

An Experimental Model of Tumor Dormancy Therapy for Advanced Head and Neck Carcinoma

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An experimental model of tumor dormancy therapy for advanced head and neck carcinoma was developed. After transplantation of KB cells into nude mice, the mice were given tiracoxib, a selective cyclooxygenase (COX)-2 inhibitor, probucol, an antioxidant, and S-1, an oral pro-drug of 5-fluorouracil (5-FU), or combinations of two of them. The combined administration of tiracoxib with probucol significantly inhibited the tumor growth. The angiogenesis in this group was markedly reduced. Tiracoxib and probucol did not affect the intratumoral concentration of 5-FU when coadministered with S-1. The combined use of tiracoxib and probucol is thus a candidate for use in maintenance therapy after the primary therapy for patients with advanced head and neck carcinoma.

Key words: COX-2 inhibitor — Antioxidant — S-1 — Tumor dormancy — Head and neck carcinoma

Most head and neck cancer patients visit the outpatient clinic at an advanced stage, and they need to have radical operations as curative therapy. The head and neck region contains many sensory organs, e.g., nose, tongue, pharynx, and larynx, and the operation may result in the loss of many functions, e.g., aphonia, swallowing disturbance, dysgeusia. The operation may also cause cosmetic problems.

To avoid radical operation, concomitant chemoradiotherapy consisting of impact chemotherapy and definitive radiotherapy has been performed for patients with advanced head and neck cancer in our department. At the end of the curative therapy, we carefully evaluate the clinical and histopathological effects using imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonography (US), and histological study. However, it is impossible to be sure that there are no remnant tumor cells in the entire locoregional field. We have already reported inhibitory effects of tiracoxib,¹⁾ probucol²⁾ and S-1³⁾ on tumor growth. According to the concept of tumor dormancy therapy, which aims to prolong the life of patients with cancer, these drugs might be efficacious for maintenance therapy to reduce the tumor-relapse rate. In this work, we studied the therapeutic effects of combinations of these agents in terms of tumor growth.

MATERIALS AND METHODS

Materials Tiracoxib, a selective cyclooxygenase (COX)-2

inhibitor, was a kind gift from Japan Tobacco Inc. (Tokyo). Probucol, an antioxidant, was a kind gift from Daiichi Pharmaceutical Industries (Tokyo), and S-1, a new oral antineoplastic agent based on biochemical modulation of 5-fluorouracil (5-FU), was a kind gift from Taiho Pharmaceutical Co. (Tokyo). They were suspended in a 0.5% (w/v) hydroxypropylmethylcellulose (Shinetsu Chemical Co., Nagano) solution for *in vivo* administration. Female 5-week-old athymic nude mice (BALB/c *nu/nu*) were purchased from Charles River Japan Inc. (Atsugi). The animals were housed in a room kept at 24±2°C and 40–70% humidity with a 12 h light/dark cycle.

Cell line KB cells,⁴⁾ a human oral floor carcinoma cell line, were grown in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

***In vivo* tumor growth assay** To evaluate the addictive effects of tiracoxib, probucol and S-1 on tumor growth inhibition, each drug was used at a not significantly effective dosage, based on our previous data.^{1–3)} KB cells (5×10⁶) were injected subcutaneously into the left flanks of mice. The tumor size was measured once a week by determining two perpendicular dimensions with calipers, and the volume in mm³ was calculated from the formula ($a \times b^2$)/2, where *a* is the longer and *b* is the smaller dimension. Two weeks after inoculation, the tumors had grown to 250 mm³ in volume. Then, the mice were randomly divided into seven groups of 5 animals: A) vehicle control; B) 5 mg/kg of S-1; C) 10 mg/kg of tiracoxib; D) 50 mg/kg of probucol; E) 5 mg/kg of S-1 and 10 mg/kg of tiracoxib; F) 5 mg/kg of S-1 and 50 mg/kg of probu-

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col; G) 10 mg/kg of tiracoxib and 50 mg/kg of probucol. Each material was administered 5 days per week for 8 weeks. At the termination of the investigation, the animals were killed under anesthesia.

