The preventive effect of salvianolic acid B on malignant transformation of DMBA-induced oral premalignant lesion in hamsters

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Salvia miltiorrhiza (SM) has been used clinically in Asian countries to improve the microcirculation in the human body. Salvianolic acid B (Sal B), a pure compound extracted from SM, has been reported to be effective against fibrosis and ischemia-reperfusion injury, possibly through its anti-lipid peroxidation action. But the effect of Sal B on oral premalignant lesion and oral carcinogenesis remains unexplored. It is our interest to investigate the chemopreventive effect of Sal B on 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters with respect to angiogenesis. Seventy male Syrian golden hamsters were randomly divided into five groups, with two of 20 and three of 10. DMBA solution (0.5% in acetone) was applied topically to the left cheek pouch of male Syrian golden hamsters in Groups A and B, while animals in Group C were painted with acetone, three times a week for 6 weeks. For the next 18 weeks, animals in Groups B and D received Sal B daily (10 mg/kg body wt/day) by gavage, animals in Groups A and C received same volume of saline. Animals in Group E received no treatment and served as blank control. At the end of the experiment, animals were killed and tissue samples were collected for histopathological and immunohistochemical examinations. The results showed that Sal B significantly decreased the squamous cell carcinoma (SCC) incidence from 64.7 (11/17) to 16.7% (3/18) (P = 0.004); angiogenesis was inhibited in dysplasia and SCC (P < 0.01), with a simultaneous decrease in the immunostaining of hypoxia-inducible factor 1α and vascular endothelium growth factor protein (P < 0.05). The results suggested that Sal B had inhibitory effect against the malignant transformation of oral precancerous lesion and such inhibition may be related to the inhibition of angiogenesis.

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; MVD, microvessel density; Sal B, Salvianolic acid B; SCC, squamous cell carcinoma; SM, Salvia miltiorrhiza; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelium growth factor.

Introduction

Oral cancer is a common neoplasm worldwide, particularly in the developing countries (1). In recent decades, oral cancer incidence and mortality rates have been increasing in some countries including China (2,3), and the survival of patients with oral cancer has not improved significantly despite recent advances in radiotherapy and chemotherapy. The answer to combat cancer may, therefore, lie with the prevention rather than cure. Oral cancer usually develops from hyperplasia through dysplasia to carcinoma (4). Oral leukoplakia is the most common premalignant lesion of oral cancer, and up to 20% of the patients with leukoplakia develop invasive carcinomas (5). Thus the possibility of addressing the issue of precancerous tissue behavior would be contributory. Preventing or treating oral premalignant lesion with natural or synthetic chemical agents is now one of the most promising approaches to prevent oral cancer.

Angiogenesis, the growth of new capillaries from preexisting blood vessels, is essential for cancer to grow beyond minimal size and metastasize (6,7). Anti-angiogenesis remains a prime therapeutic target. Increasing experimental evidences suggest that angiogenic switch occurs in premalignant lesions, and angiogenesis persists during progression to expansive solid tumors and invasive carcinomas (8–10). Thus, blocking angiogenic switch before the initial formation of solid tumor may be a promising cancer prevention modality.

The dried roots of Salvia miltiorrhiza (SM), called Danshen, is one of the most well known traditional Chinese medicines. It has the effect of ’promoting blood circulation and removing stasis’, and is widely used for the treatment of cardiovascular disorders. Salvianolic acid B (Sal B) is one of its major water soluble active components and has been reported to have significant scavenging effects on oxygen free radicals and protective effects on heart and brain injuries induced by ischemia reperfusion (11,12). But the majority of researches on Sal B have been focused on its heart protective properties, the effect of Sal B on oral carcinogenesis remains unexplored, and the published in vitro studies about its effect on angiogenesis are controversial. Qui et al. (13) reported that Sal B could strongly inhibit the hyperpermeability induced by VEGF in bovine aortic endothelial cell. Furthermore, Ding et al. (14) found that the effect of Sal B on tumor necrosis factor (TNF)-α induced endothelial permeability was likely due to a reduction of vascular endothelium growth factor (VEGF) protein expression as a result of modulation of the extracellular signal regulated kinase (ERK) signaling pathway. However, it was also reported that Sal B enhanced angiogenic processes on SVR cells through upregulation of VEGF and VEGF receptors.
To assess the angiogenesis of the squamous epithelium of the oral mucosa, tissue sections were immunostained with anti-von Willebrand factor (Dako, Carpinteria, CA). Briefly, sections were trypsinized for 20 min at 37°C. The localization of any cellular staining and its intensity were independently assessed by two observers. Grading of immunostaining was based on semi-quantitative evaluation of stain intensity from I to III. Mild stain of 5–25% of cells was classified as I, moderate stain of 25–50% of cells was graded as II, and intense staining comprising >50% of the cells was classified as III. 

