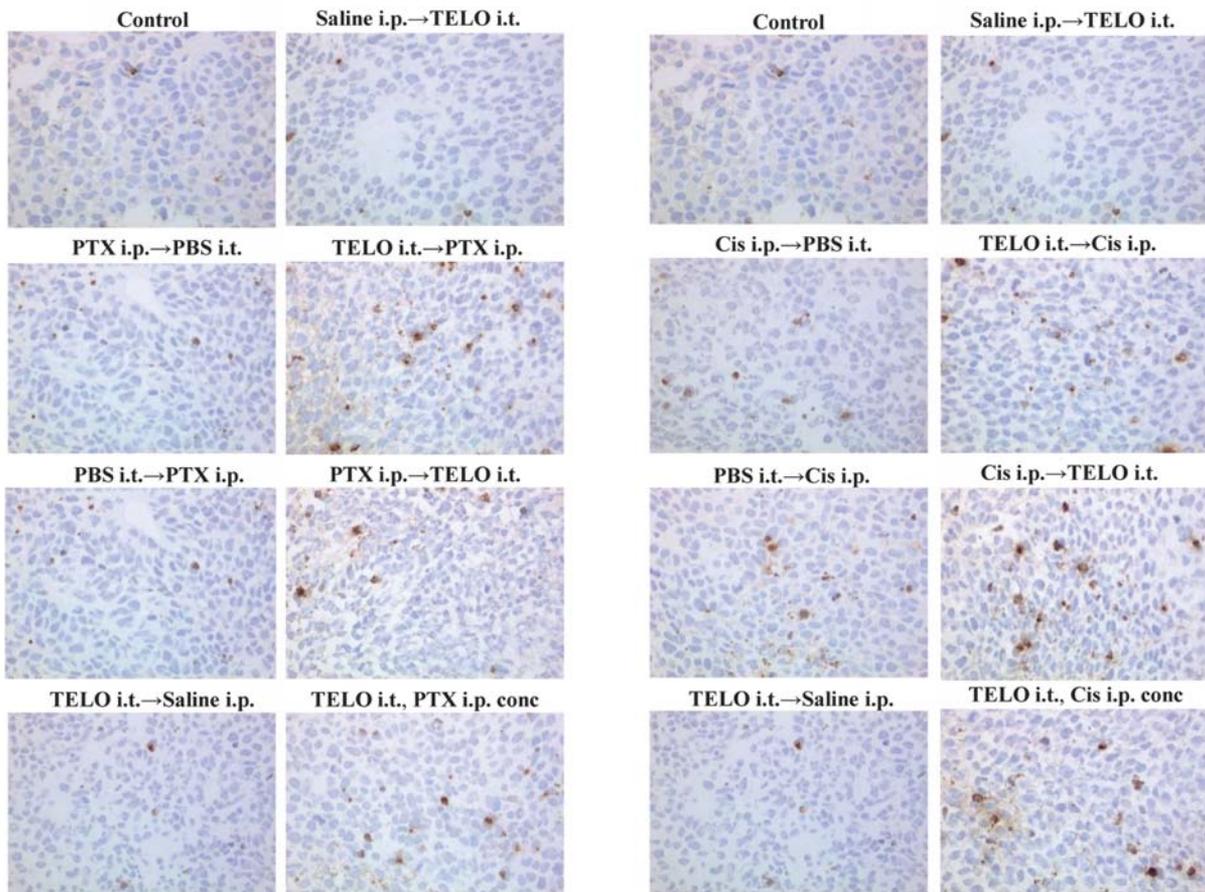
Figure 4. Effect of telomelysin and chemotherapy on the growth of HNSCC xenograft model. (A) The group of combination treatment of telomelysin and paclitaxel concurrently (telomelysin: 5×10^7 PFU/body i.t. on day 7, paclitaxel:7.5 mg/kg i.p. 2 times on day 7 and 11) and the group of combination treatment of telomelysin and then paclitaxel (paclitaxel:7.5 mg/kg i.p. 2 times on day 7 and 11, telomelysin: 5×10^7 PFU/body i.t. on day 14) demonstrated significant growth inhibition compared to telomelysin alone (telomelysin: 5×10^7 PFU/body i.t. on day 7 or 14) ($P < 0.05$). (B) The group of combination treatment of cisplatin and then telomelysin (telomelysin: 5×10^7 PFU/body i.t. on day 14, cisplatin:2.2 mg/kg i.p. on day 7) demonstrated significant growth inhibition compared to telomelysin alone (telomelysin: 5×10^7 PFU/body i.t. on day 14) ($P < 0.05$). Error bars indicates SE. Significant difference was analyzed by Student's paired t-test.

