S-1 inhibits tumorigenicity and angiogenesis of human oral squamous cell carcinoma cells by suppressing expression of phosphorylated Akt, vascular endothelial growth factor and fibroblast growth factor-2

KOJI HARADA, SUPRIATNO, YUICHIRO KAWASHIMA, HIDEO YOSHIDA and MITSUNOBU SATO

Therapeutic Regulation for Oral Tumor, Institute of Health Biosciences, University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan

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Abstract. It has been reported that S-1 can exert antitumor effects on various human cancers including oral squamous cell carcinoma (OSCC). However, little is known about the detailed mechanisms of the antitumor activity of S-1. In the present study, we determined whether S-1 could suppress the angiogenesis and growth of human OSCC cells in vitro and in vivo. The S-1 component (5-FU plus CDHP) significantly suppressed the growth and migration of OSCC cells and BAEC, which inhibited tubule formation in HUVECs in vitro. Also, S-1 inhibited the nuclear factor-κB (NF-κB) activity in human OSCC cells in vitro. Moreover, S-1 inhibited the expression of survival signal, phosphorylated Akt (p-Akt), and of two major proangiogenic molecules, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), in cells implanted into the subcutaneous tissue of nude mice. The decreased expression of p-Akt, VEGF and FGF-2 correlated with decreased tumorigenicity and decreased vascularization of lesions in vivo. These findings suggest that S-1 can suppress the angiogenesis and growth of OSCC cells by inhibiting the expression of p-Akt, VEGF and FGF-2 involved in the blockade of Akt/NF-κB pathway.

Introduction

Oral squamous cell carcinoma (OSCC), along with pharyngeal carcinoma, is the sixth most common cancer in the world (1). OSCC is a significant public health problem throughout the world because oral function is very important for breathing, eating and conversation. In spite of recent advances in surgery, radiotherapy, chemotherapy and immunotherapy, the mortality rate of OSCC is below 50% in the advanced stage (2,3). Also, the large operation or systemic chemotherapy is not often suitable for patients of advanced age or with complications. To improve the prognosis of patients with OSCC, the development of new effective chemotherapeutic agents has been anticipated.

S-1 is a novel oral fluoropyrimidine anticancer agent designed to enhance anticancer activity and reduce gastrointestinal toxicity through the deliberate combination of the following components: an oral fluoropyrimidine agent, tegafur (FT), which is a masked form of 5-fluorouracil (5-FU); a DPD inhibitor, 5-chloro-2, 4-dihydroxypyridine (CDHP), which is an inhibitor of 5-FU degradation; and an OPRT (orotate phosphoribosyltransferase) inhibitor, potassium oxonate (Oxo), which is an inhibitor of 5-FU phosphorylation, which is localized in the gastrointestinal tract. S-1 was devised as a combination drug with a molar ratio of 1:0.4:1 for FT, CDHP, and Oxo, respectively. S-1 has cytotoxic mechanisms similar to those of 5-FU, but has been shown to have less-toxic side-effects than 5-FU in clinical trials (4). Also, the response rate was 46.2% (12/26) in a late phase II study of advanced head and neck cancer (5). However, little is known about the detailed mechanisms of antitumor activity of S-1.

The progression of OSCC is dependent in part on the formation of adequate vascular support, so we focused on antiangiogenesis for OSCC treatment. It has been reported that angiogenesis showed a correlation with short survival and regional recurrence in oral cancer (6). Vascular endothelial growth factor (VEGF) has been shown to induce the proliferation of endothelial cells, increase vascular permeability, and prolong their survival (7,8). In addition, it has been reported that VEGF positivity by immunohistochemistry was a useful predictor of poor prognosis in oral cancer. VEGF status may prove to be an important prognostic factor in head and neck cancer (9,10).

Fibroblast growth factor (FGF-2) is a potent angiogenic factor in normal and malignant tissues. Expression of FGF-2 protein has been reported as higher in OSCC than in normal mucosa (11,12), with more intense expression in more poorly-differentiated tumors (13). However, Ninck et al reported...
that no overexpression of FGF-2 was found in carcinomas and there was no correlation between vascularity and the expression of FGF-2 (14). Recently, Li et al reported that FGF-2 expression was related to tumor size (T stage), but not microvessel density (15).