Pathological and histopathological examinations

The whole cheek pouch was excised, flattened on the transparency plate and fixed in 10% phosphate-buffered saline (PBS)-buffered formalin. Formalin-fixed pouches were cut into 4–6 pieces of approximately equal width, Swiss-rolled, processed and then embedded in paraffin. Thirty sections (5 μm) of each sample were cut and the 1st, 15th and 30th slides were H&E stained for histopathological analysis. Basal cell hyperplasia, dysplasia, SCC and papillomas were diagnosed with established criteria (16,17). The hyperplasia of oral epithelium was indicated by increased number of basal cells. The dysplasia was characterized by irregular epithelial stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells. Papilloma was diagnosed by stratified squamous epithelium over branching fibrovascular cores including papilloma-hyperplasia and papilloma-dysplasia. Carcinoma was diagnosed by the invasion of underlying tissues, including those originating from papilloma and those from apparently normal epithelium.

Tumor vascularity and measuring microvessel density

To assess the angiogenesis of the squamous epithelium of the oral mucosa, tissue sections were immunostained with anti-von Willebrand factor (Dako, Carpinteria, CA). Briefly, sections were trypsinized for 20 min at 37°C in 0.05 M Tris-HCl containing 0.01% trypsin and 0.01% calcium chloride. After washing, the sections were incubated in 10% normal swine serum for 10 min, followed by rabbit antihuman von Willebrand factor (1:500), biotinylated swine antirabbit antibody (1:400) for 30 min, and streptavidin-biotin complex/horseradish peroxidase for 30 min. All of the sera and avidin-biotin reagents were obtained from Dako.

Sections were analyzed using the vascular hotspot technique to obtain microvessel density (MVD) (18). Sections were scanned at low power to determine areas of highest vascular density. Within this region, individual microvessels were counted in three separate random fields at high power (0.104-mm field size). The mean vessel count from the three fields was used. A single countable microvessel was defined as any endothelial cell or group of cells that was clearly separate from other vessels, stroma, or tumor cells without the necessity of a vessel lumen or RBC within the lumen. Areas of gross hemorrhage and necrosis were avoided. In non-lesioned area and pre-invasive lesions including hyperplasia, dysplasia and benign papillomas, the blood vessels in submucosa were counted. In carcinoma, peritumoral vessels were counted.

Immunohistochemistry for hypoxia-inducible factor 1α, and VEGF

Immunohistochemistry for hypoxia-inducible factor 1α (HIF-1α) and one of its downstream genes VEGF was performed on 4-μm thick slides from the tissue samples. Tissue sections were cleared of paraffin, rehydrated, and blocked in hydrogen peroxide. Then for HIF-1α staining, sections were pressure cooked for 3 min in Tris-EDTA lysis buffer (pH 9.0) before incubation with rabbit antihuman HIF-1α (1:100, Dako). For VEGF staining, after 10 min microwaving in a 0.01 M citrate buffer (pH 6), sections were stained with an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution. Then sections were incubated with biotinylated swine anti-rabbit antibody (1:100) for 30 min, and streptavidin-biotin complex/horseradish peroxidase for 30 min. All of the sera and avidin-biotin reagents were obtained from Dako. Appropriate negative (obtained by omission of the primary antibody) and positive controls were used throughout.

The localization of any cellular staining and its intensity were independently assessed by two observers. Grading of immunostaining was based on semi-quantitative evaluation of stain intensity from I to III. Mild stain of <25% of cells was called I, moderate stain of 25–50% of cells was graded as II, and intense staining comprising >50% of the cells was classified as III. Section grading was based on stain intensity per field (for tumors) or epithelial layer (for epithelia). Staining evaluation of the slides was performed in a blind fashion.

