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Anti-invasion and anti-tumor growth effect of doxycycline treatment for human oral squamous-cell carcinoma - In vitro and in vivo studies

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SUMMARY

Regional lymph node and distant organ metastasis of oral squamous-cell carcinoma (OSCC) has been associated with increased production of matrix metalloproteases (MMPs), and scientific data showed that doxycycline (Dox) could down-regulate the expression of MMPs. The objective of this study was to evaluate the effect of Dox on the expression of MMPs in vitro using the SCC-15 cell line and in vivo SCC-15 xenografted nude mice. SCC-15 cells maintained under distinct culture conditions expressed high levels of pro-MMP-2 and pro-MMP-9; however, as determined by zymography and Western blot analysis, Dox significantly reduced the production of pro-MMP-2 and pro-MMP-9 after 24 h of treatment in a dosedependent manner (2.5–40 $\mu g/ml).$ Dox (10 $\mu g/ml)$ decreased the expression of MMP-9 mRNA but did not alter the level of MMP-2 mRNA after 24 h of treatment. In addition, this drug significantly inhibited the invasive and migration activities of SCC-15 cells in vitro (>75% inhibition at 10 µg/ml). On the other hand, daily administration of Dox (3 mg/mice) restrained tumor growth in SCC-15 xenografted nude mice, with an inhibition rate of 85.6%. Compared with the control group (treated with normal saline), MMP-9 mRNA levels in the fresh tumor tissue decreased upon Dox treatment (P < 0.01) while MMP-2 mRNA levels were unchanged. In conclusion, reduced expression of MMP-9 at the transcriptional level and MMP-2 at the post-transcriptional level caused by Dox was found to be associated with decreased invasion of oral SCC in vitro. Moreover, Dox exerted a significant suppressive effect on tumor growth in an *in vivo* nude mice model. Taken together, these results, to our knowledge, may first imply that Doxycycline has an adjuvant therapeutic effect on OSCC that is associated with inhibition of MMPs expression. © 2009 Elsevier Ltd. All rights reserved.

Introduction

Survival of oral squamous-cell carcinoma (OSCC) patients has not improved significantly despite recent advances in treatments.¹ One of the major prognostic factors for OSCC patients is regional lymph node metastases.² Cascade of tumor cell invasion and metastasis involves alterations in cell-cell and extracellular-cell matrix (ECM) adhesion capacity.³ Matrix metalloproteases (MMPs) degrade ECM and basement membrane, which are critical steps for invasion and metastasis.⁴ Elevated production of gelatinases (MMP-2 and MMP-9) has been observed in pulmonary adenocarcinoma,⁵ ovarian carcimoma,⁶ and OSCC.^{7,8} Therefore, inhibition of gelatinases expression by agents may be a potential way of enhancing the prognosis of OSCC patients.

Doxycycline hyclate (Dox) is a semi-synthetic tetracycline. Besides its antibiotic activity, it inhibits MMP synthesis, and is the only MMP inhibitor approved by Food and Drug Association for dental applications.⁹ Besides treatment for periodontitis,¹⁰ Dox reduced proliferation, bone metastasis and gelatinolytic activity of MMP-2 and MMP-9 in breast cancer.^{11,12} Moreover, Dox inhibited tumor cell proliferation, invasion, and metastasis in prostate cancer.^{13,14} Rubins et al.¹⁵ reported malignant mesothelioma proliferation could be inhibited by Dox, and Onoda et al.¹⁶ found that Dox inhibited invasion of colorectal cell line. Hence, accumulated data revealed that Dox is a well-accepted MMP inhibitor, which chiefly inhibits MMP-2 and MMP-9.¹⁰⁻¹⁶ However, mechanism of anti-invasive effect of Dox on MMPs in OSCCs remains to be elucidated.17

We used an in vitro model of a human tongue SCC cell line to investigate the inhibitory action of Dox on the expression of MMP-2 and MMP-9. Anti-invasive and anti-migration effects were tested under such condition. In vivo anti-tumor effect of Dox was also investigated in a xenograft nude mice model.





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Table 1

Oligoprimers for real-time quantitative reverse transcriptase polymerase chain reaction.