Akt has been recognized as an important survival signal for cancer cells. Akt is frequently activated in various cancers (16). In addition, Akt activation may enhance the risk of progression to malignancy (17), and thus become a prognostic factor (18). Briefly, suppression of Akt activation may play an important role in cancer therapy.

Studies have demonstrated that inhibition of NF-kB activity in ovarian cancer cells can suppress angiogenesis and progressive growth by suppressing the expression of VEGF (19). Moreover, NF-κB can stimulate the angiogenesis of ovarian carcinoma by up-regulating the expression of FGF-2 (20). Furthermore, we have already reported that 5-FU induces apoptosis through the suppression of NF-κB activity (21), and that 5-FU may exert an antiangiogenic effect by inhibiting NF-κB activity in head and neck cancer cells (22).

In this study, we explored whether S-1 could suppress the angiogenesis and growth of OSCC cells by suppressing the expression of VEGF, FGF-2 and p-Akt involved in the blockade of NF-κB activity in vitro and in vivo. We show that S-1 might exert an antiangiogenic effect and antitumor effect against OSCC by decreasing the expression of p-Akt, VEGF and FGF-2.

Materials and methods

Cell lines and cell culture. B88 cells were isolated from an oral SCC patient in our laboratory. HSC3 cells were obtained from an oral SCC patient in our laboratory. HSC3 cells were obtained from an oral SCC patient in our laboratory. B88 and HSC3 cells were cultured in DMEM supplemented with 15% FBS, 1 mM sodium pyruvate, non-essential amino acids (18.4 g/l), 2 mM glutamine, 50 mg/ml ascorbic acid, penicillin (100 units/ml) and streptomycin (100 μg/ml). All cells were incubated in a humidified room temperature in 70% ethanol and stained with anti-CD31 antibody according to the manufacturer’s instructions. Briefly, the basal medium including VEGF (final concentration, 10 ng/ml) was used for the human umbilical vein endothelial cell (HUVEC)/fibroblast co-culture. The medium including 5-FU, CDHP, Oxo, or 5-FU plus CDHP was changed every 3 days. After 11 days, cells were fixed at room temperature in 70% ethanol and stained with anti-CD31 antibody according to the manufacturer’s protocol.

Vascularity in vitro and in vivo. Vascularity was determined by the stereological method of point counting, which estimates the volume occupied by the vasculature (25). An eyepiece graticule that contained 100 points was used. CD31 (in vitro) or CD34 (in vivo)-positive capillaries or vessels intersecting the points were counted in 15 random fields across each section (i.e. 1500 points examined), and the results are expressed as the mean percentage ± SD. Vascularity was quantified in the stroma close to the epithelium, up to approximately 750 μm from the basal lamina. Vessels larger and smaller than 50 μm in diameter were counted separately (25). The sum of small and large vessels is referred to as ‘total’ vessels.

Microwell colorimetric NF-κB assay. The assay was performed using TransAM® NF-κB p65/NF-κB p50 transcription factor assay kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, nuclear extracts were prepared using nuclear and cytoplasmic extraction reagents (NE-PER, PIERCE Biotechnology, IL, USA) and placed on ice. Step 1: binding of NF-κB to its consensus sequence. Twenty microliters of p65 diluted in lysis buffer or 20 μl of cell extract were incubated with 30 μl of binding buffer [4 mM HEPES pH 7.5, 100 mM KCl, 8% glycerol, 5 mM DTT, 0.2% BSA, 0.016% poly d(IC)] in microwells coated with the probes containing the NF-κB binding consensus. After 1-h incubation at room temperature with mild agitation (200 rpm on an IKA MS2 vortex), microwells were washed three times with PBS (-) + 0.1% Tween-20. Step 2: binding of primary antibody. Rabbit anti-NF-κB antibodies (100 μl), diluted 1000 times in a 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl and 1% non-fat dried milk, were incubated in each well for 1 h at room temperature. Microwells were then washed three times with 200 μl PBS (-) + 0.1% Tween-20. Step 3: binding of secondary antibody. Peroxidase-conjugated goat anti-rabbit IgG (100 μl), diluted 1000 times in a 10 mM...
phosphate buffer (pH 7.4) containing 50 mM NaCl and 1% non-fat dried milk, were incubated in each well for 1 h at room temperature. Microwells were then washed four times with 200 μl PBS (-) + 0.1% Tween-20. Step 4: colorimetric reaction. Tetramethylbenzidine (100 μl) was incubated for 10 min at room temperature before adding 100 μl of stopping solution. Optical density was then read at 450 nm, using a 650 nm reference wavelength, with a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA) (26).