Statistical analysis

The tumor incidence of different groups was compared using χ²-test. One way ANOVA followed by Dunn’s multiple test was used to compare the number of various oral lesions and MVD among these groups. Correlation of the HIF-1α and VEGF protein expression in different lesions was evaluated by Spearman’s test. All statistical analysis was used bySPSS. Differences with calculated P-values <0.05 were regarded as significant.

Results

Inhibition of Sal B against DMBA-induced oral carcinogenesis

At Week 6, some DMBA-treated animals had a visibly roughened granular surface on the mucosa with varying degrees of erythema and occasional white plaque-like lesion. No SCC was observed in these animals at this time.

At Week 24, the left buccal pouch of DMBA-treated animals histologically presented areas of hyperplasia and dysplasia as well as papillomas (papilloma-hyperplasia and papilloma-dysplasia) and SCC. Sal B treatment significantly decreased the oral SCC incidence from 64.7 (11/17, Group A) to 16.7% (3/18, Group B) (P = 0.004) (Table I). In comparison with Group A, the incidence of dysplasia in Sal B treated group (Group B) was higher than that in Group A, which were 66.7 and 29.4% respectively, reaching statistical difference (P = 0.028). But when taking the degree of dysplasia into account, a majority of dysplastic lesions in Sal B treated group were of mild dysplasia (9/12), and the number of lesions with moderate or severe dysplasia was not statistically different between Groups A and B. All animals in Groups C, D and E appeared normal, except three animals in Group C showed inflammatory exhibition (Table I).

MVD associated with different lesions of different groups

In DMBA-induced oral lesions, the neovasculatures primarily concentrated in the stromal areas and spread along stromal...
ridges on the periphery of epithelial lesions (Figure 1). Normal epithelium contained few capillaries, contiguous to the epithelial basal cell layer (Figure 1A). The number and distribution were altered in hyperplasia (Figure 1B), dysplasia and carcinomas (Figure 1C). The vessels were more densely packed and closer to the basal layer of the lesions than in the normal epithelium. Quantification of vascularization in different lesions revealed a statistically significant increase in MVD when normal epithelium (278 ± 17.27) was compared with hyperplasia (372 ± 21.21) or dysplasia (402 ± 18.06) or carcinoma (504 ± 17.24). There was no significant difference between MVD of hyperplasia (372 ± 21.21) and dysplasia (402 ± 18.06).

Compared with Group A, Sal B treatment led to a decrease in MVD of hyperplasia, dysplasia and SCC (311 ± 27.77 versus 402 ± 18.06 and 392 ± 24.81 versus 504 ± 17.24, respectively; vessels/high-power field; P < 0.05) (Table II).

Expression of HIF-1α in tissue samples of different groups
Little expression of HIF-1α protein was found in normal epithelium (Figure 2A). In the positive control group (Group A), hyperplasia showed mild staining (Grade I, Figure 2B). Four samples of dysplasia showed moderate staining (Grade II), and the other one showed strong staining (Grade β; Figure 2C). All of the SCCs showed strong staining (Grade β; Figure 2D).

In the Sal B treated group, little staining of HIF-1α protein was detected in hyperplasia. Three samples of dysplasia showed moderate staining (Grade II, Figure 2e) and mild immunostaining was detected in the other nine samples (Grade I). All of the SCCs showed moderate staining (Grade II, Figure 2F) (Table III).

Expression of VEGF in tissue samples of different groups
Low or mild expression of VEGF was found in normal epithelia (Grade I, Figure 3A). In the positive control group (Group A), strong staining was detected in three samples of

### Table I. Effect of Sal B on DMBA-induced oral carcinogenesis in hamsters

| Group | Treatment          | No. of animals | Microscopic lesions |
|-------|--------------------|----------------|---------------------|
|       |                    |                | No. of inflammation | No. of hyperplasia | No. of dysplasia | Oral SCC no. | Incidence (%) |
| A     | DMBA+ saline       | 17             | —                   | 3.80 ± 1.57        | 2.80 ± 1.75      | 4.55 ± 2.71  | 64.7          |
| B     | DMBA+ Sal B        | 18             | —                   | 2.33 ± 0.94        | 1.17 ± 0.37      | 1.33 ± 0.47  | 16.7          |
| C     | Acetone+ saline    | 10             | 1.33 ± 0.47         | —                   | —                | —            | —             |
| D     | Sal B only         | 10             | —                   | —                   | —                | —            | —             |
| E     | Blank control      | 10             | —                   | —                   | —                | —            | —             |