	Sense	Antisense
MMP-2	5'-CCGTGCCTTCAGCTCTACA-3'	5'-CGCCTGGGAGGAGTACAG-3'
MMP-9	5'-GCTTGCCCTGGTGCAGTAC-3'	5'-ATTGCCGTCCTGGGTGTAG-3'
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'

Materials and methods

Cell line and culture

Human lingual SCC cell line (SCC-15) was cultured as previously described.¹⁸

Drug treatment

Dox-HCl powder (purity 99.99%) (#D9891; Sigma) was dissolved in double-deionized water to make a 50 mg/ml stock

solution. A concentration of 20 mg/ml was used in the xenograft nude mice model.

MTS assay¹⁹

SCC-15 cells were seeded into 96-well plate and cultured for 2day, following which culture medium was replaced with serum-free medium containing various dosages of Dox. After further culturing for 24 h, MTS reagent (G3582; Promega, Madison, WI, US) was added to each well followed by incubation at 37 °C for 1 h. Cell viabilities were determined by measuring absorbance at 490 nm with a spectrophotometer (Multiskan RC, Labsystems, Finland).

Gelatin zymography²⁰

A 15 μ l of condition medium was treated with SDS–PAGE sample buffer with neither a reducing agent nor boiling, and samples were fractionated in 8% polyacrylamide gel containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated in an activation buffer (10 mM Tris-HCl, pH 7.4, 5 mM CaCl₂, 1 μ M ZnCl₂ and 1.25% Triton X-100). Then



Figure 1 Gelatine zymography of gelatinase secreted by SCC-15 cells. (A) Gelatinolytic bands and molecular weight positions can be clearly observed for pro-MMP-2 (72-kD) and pro-MMP-9 (92-kD) after various periods (3–48 h) of incubation. (B) APMA-dependent reduction of gelatinase activity in conditioned media after incubation for 30 min. Effect of Dox on pro-MMP-2 and pro-MMP-9 production by cultured SCC-15 cells. Cells were treated with Dox at 0–40 μ g/ml for 24 h, following which culture media samples were collected and subjected to gelatin zymography (C) and Western blot analysis (F). Bar graphs (D and E) show the dose–response effect of Dox on pro-MMP-2 and pro-MMP-9 production. The expression level of -actin protein in equal amounts of total cell lysate was measured at the same time as that of the internal control. Means and standard errors were obtained from three independent experiments. Different letters represent significant differences between groups by Tukey's analysis of *P* < 0.05.

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the gels were stained with 0.5% Coomassie brilliant blue R-250 in 20% acetic acid-50% methanol.

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA samples were prepared from treated and untreated SCC-15 cells or xenograft tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was generated from 500 ng total RNA template using Taqman reverse transcription reagent. The cDNA was amplified by PCR with oligoprimers for MMP-2, MMP-9 and GAPDH (Table 1). Reaction was performed on a thermal cycler (Mx3000PTM; StratageneTM) with MaximaTM SYBR Green qPCR Master Mix (2×) added to the PCR reaction mixture. After normalization to expression level of GAPDH mRNA, relative expression levels of MMP-9 and MMP-2 mRNA were presented as a percentage change compared with the control.

Western blot

Proteins concentration in collected condition media samples was determined. The denatured proteins were resolved on SDS– PAGE, transferred onto nitrocellulose membrane. Western blot was done with standard procedures and probed with MMP-2 (Abchem Corporation, UK) and MMP-9 (R&D, MN, USA) antibodies.

In vitro invasion/migration assays¹²

In vitro invasive/migrating activity was determined using a BD BioCoat[™] Matrigel[™] Invasion Chamber (BD Biosciences, San Jose, CA, USA). Invasion assay was performed with Falcon cell culture inserts containing an 8-µm pore size poly(ethylene terephthalate) (PET) membrane with a thin layer of matrigel-reconstituted basement membrane. In total, 2.5×10^4 cells were seeded into each insert with serum-free culture medium with/without 10 µg/ml Dox, and each insert was placed into 24-well plate. Serum-containing medium with/without 10 µg/ml Dox was added to each well external to the insert, which acted as a chemo-attractant. After 24 h of incubation at 37 °C in a humidified 5% CO2 atmosphere, non-invading cells were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab. Invading cells were fixed with methanol and stained with crystal violet and counted under a microscope. In vitro migration assay was performed following the same procedure but using PET membrane not coated with matrigel.