**Nude mice and tumor inoculations.** Female athymic nude mice with CAnN.Cg-Foxn1nu/CrlCrlj genetic background (CLEA Japan, Inc. Tokyo, Japan) were purchased at 4 weeks of age and maintained under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food, and all manipulations were carried out aseptically inside a laminar flow hood. B88 cells were used as a xenograft model in the nude mice. Briefly, tumor cells (1x10^6) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice using a 27-gauge needle. Tumors were allowed to grow for 10 days before treatment. Then, the mice were divided into 2 groups, each consisting of 3 mice with almost equal mean tumor volumes (~40-50 mm^3 in volume).
Reagents and treatment protocol. S-1 was obtained from Taiho Pharmaceutical Co., Ltd., (Tokyo, Japan). The drug was suspended in autoclaved 0.5% sodium hydroxypropyl-methylcellulose (HPMC, Daiichi seiyakukogyo, Kyoto, Japan) in sterile conditions, at 1.0 mg/ml, and subsequently homogenized by stirring. The suspension was given to mice by a gastric tube in a volume of 0.1 ml/10 g body weight for 7 days. Control mice were administered orally with an equal volume of 0.5% HPMC for 7 days.

Tumor measurements. The size of the tumors was determined by measuring the length (L) and width (W) and then calculating the volume \(0.4 \times L \times W^2\) every 2 days. The body weight of the mice was also measured every 2 days. The mice were sacrificed 2, 4 and 6 weeks after inoculation by cervical dislocation. The tumors were dissected, fixed in neutral-buffered formalin, and embedded in paraffin for further study. Also, experiments were started when tumors reached an average volume of \(40-50\) mm\(^3\).

Immunohistochemistry. The avidin-biotin complex immuno-histochemical technique was used to detect VEGF, FGF-2, p-Akt and CD34 in tissue specimens, using the Vectastain kit (Vector Laboratories). Paraffin-embedded tissue sections, 4 \(\mu\)m thick, were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide/methanol mixture for 30 min. Sections were rinsed and preincubated with 2% blocking serum for 30 min, followed by incubation with a rabbit anti-VEGF polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a rabbit anti-FGF-2 polyclonal antibody (Santa Cruz), a rabbit anti-p-Akt 1/2/3 (Thr308) polyclonal antibody (Santa Cruz), and a mouse anti-CD34 monoclonal antibody (Santa Cruz) for 8 h at 40˚C. After rinsing the tissue sections in PBS for 30 min, followed by incubation with a rabbit anti-VEGF polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a rabbit anti-FGF-2 polyclonal antibody (Santa Cruz), a rabbit anti-p-Akt 1/2/3 (Thr308) polyclonal antibody (Santa Cruz), and a mouse anti-CD34 monoclonal antibody (Santa Cruz) for 8 h at 40˚C. After rinsing the tissue sections in PBS for 10 min, 100 \(\mu\)l of secondary antibody (biotinylated anti-rabbit or anti-mouse) was added for 30 min. Tissue sections were then rinsed in PBS and incubated with diaminobenzene substrate (Sigma) for 10 min. Tissues were finally rinsed in PBS for 5 min and tap water for 5 min, and counterstained with Mayer’s hematoxylin (Sigma) for 1 min. The tissue sections were subsequently dehydrated in graded ethanol, cleared in Histoclear, and mounted with glass coverslips using DPX. Each run included positive and negative controls.