5-FU concentration At the conclusion of treatment, S-1-treated nude mice (S-1 alone or with tiracoxib or probucol) were sacrificed and tumor tissue was collected. 5-FU was assayed as described previously.³⁾

Immunohistochemistry Paraffin-embedded tumor sections were deparaffinized in xylene and rinsed in absolute ethanol. After treatment with 0.3% hydrogen peroxide, the sections were incubated in 1% *Bandeiraea simplicifolia* agglutinin for 20 min, and then incubated with an anti-factor VIII rabbit monoclonal antibody (Nichirei, Tokyo) and an anti-Ki-67 rabbit monoclonal antibody (Immunoteck, Marseille, France). Next the sections were incubated with biotinylated goat antirabbit immunoglobulin, followed by incubation with peroxidase-conjugated streptavidin (ABC kit; Nichirei). Reaction products were visualized by treatment with diaminobenzidine and the sections were examined under a microscope after counterstaining with hematoxylin.

Investigation of apoptosis The terminal deoxynucleotidyl transferase-mediated cUDP nick end labeling (TUNEL) method was performed using an Apop Taq Plus kit (Oncor, Gaithersburg, MD) for tumor sections. Counting of immunoreactive tumor cells was done in three different fields for each section, and the apoptotic index was expressed as the percentage of TUNEL-positive cells relative to the total number of cells.

Assay of telomerase Telomerase activity in tumor extracts was assayed by means of hybridization protection assay coupled with a telomeric repeat amplification protocol (TRAP/HPA), as described before.¹⁾

Tumor dormancy clinical model For head and neck squamous cell carcinoma, *cis*-diamminedichloroplatinum (CDDP) and 5-FU based chemotherapy (CF therapy) is commonly performed. In clinical use, CDDP and 5-FU are given by intravenous drip infusion, but in this model, CDDP is injected intratumorally and 5-FU is substituted for S-1. CF model therapy was performed as follows. On treatment day 1, we injected 10 mg/kg of CDDP into the local tumor, and from day 1 to 5, we administered S-1, 7.5 mg/kg/day as tegafur, orally. After confirming the additive effect of tiracoxib and probucol, the dosages were set at 30 mg/kg for tiracoxib and 100 mg/kg for probucol. These set points were significantly effective dose levels when each material was given individually, and we expected a more significant effect on tumor growth suppression than in the case of low-dose administration. KB cells (5×10^6) were injected in the same way as above. When the tumors had grown to 250 mm³ in volume, the mice were randomly divided into five groups of 5 animals: A) no treatment but with control vehicle; B) CF model

therapy alone; C) CF model therapy following tiracoxib administration; D) CF model therapy following probucol administration; E) CF model therapy following tiracoxib and probucol combined administration. After two courses of the CF model therapy, maintenance therapy with tiracoxib (30 mg/kg), probucol (100 mg/kg) or both of them was given. Tiracoxib and probucol were administered 5 days per week for 8 weeks, orally. The tumor size was measured as described above.

Statistical analysis Student's *t* test was used to compare different treatment groups and to obtain *P* values, with $P \leq 0.05$ being considered statistically significant.

RESULTS

Antitumor effect Female nude mice were implanted with

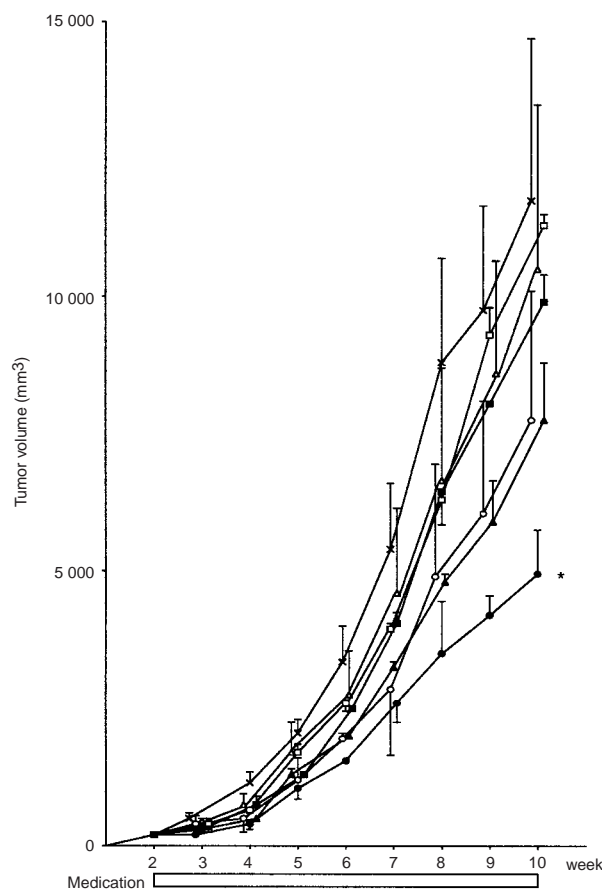


Fig. 1. Anti-tumor effect on tumor xenografts in nude mice. After implantation of KB cells, mice were given the test materials p.o. from day 14 (5 times/week). No mark, NS; *, $P < 0.05$; bars, SD; ×, control; ○, 10 mg/kg of tiracoxib; △, 50 mg/kg of probucol; □, 5 mg/kg of S-1; ●, 10 mg/kg of tiracoxib and 50 mg/kg of probucol; ▲, 10 mg/kg of tiracoxib and 5 mg/kg of S-1; ■, 50 mg/kg of probucol and 5 mg/kg of S-1.