Nude mouse model for tumor growth analysis²¹

A SCC-15 cell suspension $(3 \times 10^6 \text{ vital cells/ml})$ was transplanted subcutaneously into the right back of mice (0.1 ml/mice), and 7-day after implantation, induced tumors dimensions were recorded. Dox solution (3 mg/mice/day) was administered to each mouse in Dox-treated group (10 mice) subcutaneously near to the tumor; a 0.9% normal saline solution was administered similarly to all animals in the control normal saline-treated group (10 mice). Length and width of each tumor were measured every day. Following 12-day of consecutive administration of Dox and normal saline, the mice were killed and the tumors removed. One-half of the tumor was stored at -80 °C for real-time PCR analysis, and the other half for hematoxylin-eosin staining. Tumor volume²² was calculated: tumor volume $(mm^3) = 1/2 \times \text{short}$ diameter² \times long diameter. Inhibition rate (IR) of xenograft tumor was calculated: IR(%) = [1 - (tumor volume of doxy-treated group/tumor volume of normal saline-treated group)] \times 100%.

Statistical analysis

Statistical analyses were performed using Jump 7.0 software (SAS, Cary, NC, USA). P < 0.05 was indicated as significant.

Results

Expression of pro-MMP-2 and pro-MMP-9 in SCC-15 cell lines

Gelatin zymography was performed to detect secretion of MMPs into the culture medium, and gelatin zymograms showed that SCC-15 cells secreted MMPs into culture medium after various culture durations (3, 6, 12, 24, and 48 h) (Fig. 1A). Furthermore, upon treating culture medium with p-aminophenylmercuric acetate (APMA) to activate pro-MMPs, a decrease in pro-MMP-2 and pro-MMP-9 activity in a dose-dependent manner was noted (Fig. 1B). We confirmed that SCC-15 cells secrete pro-MMP-2 (72-kD) and pro-MMP-9 (92-kD), respectively.

Effect of Dox on pro-MMP-2 and pro-MMP-9 production (synthesis and/or secretion) in SCC-15 cells

Different pro-MMP-2 and pro-MMP-9 levels were produced and released into culture medium by SCC-15 cells. Pro-MMM-2 and pro-MMP-9 activity were evaluated by gelatin zymography and Western blot and levels of pro-MMP-2 and pro-MMP-9 production were measured. As shown in Fig. 1C–F, when SCC-15 cells were treated with Dox at doses ranging from 0 to 40 μ g/ml for 24 h, latent forms of MMP-2 and MMP-9 were detected in both assays. Using gelatin zymography, it was shown that Dox treatment effected a significant reduction (*P* < 0.05) in pro-MMP-2 and pro-MMP-9 activity in a dose-dependent manner (Fig. 1C– E). Moreover, Western blotting showed that Dox treatment decreased pro-MMP-2 and pro-MMP-9 protein expression in a dose-dependent manner (Fig. 1F). Latent activity and production of MMPs were therefore significantly inhibited by Dox.



Figure 2 (A) Effects of various doses of Dox on SCC-15 cell viability. SCC-15 cells were cultured in serum-free medium and treated with 0–40 µg/ml Dox for 24 h before being subjected to MTS assay to ascertain the cell viability. (B) The stability of proteolytic enzymes obtained from culture media treated with Dox at 10 µg/ml (0–24 h) was analyzed by gelatin zymography. No inhibition of pro-MMP-2 and pro-MMP-9 was observed. Means and standard errors were obtained from triplicate independent experiments. Different letters represent significant differences between groups by Tukey's analysis following one-way ANOVA of P < 0.05.

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Effects of Dox on SCC-15 cell viability

It was critical to ensure that sensitivity of Dox towards SCC-15 cells has no relationship with the effect of Dox on MMP activity. Common sensitivity of Dox at different concentrations $(0-40 \ \mu g/m)$ toward SCC-15 cell viability was evaluated *in vitro*. As revealed in Fig. 2A, it was apparent that after 24 h of Dox treatment, cell viability decreased in a dose-dependent manner. It was also found that concentration of Dox $(10 \ \mu g/m)$ had a significant influence on growth inhibition. Therefore, in subsequent analyses, Dox was used at a concentration of $10 \ \mu g/ml$ in SCC-15 cells experiments. It was also noted that Dox induced growth inhibition in a time-dependent manner (data not shown).

Effect of Dox in a cell-free system

Effect of Dox on the latent activity of MMPs in culture medium was evaluated in a cell-free system. As shown by zymography, Dox did not interfere with the stability and activity of pro-MMP-2 and pro-MMP-9 in culture medium (Fig. 2B).