The animals were given saline (Group A), or 10 mg/kg body wt Sal B by gavage (Group B), for 12 weeks after topical application of 0.5% DMBA to the left oral pouch of hamsters three times/week for 6 weeks. During the study period, three animals in Group A and two animals in Group B died. The different oral lesions were expressed as the number of lesions per animal (mean ± SD) or incidence (%) in each group. Statistically different from Group A, P < 0.05, based on χ²-test.
dysplasia (Grade b, Figure 3C), the other two samples showed moderate staining (Grade II). All of the SCCs showed strong staining (Grade b, Figure 3D).

In the Sal B treated group, mild staining was detected in nine samples of dysplasia (Grade I, Figure 3E), the other three samples showed moderate staining (Grade II). The SCC showed moderate staining (Grade II, Figure 3F) (Table III).

MVD (vessels/mm²) quantitated at 200× magnification. Mean ± SE.

Table II. Microvessel density data of different groups

| Group | Treatment       | MVD     |
|-------|-----------------|---------|
|       |                 | Normal  | Inflammation | Hyperplasia | Dysplasia | OSCC    |
| A     | DMBA+saline     | —       | —            | 372 ± 21.21 | 402 ± 18.06 | 504 ± 17.24 |
| B     | DMBA+Sal B      | —       | —            | 304 ± 29.40 | 311 ± 27.77 | 392 ± 24.81 |
| C     | Acetone+saline  | 279 ± 19.94 | 288 ± 27.27 | —          | —         | —       |
| D     | Sal B only      | 269 ± 21.63 | —            | —          | —         | —       |
| E     | Blank control   | 273 ± 17.2 | —            | —          | —         | —       |

MVD (vessels/mm²) quantitated at 200× magnification. Mean ± SE.

Discussion

It was once considered that protocatechuic aldehyde and danshensu were the major biologically active components of SM. Comparative studies of these depsides and phenolic components indicated that the antioxidant effects as well as antiplatelet aggregation and antithrombic activities of Sal B were stronger than those of protocatechuic aldehyde and danshensu.
danshensu. Further studies showed that Sal B had significant scavenging effects on oxygen free radicals and protective effects on heart and brain injuries induced by ischemia reperfusion (19,20). Clinical practice for years has proven that SM has anti-cancer potential and application of it in the treatment of a variety of cancers has achieved surprising effects (21,22). However, most of the studies about Sal B, one of the major biologically active components of SM, were focused on its

Table III. Results of immunostaining for VEGF and HIF-1a in different groups

| Diagnosis | Group | I | II | β | Samples expressing HIF-1a and VEGF (immunostaining intensity) |
|-----------|-------|---|----|---|-------------------------------------------------------------|
| Hyperplasia | A | 1 | — | — | — | 3 | 1 | — |
| B | 3 | — | — | — | — | 2 | 3 | — |
| Dysplasia | A | — | 4 | 1 | — | 9 | 3 | — |
| B | 9 | 3 | — | — | — | 8 | — | — |
| OSCC | A | — | — | 8 | — | — | — | — |
| B | 9 | 3 | — | — | — | 8 | — | — |
| Normal | C, D, E | 27 | — | — | — | 27 | — | — |

Fig. 3. VEGF immunostaining in DMBA-induced oral lesions. (A) normal epithelium; (B) hyperplasia of the positive group; (C) dysplasia of the positive control group; (D) SCC of the positive control group; (E) dysplasia of the Sal B treated group; (F) SCC of the Sal B treated group.
effects on cardiovascular disorders and there is few data about the effect of this component on cancer. It is for this reason that Sal B was evaluated in this study for its chemopreventive potential against oral SCC.