resulted in almost the same number of microvessels positive for CD31 staining compared to the control group (Fig. 6C).

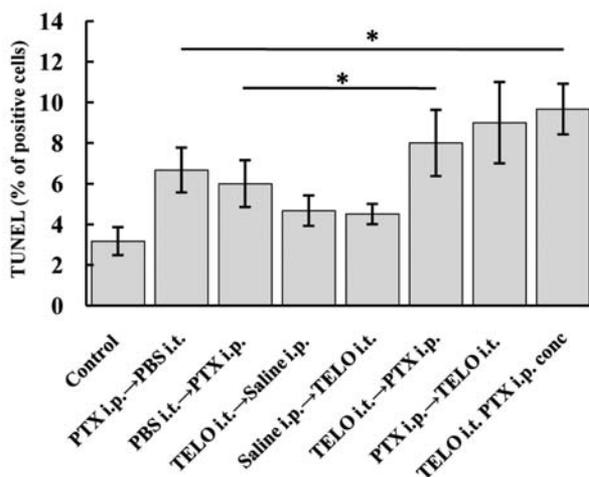
Discussion

Telomelysin has been clarified to be effective against human cancers (12,13,15). We also reported that telomelysin has a strong antitumor effect against human HNSCC cell lines *in vitro* and against a murine HNSCC cell line *in vitro* and *in vivo* (26). Telomelysin has a human telomerase reverse

A

TUNEL ($\times 400$)