Results

Effect of S-1 component on in vitro cell growth. We tested the effect of the S-1 component at seven concentrations on the in vitro cell growth of B88 and HSC3 cells. A low concentration of 5-FU (0.01-0.5 \(\mu\)g/ml) did not suppress the cell growth of B88 cells. However, a high concentration of 5-FU (1.0-10.0 \(\mu\)g/ml) significantly suppressed the growth of B88 at 48 h of treatment when compared with that of the untreated control. CDHP (0.01-5.0 \(\mu\)g/ml), and Oxo were rinsed in PBS and incubated with dianiminobenzene substrate (Sigma) for 10 min. Tissues were finally rinsed in PBS for 5 min and tap water for 5 min, and counterstained with Mayer’s hematoxylin (Sigma) for 1 min. The tissue sections were subsequently dehydrated in graded ethanol, cleared in Histoclear, and mounted with glass coverslips using DPX. Each run included positive and negative controls.
(0.01-10.0 μg/ml) did not suppress the cell growth of B88 cells (Fig. 1). On the other hand, 5-FU (0.05-1.0 μg/ml) did not significantly inhibit the cell growth of HSC3 at 48 h of treatment when compared with that of the control. In addition, both CDHP (0.01-10.0 μg/ml) and Oxo (0.01-10.0 μg/ml) did not suppress the cell growth of HSC3 (Fig. 2). Briefly, the S-1 component (5-FU and CDHP) preferentially suppressed the growth of B88 cells when compared with that of HSC3 cells.

The combined treatment of 5-FU (2.0 μg/ml) and CDHP (0.01-1.0 μg/ml) did not significantly suppress the growth of B88 cells when compared with that of 5-FU (2.0 μg/ml) treatment alone. However, a combination of 5-FU (2.0 μg/ml) and CDHP (5.0 or 10.0 μg/ml) significantly suppressed the growth of B88 when compared with that of 5-FU (2.0 μg/ml) treatment alone (Fig. 3). As CDHP (5.0 μg/ml) did not significantly suppress the B88 cell growth (Fig. 1), we hypothesized that the growth inhibitory effect of 5-FU (2.0 μg/ml) plus CDHP (5.0 μg/ml) might mimic the effect of S-1 in vivo in the case of B88 cells. Furthermore, 5-FU (2.0 μg/ml) or 5-FU (2.0 μg/ml) plus CDHP (5.0 μg/ml) significantly inhibited the growth of BAEC at 2 and 3 days of treatment when compared with that of the control. In addition, the combined treatment of 5-FU (2.0 μg/ml) and CDHP (5.0 μg/ml) significantly suppressed the growth of BAEC when compared with that of 5-FU (2.0 μg/ml) treatment alone (Fig. 4).

**Effect of S-1 component on in vitro cell migration.** Cell migration is an essential process involved in tumor angiogenesis as well as tumor invasion and metastasis. CDHP (5.0 μg/ml) did not suppress the cell migration of B88 and

### Table I. Microvessel density.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2±3.3</td>
</tr>
<tr>
<td>5-FU (1.0 μg/ml)</td>
<td>10.2±1.8*</td>
</tr>
<tr>
<td>5-FU (2.0 μg/ml)</td>
<td>6.00±1.0*</td>
</tr>
<tr>
<td>5-FU (5.0 μg/ml)</td>
<td>2.40±0.55*</td>
</tr>
<tr>
<td>CDHP (1.0 μg/ml)</td>
<td>21.8±1.9</td>
</tr>
<tr>
<td>CDHP (5.0 μg/ml)</td>
<td>27.6±3.3</td>
</tr>
<tr>
<td>Oxo (1.0 μg/ml)</td>
<td>22.6±2.1</td>
</tr>
<tr>
<td>Oxo (5.0 μg/ml)</td>
<td>20.8±2.1</td>
</tr>
<tr>
<td>5-FU (1.0 μg/ml) + CDHP (5.0 μg/ml)</td>
<td>6.60±2.2*</td>
</tr>
<tr>
<td>5-FU (2.0 μg/ml) + CDHP (5.0 μg/ml)</td>
<td>4.40±1.7*</td>
</tr>
</tbody>
</table>

*p<0.01 when compared with that of control by Mann-Whitney U test. 5-FU significantly inhibited tubule formation, but CDHP and Oxo did not significantly inhibit tubule formation. Also, 5-FU (1.0 or 2.0 μg/ml) plus CDHP (5.0 μg/ml) treatment significantly inhibited tubule formation compared to each treatment alone.
Effect of S-1 component on angiogenesis in vitro. To investigate the effect of S-1 on induced angiogenesis in B88 tumors, we examined the effects of the S-1 component on HUVECs. *In vitro* angiogenesis assays showed that the basal medium including VEGF (final concentration, 10 ng/ml) promoted tubule formation in HUVECs 11 days after treatment, which was ~60% inhibited by the addition of 5-FU (1.0 μg/ml) and ~75% inhibited by the addition of 5-FU (2.0 μg/ml). Also, the addition of CDHP (1.0, 5.0 μg/ml) did not significantly inhibit the tubule formation in HUVECs. However, the addition of 5-FU (1.0 or 2.0 μg/ml) plus CDHP (5.0 μg/ml) significantly inhibited the tubule formation compared to each addition alone (Fig. 6 and Table I).