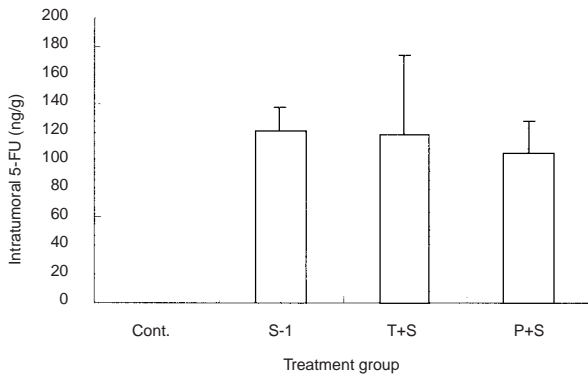


Fig. 2. Intra-tumoral 5-FU concentrations of S-1 treatment groups. 5-FU was not detected in the control group, as expected. There are no significant differences between S-1 alone and S-1 and tiracoxib or probucol treatment groups. Cont., control; T+S, tiracoxib and S-1; P+S, probucol and S-1. The Y-axis indicates concentration of 5-FU (ng/g).

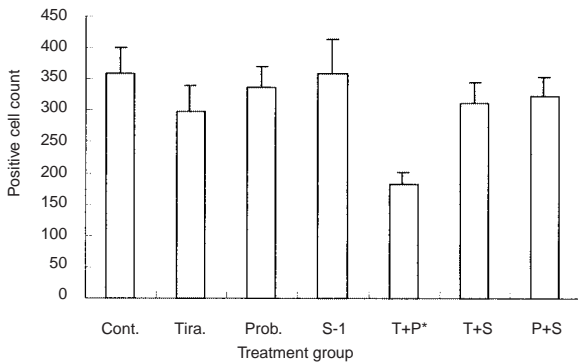


Fig. 3. The number of microvessels in the peripheral part of the tumor, positive for anti-human factor VIII, is significantly reduced when mice receive tiracoxib and probucol concomitantly. No mark, NS; *, $P < 0.05$; bars, SD; Cont., control; Tira., tiracoxib; Prob., probucol; T+P, tiracoxib and probucol; T+S, tiracoxib and S-1; P+S, probucol and S-1. The Y-axis indicates TUNEL-positive cells (%).

KB cell xenografts on day 0, and materials were administered orally, beginning on day 14 (5 times/week), for 8 weeks as follows: 5 mg/kg of S-1, 10 mg/kg of tiracoxib, 50 mg/kg of probucol, 5 mg/kg of S-1 and 10 mg/kg of tiracoxib, 5 mg/kg of S-1 and 50 mg/kg of probucol, and 10 mg/kg of tiracoxib and 50 mg/kg of probucol. The results are shown in Fig. 1. Administration of 10 mg/kg of tiracoxib and 50 mg/kg of probucol resulted in significant inhibition of tumor growth ($P < 0.05$), but other methods did not inhibit tumor progression.

5-FU concentration Intra-tumoral 5-FU concentration

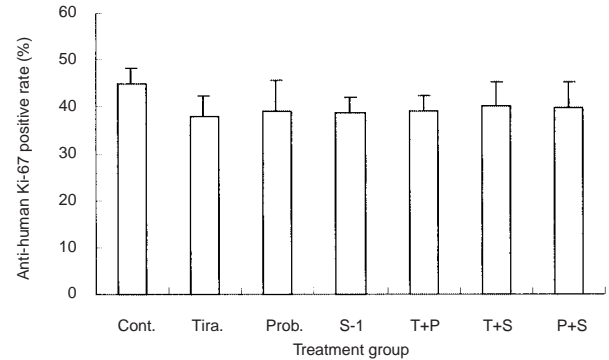


Fig. 4. Positive rate for anti-human Ki-67 antibody. There are no significant differences among the groups. No mark, NS; bars, SD; Cont., control; Tira., tiracoxib; Prob., probucol; T+P, tiracoxib and probucol; T+S, tiracoxib and S-1; P+S, probucol and S-1.