B



C

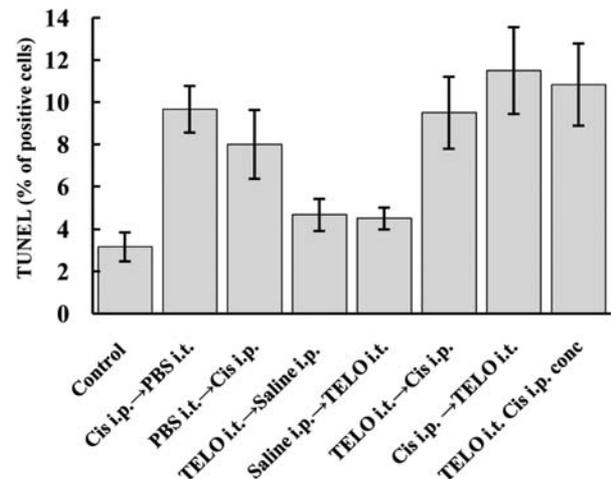
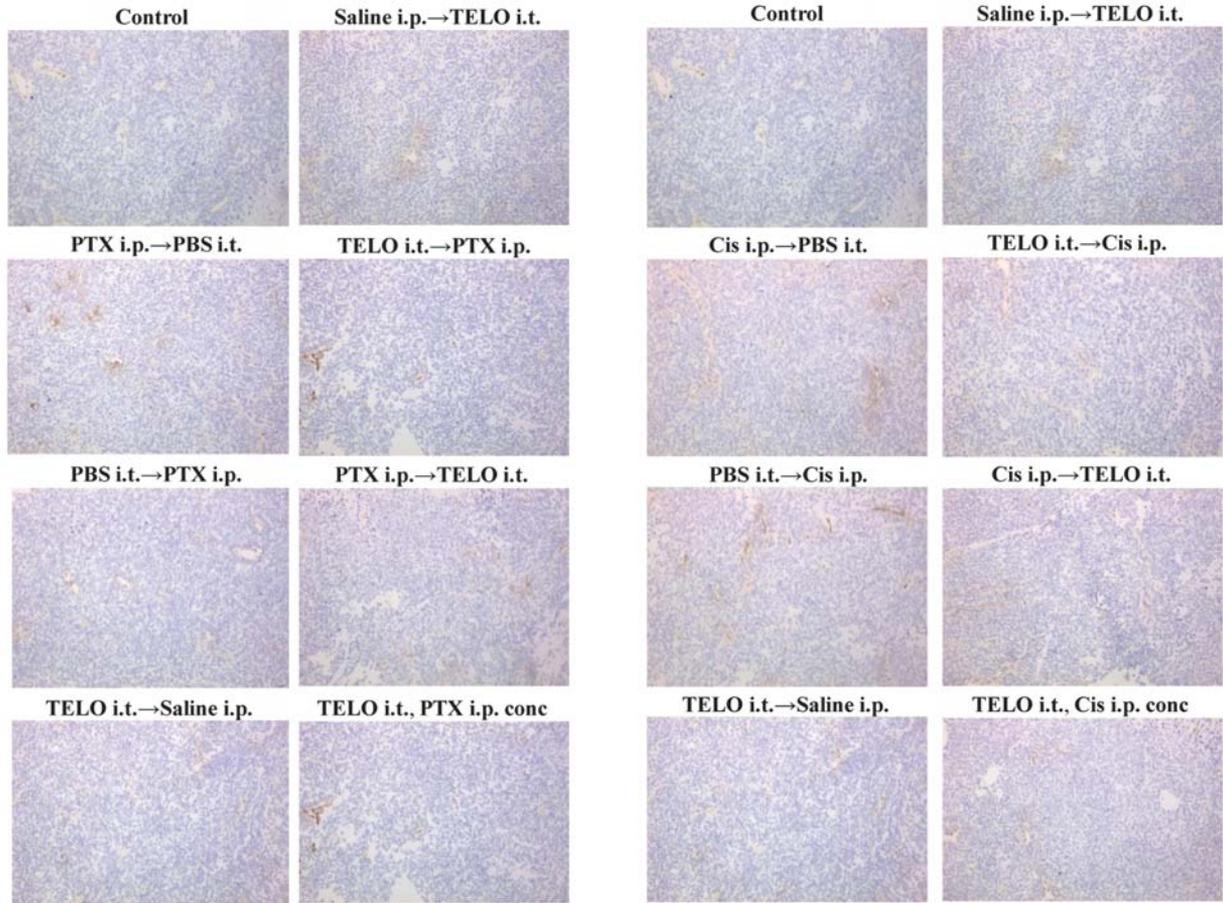


Figure 5. Apoptotic events in YCU-H891 tumor xenografts. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was utilized. (A) Histopathological analysis of apoptosis by TUNEL about the group of combination treatment of telomelysin and paclitaxel or cisplatin ($\times 400$). (B) Effects of telomelysin and paclitaxel on apoptotic index (TUNEL). Statistical significance was observed in the group of telomelysin and paclitaxel concurrently and the group of telomelysin and then paclitaxel compared to paclitaxel alone ($P < 0.05$). (C) Effects of telomelysin and cisplatin on apoptotic index (TUNEL). Statistical significance was not observed in the group of combination treatment of telomelysin and cisplatin compared to cisplatin alone ($P < 0.05$). Data represent mean values (\pm SD). Significant difference was analyzed by Student's paired t-test.