In the DMBA-induced hamster cheek pouch carcinogenesis model, Sal B, dosed since the post-initiation stage, markedly reduced the incidence of chemically induced cancer (from 64.7 to 16.7%, $P = 0.004$). Although the incidence of dysplasia in Sal B treated group was higher than that in the positive control group (66.7 and 29.4%, respectively), most of them were of mild dysplasia. There is a possibility that these dysplastic lesions will progress to carcinoma in the future. In that case, the conclusion can still be made that Sal B does have some chemopreventive effect on oral carcinogenesis, at least it can delay the malignant conversion of premalignant lesions. Further study is needed in this regard.

MVD, as quantified in histological sections of tumors, has proven to be an independent prognostic indicator in various types of solid tumors, including SCC of the head and neck (23–25). VEGF is regarded as the major angiogenic factor during epithelial carcinogenesis in many malignant human cancers and in tumor metastases (26–28). Most human cancers have been characterized as containing VEGF-overexpressing tumor cells. In many such malignant tumors, VEGF/VEGFmRNA is expressed at the boundary of necrotic area, indicating that hypoxia stimulates the production of VEGF (29–32). Hayahito et al. (33) reported that hypoxia in SCC of the cervix appeared to upregulate VEGF expression and led to high vascularity. HIF-1 is a transcription factor, which plays a central role in biologic processes under hypoxic conditions, especially concerning tumor angiogenesis. It is a heterodimeric protein consisting of an α- and β-subunits, in which the HIF-1α subunit mediates HIF-1 function as a transcription factor in response to cellular hypoxia. Being stabilized under decreased tissue oxygen concentration, it works as a cellular oxygen-sensing system, and transactivates a large number of genes whose protein products either increase oxygen availability or mediate metabolic adaptation to reduced oxygen tensions. Included among these are erythropoietin, glucose transporters, glycolytic pathway enzymes, VEGF and inducible nitric oxide synthase (34–36). Alteration and overexpression of HIF-1α has been detected in a variety of solid tumors, including breast, lung, ovarian and oral cancer with varying (diffuse and perinecrotic) staining patterns (31,32). Therefore, this study focused on evaluating these factors.

The results presented in this study demonstrated a significant increase in vascularity during the transition from normal tissue through different degrees of dysplasia to carcinoma. This finding is in agreement with the hypothesis that ‘angiogenic switch turns on at premalignant stage’. At the same time, we also observed that HIF-1α showed increased expression during this transformation, with a simultaneous increase in VEGF immunostaining. A relationship was seen between the expression of these two markers (Spearman’s test, $p < 0.05$). Thus, hypoxia in oral SCC appeared to upregulate VEGF expression, leading to high vascularity.

The present study also showed that Sal B, given after DMBA treatment, effectively inhibited oral carcinogenesis. It potently retarded the progression of existing precancerous lesions and the growth of tumors in the oral mucosa. The concentration of Sal B used in this study was designed to be relevant to the average dosage commonly prescribed in clinical practice. Higher concentrations of this agent are expected to be more efficacious; and dosing Sal B before exposure to DMBA (e.g., for 2 weeks) may be more able to maximize the chemopreventive effect, since it takes some time for Sal B to reach a pharmacologically steady state in vivo. The dose-response relationship and timing of dosing need to be further studied.

We observed in this study that the formation of microvessels, as well as the expression of pro-angiogenic factors HIF1α and VEGF, was inhibited in dysplasia and SCC by Sal B, suggesting Sal B may inhibit the malignant conversion of precancerous lesions and further growth of tumors through anti-angiogenesis. A bundle of previous studies and clinical practice have indicated that Sal B dilates arteries and blood vessels, promoting blood flow. Thus it is reasonable to speculate that the potent effect of Sal B on blood circulation reduced the hypoxia stress in the local tissues, which further contributed to the inhibitory effect on angiogenesis. Many studies have demonstrated that Sal B has significant scavenging effects on oxygen free radicals. Further research is required to investigate whether this antioxidant mechanism also contributes towards the chemopreventive effect on oral carcinogenesis.

In conclusion, our results demonstrated that salvianolic acid B inhibited oral carcinogenesis at the post-initiation stage. Anti-angiogenesis may be one of the possible mechanisms behind this preventive effect. SM is a kind of cheap and safe Chinese herb medicine. Nowadays, with advanced processing technique, salvianolic acid B can be extracted effectively and conveniently. Thus this agent is a promising chemopreventive agent for humans at high risk of oral cancer such as those with leukoplakia and erythroplakia.

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