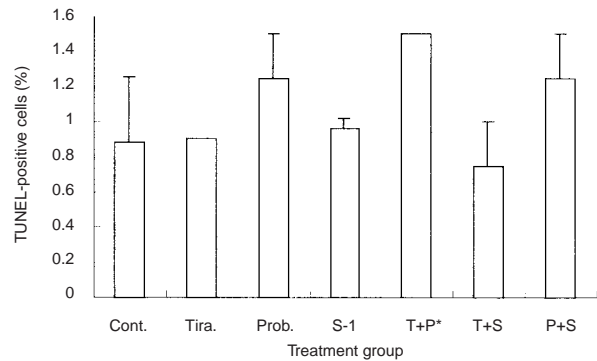


Fig. 5. TUNEL-positive cell rate is significantly increased when mice receive tiracoxib and probucol concomitantly. No mark, NS; bars, SD; Cont., control; Tira., tiracoxib; Prob., probucol; T+P, tiracoxib and probucol; T+S, tiracoxib and S-1; P+S, probucol and S-1.

was determined among S-1 treatment mice (Fig. 2) and significant differentiation was not detected.

Angiogenesis The number of microvessels peripheral to the site of the tumor, positive for anti-human factor VIII, was significantly less when mice were concomitantly given tiracoxib and probucol (Fig. 3).

Cell viability analysis Ki-67 positive cell rate showed no significant change (Fig. 4). Tiracoxib or probucol had no effect on cell viability at the doses used here. Concomitant administration of tiracoxib and probucol suppressed tumor growth, but the effect did not appear to be due to a decrease of cell viability.

Apoptosis Apoptotic cells were stained with the TUNEL method and the results of quantitative analysis for

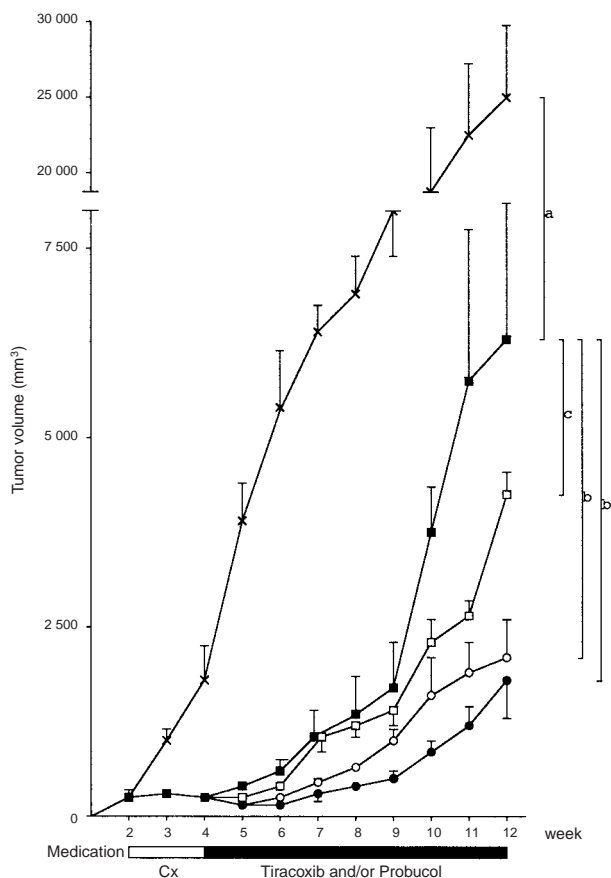


Fig. 6. The results of maintenance therapy following clinically based chemotherapy. CF modified chemotherapy inhibits tumor growth compared with the control. Treatment with 30 mg/kg of tiracoxib alone or with 100 mg/kg of probucol suppressed the tumor growth compared with the CF modified chemotherapy group. Treatment with 100 mg/kg of probucol alone tended to suppress the growth, but without significance. Cx, CF modified chemotherapy; ×, control; ■, Cx-control; ○, 30 mg/kg of tiracoxib; □, 100 mg/kg of probucol; ●, 30 mg/kg of tiracoxib and 100 mg/kg of probucol; a, $P < 0.01$ vs. control; b, $P < 0.05$ vs. Cx-control; c, NS vs. Cx-control; bars, SD.

TUNEL-positive cells are summarized in Fig. 5. The apoptotic index was significantly higher in tumors from mice given combined treatment with tiracoxib and probucol.