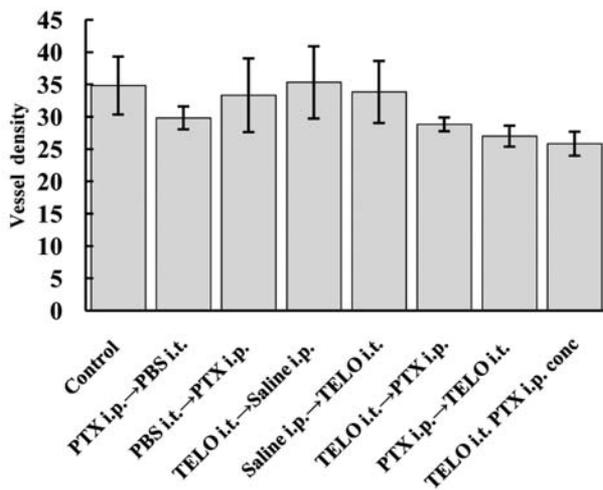
transcriptase promoter that regulates viral replication and efficiently kills human cancer cells. Human telomerase reverse transcriptase (hTERT) positively regulates telomerase at the transcriptional level and shows a selectively high inhibitory

activity against growing neoplastic tissues and cells. Therefore, we investigated the relative mRNA expression of hTERT in 17 HNSCC cell lines and evaluated the correlation of these mRNA species with the telomelysin ID_{50} reported by us

CD31 (× 100)



B



C

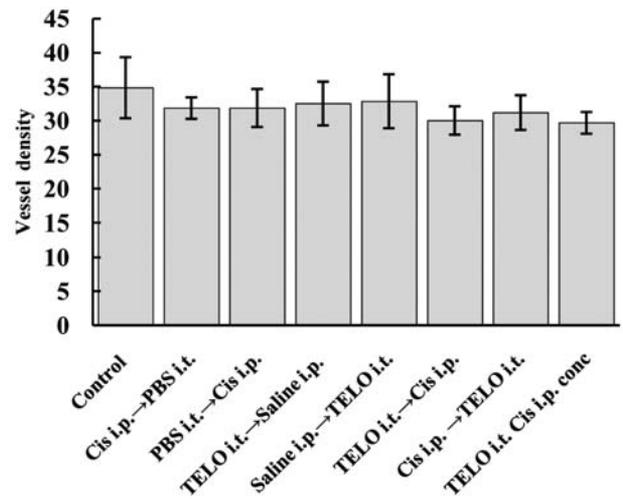


Figure 6. Vessel density in YCU-H891 tumor xenografts. The vessels of tumors were stained with anti-CD31 antibody (x100). (A) Vascular attaining about the group of combination treatment of telomelysin and paclitaxel or cisplatin. (B) Effects of telomelysin and paclitaxel on vessel density. Statistical significance was not observed in the group of combination treatment of telomelysin and paclitaxel compared to paclitaxel alone ($P < 0.05$). (C) Effects of telomelysin and cisplatin on vessel density. Statistical significance was not observed in each group ($P < 0.05$). Data represent mean values (\pm SD). Significant difference was analyzed by Student's paired t-test.

previously (26). Similarly to the other results (12), elevated levels of telomerase activity were observed in the all cell

lines, but the extent of mRNA expression of hTERT was not significantly correlated with telomelysin ID₅₀.

The infection efficiency of the currently available adenovirus agent, which is derived from human adenovirus serotype 5, varies widely depending on expression of the coxsackievirus-adenovirus receptor (CAR) (28). Therefore, the interaction of the adenovirus with CAR on the cell surface is a key mechanism by which adenoviral agents enter cells. Some studies have shown that anticancer agents such as FR901228, etoposide and topotecan, increase adenoviral infection via enhancement of CAR expression on cancer and normal cells (14,29,30). Based on these studies, we investigated the relative mRNA expression of CAR in 17 HNSCC cell lines and evaluated the correlation of CAR with telomelysin ID₅₀. The mRNA expression of CAR was not significantly correlated with the efficacy of telomelysin, although the mRNA expression of CAR was observed in all 17 HNSCC cell lines. For the telomelysin ID₅₀ for day 3 after the treatment, cell lines that showed relative mRNA expression of CAR that was >0.4 of, tended to low telomelysin ID₅₀. A statistical correlation was not observed between mRNA expression and telomelysin ID₅₀ for day 3, but a relative mRNA expression of CAR appears to the efficacy of telomelysin infection and the antitumor activity at early stages after telomelysin treatment. For day 5 or 7 after telomelysin treatment, no significant correlation was observed between mRNA expression of CAR and telomelysin ID₅₀. Therefore, another factor must be involved in the long-term efficacy of telomelysin treatment.

