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Anti-tumor effect of radiation response by combined treatment with angiogenesis inhibitor, TNP-470, in oral squamous cell carcinoma

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Summary Blocking angiogenesis may enhance conventional anticancer treatments such as radiation therapy. In this study, we examined the effects of the angiogenesis inhibitor TNP-470 on human OSCC cell lines HSC2 and KB, with combining radiation therapy in the nude mouse. We evaluated cell-induced neovascularization with dorsal air sac assay, and selected two cells (HSC2: low, KB: high) with different level of cell-induced angiogenesis. The angiogenesis inhibitor TNP-470 was given 30 mg/kg s.c. daily on day 1–5, and irradiation, 8 Gy × 1, was administered on day 1 each week for 3 weeks. Significant inhibition of tumor growth relative to untreated controls was achieved in KB cells showing high induced angiogenesis with both TNP-470 ($P < 0.01$) and radiation ($P < 0.01$) and combining TNP-470 and radiation ($P < 0.01$). We saw little effect of TNP-470 either alone or in addition to the effect of radiation on the HSC2 cells showing low induced angiogenesis.

These results suggested that TNP-470 significantly enhanced the effect of radiation on the cells with high neovascularization. These findings indicated that individual evaluation of each tumor neovascularization potential will be important before deciding the anti-angiogenesis treatment.

Abbreviations: OSCC; oral squamous cell carcinoma; SD; standard deviation.

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Introduction

Angiogenesis, the formation of the microvasculature, has important roles in metabolic function and solid tumor growth.[1] Recently, inhibition of angiogenesis has been used as a strategy for cancer therapy.[2] The anti-angiogenic compound TNP-470 strongly inhibits angiogenesis by the suppression of vascular endothelial cell proliferation.[3] TNP-470 also has a direct inhibitory effect on cancer cells,[4,5] although the mechanism of the action is not fully elucidated. We have previously reported a correlation between increased tumor vascularity and tumor progression in OSCC.[6] OSCC cell lines or tissues also express large amounts of angiogenic factors, such as vascular endothelial growth factor (VEGF).[7] VEGF expression was related to the pathological effects of radiation therapy and its expression can predict the therapeutic sensitivity of preoperative radiation therapy.[8] Therefore, anti-angiogenesis may contribute to the outcome of radiation therapy of OSCC.

The combination of radiation and TNP-470 has been investigated in some tumors but not in oral cancer.[9,10] TNP-470 in combination with other anti-angiogenic agents, given during fractionated radiation therapy, increased the inhibitory effect of radiation on tumor growth.[9,10]

On this basis, we evaluated the effect of combination therapy with TNP-470 and ionizing radiation on human OSCC xenografts in the nude mouse.

Materials and methods

Chemicals

The angiogenesis inhibitor TNP-470 was provided by Takeda Chemical Industries, Ltd. (Osaka, Japan).

Dorsal air sac assay

We evaluated the neovascularization potential of six OSCC cell lines (HSC2, HSC3, HSC4, KB, SAS, Ho-1-N1). All OSCC cells were obtained from the Japanese Collection Research Bioresources. The cell lines were maintained in DMEM/F12 medium (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA, St. Louis, MO, USA), and 50 units/ml penicillin and streptomycin at 37 °C in 95% air/5% CO_2. Male BALB/cA mice (6 weeks old) were purchased from Charles River Japan (Tokyo, Japan). The dorsal air sac assay was used in the mice to examine the angiogenic response triggered by OSCC cells as described by Oikawa et al.[11] Briefly, both sides of a Millipore ring were covered with Millipore filters with a 0.45 μm pore size, and the resulting Millipore chamber was filled with 1×10^6 OSCC cells suspended in 0.15 ml of PBS. The chamber was then implanted into a preformed air sac in the dorsum of anesthetized (50 mg/kg pentobarbital, i.v.) male BALB/cA mice. The animals were divided into a treated group and a negative control group (treated with chambers containing only PBS $n = 8$) on day 0. On day 5, the implanted chambers were removed from the subcutaneous fascia of the mice, and black rings with the same internal diameters as the Millipore rings were placed at the treated sites, directly in contact with the chamber. The angiogenic response was assessed by determining the areas of blood vessels with NIH image 1.56 on photographs taken with the use of a dissecting microscope.

Assay of tumor growth in athymic nude mice

Athymic BALB/c nude mice (4—5 week old males) were obtained from Charles River Laboratories (Shizuoka, Japan). The care and treatment of experimental animals was in accordance with institutional guidelines. Mice were injected s.c. with 1×10^6 cells into their dorsal flank. For each cancer cell line, after 3 weeks (when tumors were established with a mean volume of 100 mm^3), 10 mice/group were treated with TNP-470 (30 mg/kg s.c. on days 1 to 5 each week for 3 weeks) and/or received radiation treatment (8 Gy on day 1 each week for 3 weeks). Animal experiments included four treatment groups: control, radiation alone, TNP-470 alone and radiation in combination with TNP-470. Tumor volume was determined by direct measurement with calipers and calculated using the formula: $\pi/6 \times (\text{large diameter}) \times (\text{small diameter})^2$.

Immunohistochemistry

Tissue was frozen in cooled isopenthane, and frozen sections were fixed in formalin and postfixed in ethanol:acetic acid (2:1). CD31 and proliferation cell nuclear antigen (PCNA) immunostaining were performed on sections from tumors in each group. Sections were washed in PBS and TBS and incubated with 10% normal rabbit serum for 30 min. They were then incubated with a monoclonal rat antimouse CD31 antibody (1:100, MEC 13.3, PharMingen) or anti PCNA antibody (1:100, PC-10, Dako) overnight at 4 °C. Peroxidase activity was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxidase. The sections were then counterstained with methylgreen,