Telomerase activity We previously reported that treatment of KB cell xenografts with 30 mg/kg of tiracoxib inhibited telomerase activity, but 10 mg/kg was ineffective. In this study, telomerase activity was not inhibited by any treatment (data not shown). Even though combined administration of tiracoxib and probucol suppressed tumor growth, it did not inhibit telomerase activity.

Clinical model The results of maintenance therapy (30 mg/kg of tiracoxib or 100 mg/kg of probucol or both) fol-

lowing clinical-based chemotherapy are shown in Fig. 6. Compared with the control group, CF modified therapy treated groups showed significant inhibition of tumor growth ($P < 0.01$). Tiracoxib alone caused significant tumor growth inhibition ($P < 0.05$), but probucol alone seemed ineffective. Combined therapy with tiracoxib and probucol had the greatest inhibitory effect.

DISCUSSION

We previously investigated anti-tumor effects on the growth of head and neck carcinoma cells in animal models using tiracoxib,¹⁾ probucol²⁾ and S-1.³⁾ Tiracoxib, a selective COX-2 inhibitor, causes cell-cycle arrest in the early G1 or G0 phase, inhibits angiogenesis through a reduction of platelet-derived growth factor (PDGF), and suppresses telomerase activity. It has a tumor growth-inhibitory activity. Further, the decrease of tumor blood supply, via the anti-angiogenic effect, may lead to tumor cell apoptosis *in vivo*.

Probulcol is a strong antioxidant and has been clinically used for the treatment of hyperlipidemia in Japan since 1985. It has been demonstrated⁵⁾ that lipid oxidation products can alter growth factor production, which could influence smooth muscle cell proliferation. Oxidized low density lipoprotein (LDL) has been shown⁶⁾ to be a chemoattractant for smooth muscle cells and a stimulant of smooth muscle cell proliferation. It enhances *PDGF-AA* gene expression and PDGF receptor expression in vascular smooth muscle cells.⁷⁾ Probulcol has a superoxide scavenging effect, inhibits the production of PDGF, and also protects LDL from oxidation, which potentially reduces smooth muscle cell proliferation. Probulcol does not directly act on tumor cells *in vitro*,²⁾ but showed an anti-tumor effect in tumor-bearing nude mice via an anti-angiogenic action, which may induce cellular apoptosis as a result of decreased blood supply.

S-1 is an oral anti-neoplastic agent based on biochemical modulation of 5-FU. It is a combination of tegafur (FT), 5-chloro-2,4-dihydropyridine (CHDP), and potassium oxonate (Oxo) in a molar ratio of 1:0.4:1.⁸⁾ FT is a prodrug of 5-FU. Neither CHDP nor Oxo has any anti-tumor activity itself, and they act solely as modulators of 5-FU. CHDP competitively inhibits dihydropyridine dehydrogenase, an enzyme that degrades 5-FU,⁹⁾ thereby prolonging high 5-FU concentration in the circulation. Oxo is mainly distributed in the gastrointestinal tract and acts to reduce the toxicity of 5-FU.¹⁰⁾ S-1 administration provides prolonged high concentrations of 5-FU in tumor tissue, having a superior inhibitory effect on tumor growth. In our previous study,³⁾ the minimum 5-FU concentration in the tumor for significant tumor growth suppression was 300 ng/g. In this study, we administered S-1 at 5 mg/kg, which did not afford significant tumor growth

inhibition because of the low concentration of 5-FU in the tumor (120 ng/g).

The effect of administration of combinations of two among these three drugs, which have different mechanisms of action, was investigated in tumor-bearing nude mice. A significant suppressive effect on tumor growth was obtained with the combination of tiracoxib and probucol, as a result of a significant anti-angiogenic effect, which induces apoptosis of the tumor cells. The dose of S-1 used in this study does not provide an effective intratumoral 5-FU concentration and can not inhibit tumor growth. No anti-tumor effect of S-1 combined with tiracoxib or probucol was observed, and neither tiracoxib nor probucol alone reduced the intra-tumoral 5-FU concentration. Further investigation of the combination of S-1 and tiracoxib or probucol at different doses might be worthwhile.

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There was no significant difference in Ki-67 positive cell rate or telomerase activity between the control and the treatment groups. This is not in conflict with our previous reports,^{1,2)} because the drugs at the doses used in the present study are not expected to be effective.

In conclusion, we suggest that the concomitant administration of tiracoxib and probucol is a candidate for maintenance therapy after the primary treatment for patients with advanced head and neck carcinoma.

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