Effect of S-1 component on NF-κB activation. In the case of untreated B88 cells, NF-κB activation was not detected. When B88 cells were treated with TNF-α (50 ng/ml), NF-κB binding activity was markedly increased at 0.5 h after treatment. Moreover, incubation with the S-1 component (5-FU+CDHP) as well as 5-FU fully suppressed the TNF-α-induced NF-κB activation. However, CDHP alone did not suppress the TNF-α-induced NF-κB activation (Fig. 7).

Effect of S-1 on nude mouse tumor growth. The therapeutic effect of S-1 on B88 tumor-bearing nude mice is shown in Fig. 8. When the S-1 was administered per OS daily at the dose of 10 mg/kg for 6 weeks, tumor growth was significantly suppressed when compared with that of the control (0.5% HPMC). During the experimental period, no loss of body weight was observed in the S-1-treated group or the control group (data not shown).

Effect of S-1 on p-Akt expression in vivo. As shown in Fig. 9, higher expression of p-Akt was found in untreated control tumors than in S-1-treated tumors. Also, expression of p-Akt was markedly decreased in the tumors administered S-1 for 2-6 weeks.
Figure 9. Expression of p-Akt in S-1-treated B88 tumors at different time intervals. B88 tumors were treated or untreated with 10 mg/kg S-1. The expression of p-Akt was detected with immunohistochemical staining.

Figure 10. Expression of VEGF in S-1-treated B88 tumors at different time intervals. B88 tumors were treated or untreated with 10 mg/kg S-1. The expression of VEGF was detected with immunohistochemical staining.
Figure 11. Expression of FGF-2 in S-1-treated B88 tumors at different time intervals. B88 tumors were treated or untreated with 10 mg/kg S-1. The expression of FGF-2 was detected with immunohistochemical staining.

Figure 12. Expression of CD34 in S-1-treated B88 tumors at different time intervals. B88 tumors were treated or untreated with 10 mg/kg S-1. The expression of CD34 was detected with immunohistochemical staining.
to those of 5-FU. However, little is known about the detailed antiangiogenic effect of S-1 against unresectable advanced carcinomas in phase II studies. S-1 has shown the highest response rate among many oral anticancer agents. Also, S-1 has shown an antiangiogenic effect on OSCC as well as gastric cancers. Moreover, S-1 has shown the suppression of NF-κB transcriptional activity (32). So, we investigated whether the S-1 component can decrease NF-κB binding activity than normal oral epithelial and salivary gland cells (21). Furthermore, we have reported that 5-FU may suppress NF-κB transcriptional activity (32). So, we investigated whether the S-1 component can decrease NF-κB binding activity on B88 cells. In the results, the S-1 component (5-FU plus CDHP) as well as 5-FU suppressed TNF-α-induced activation of NF-κB in OSCC cells though CDHP did not suppress the NF-κB activation. S-1 may be a potent inhibitor of NF-κB activation induced by TNF-α.

We have also reported that S-1 can enhance radiosensitivity by suppressing the activation of Akt (33). In this study, we found that S-1 administration inhibited p-Akt expression in nude mouse tumor. So, inhibition of p-Akt could induce suppression of NF-κB activation, which might lead to anti-angiogenesis via inhibition of VEGF and FGF-2 expression. The inhibition of angiogenesis or tumorigenicity by suppression of NF-κB activity may be related to other mechanisms, such as inhibition of cell adhesion (34), pro-inflammatory cytokine production (35), or plasminogen activator and matrix metalloproteinase (36,37), which contribute to neoplastic angiogenesis, growth, and metastasis. Briefly, suppression of NF-κB activity may induce antiangiogenesis without down-regulation of VEGF and FGF-2. However, we found that S-1 inhibited the expression of p-Akt, VEGF and FGF-2 in cells implanted into the subcutaneous tissue of nude mice.

In summary, we showed that S-1 decreased angiogenesis, delayed tumor growth, and reduced formation of malignant ascites, in part through the down-regulation of survival signal p-Akt and angiogenic molecules VEGF and FGF-2 involved in the blockade of Akt/NF-κB pathway.

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