Next we investigated the antitumor effect of telomelysin combined with the chemotherapeutic agents paclitaxel or cisplatin. Fujiwara *et al* reported that combination therapy of telomelysin and docetaxel produced additive therapeutic benefits over either individual modality and that docetaxel was considered to be innocuous for replication of telomelysin because this agent did not inhibit DNA synthesis (31). Watanabe *et al* reported that FR901228 with or without telomelysin had no effect on cell cycle distribution and that FR901228 might be an appropriate partner for this oncolytic adenovirus because it does not affect the virus life cycle. To clarify the influence of paclitaxel and cisplatin on the antitumor effects of telomelysin, we studied combination therapy of telomelysin with paclitaxel or cisplatin in three protocols: telomelysin and chemotherapy concurrently, telomelysin then chemotherapy, chemotherapy then telomelysin. For the combination of telomelysin and paclitaxel, the all three protocols showed an additive antitumor effect in the xenograft model. Among these three protocols, concurrent administration was most effective. This result supports the idea that paclitaxel is an appropriate partner with telomelysin therapy for HNSCC and has no influence on the distribution of telomelysin in cancer cells. Regarding cisplatin, cisplatin treatment then telomelysin, but not the other protocols, showed an additive antitumor effect. Based on this result, we conclude that cisplatin will affect the negative influence on the antitumor activity of telomelysin. One reason for this result might be that cisplatin effects the cell cycle. Cisplatin arrests the cell cycle in G1 phase. Adenoviral E1A directs the progression of cells from G1/G2 phase to S phase to optimize viral DNA replication (31). So, cisplatin might have deleterious effects on viral replication. Furthermore, cisplatin may affect the DNA

of adenoviruses directly, because cisplatin is an anti-DNA synthesis agent.

On the other hand, paclitaxel is an anticancer agent that targets microtubules and arrests the cell cycle in G2 and M phases. These mechanisms induce apoptosis and does not inhibit DNA synthesis of host cells.

To evaluate apoptotic events of antitumor effect *in vivo*, the TUNEL method was utilized. Regarding telomelysin alone, few apoptotic events were observed. We previously reported that telomelysin induced apoptotic cell death in the murine SCC model (26). In the present study we used BALB/c nu/nu mice, in which the immune system does not have active CD4⁺ and CD8⁺ T cells.

The apoptotic indexes in the combined treatment were higher compared with paclitaxel or cisplatin alone. Especially, concurrent telomelysin and paclitaxel and for telomelysin then paclitaxel showed significantly higher apoptotic indexes.

To evaluate vessel density *in vivo*, immunohistochemical staining was used. Vessels were detected by staining with an anti-CD31 antibody. Paclitaxel decreased the number of CD31-positive endothelial cells (32-34) and this supports the antiangiogenic activity of paclitaxel. But the combined treatment of telomelysin with cisplatin induced no antiangiogenic activity. Regarding angiogenesis, synergistic effects were not observed in either combination therapy of telomelysin and paclitaxel or cisplatin.

In conclusion, our results demonstrate that telomelysin and paclitaxel lead to a higher antitumor effect than the treatment of either agent alone on HNSCC xenografts. Therefore, paclitaxel is a promising agent for combination therapy with telomelysin. In contrast, cisplatin interfere with the replication and antitumor activity of telomelysin.

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