Effect of Dox on expression of MMPs

Real-time PCR was used to determine the expression levels of MMP-2 and MMP-9 mRNA. After treatment of SCC-15 cells with Dox (10 μ g/ml) for 3, 6, 12, and 24 h, it was observed that MMP-9 mRNA expression was moderately inhibited after the 12 h time-point (*P* < 0.05), but no significant effects were noted before the 12 h time-point (Fig. 3A). No change in MMP-2 mRNA

expression was observed at any time-point (Fig. 3B), even after treatment with Dox at 2.5 and 40 μ g/ml for 12 h (data not shown). For nude mice model, MMP-9 mRNA expression in Dox-treated group was significantly decreased as compared with normal saline-treated control group (*P* < 0.01) (Fig. 3C), but Dox treatment had no significant effect on MMP-2 mRNA expression (Fig. 3D).

Effect of Dox on invasion/migration capabilities

An invasion assay (MatrigelTM) was used to examine inhibitory effect of Dox on invasion potency. Treatment with Dox ($10 \mu g/ml$) inhibited invasive potential of SCC-15 cells up to 75% (Fig. 4C). Migration ability was also significantly inhibited by Dox up to 80% (Fig. 4A, B and D).

Morphology and histology of SCC-15 xenografts

Seven-day after SCC-15 cell implantation, globular tumors were visible in transplanted areas of all nude mice. Following Dox (Dox-treated group) or normal saline (control normal saline group) for 12 consecutive days, tissue samples were removed from both the Dox-treated and normal saline-treated mice. Macroscopic appearance of xenografted tumors revealed a smaller tumor mass in Dox-treated group (Fig. 5B) than in normal saline-treated group (Fig. 5A). Histologically, moderately differentiated SCCs were noted in tumors of the normal saline-treated group and in tumor remnants of Dox-treated group (Fig. 5C–F). Furthermore, tumor necrosis was observed in Dox-treated group (Fig. 5E and F), displaying



Figure 3 Effect of Dox on expression of MMP-9 and MMP-2 mRNA in SCC-15 cells (A and B) and nude mice xenograft tissue (C and D). SCC-15 cells were treated with Dox (10 μ g/ml) for 3, 6 12 and 24 h and the expression levels of MMP-9 mRNA (A) and MMP-2 mRNA (B) were determined by real-time PCR. Expression levels of MMP-9 (C) and MMP-2 (D) mRNA in normal saline-treated (control) and Dox-treated (Dox) nude mice were also evaluated by real-time PCR, and the percentages of expression inhibition are presented after normalizing to GAPDH. Means and standard errors were obtained from three independent experiments. Different letters represent significant differences between groups by Tukey's analysis following one-way ANOVA of P < 0.05; "P < 0.01.

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Figure 4 *In vitro* invasion/migration of SCC-15 cells treated with $10 \mu g/ml$ Dox for 24 h. Invaded/migrated cells were stained with crystal violet and the numbers of cells enumerated microscopically. Microscopic images of migration ($100 \times$) are shown following (A) no Dox treatment and (B) $10 \mu g/ml$ Dox treatment. The percentage differences between the Dox-treated group and the control group are shown for (C) the invasion assay and (D) the migration assay. Means and standard errors were obtained from three independent experiments. Differences were analyzed by the *t*-test. P < 0.05.

degenerative changes with vacuoles within cytoplasm (Fig. 5E and F).

Effect of Dox on tumor growth

Our results showed that the growth of SCC-15 xenografts was significantly affected by Dox treatment. Average tumor size in Dox-treated group was 70.5 mm³; however, that in normal saline-treated group was 489.7 mm³ (Fig. 5G). Rate of inhibition (tumor volume) by Dox treatment was determined to be 85.6%.

Discussion

Poor prognosis for OSCC patients is mostly due to metastasis to distant organs or lymph nodes.² Invasion by degradation of ECM and basement membrane is one of the critical steps in cascade of metastasis.²³ Therefore, agents that down-regulate MMPs could be used in the treatment of this disease. Our study indicated that Dox could reduce many activities of oral cancer cells such as cell proliferation, migration and invasion, synthesis of MMPs, and primary tumor growth. To our knowledge, this is the first study to report upon tumor-suppressive effect of Dox in the treatment of OSCCs by inhibiting MMP synthesis. Tetracycline and its analogues have been employed in the treatment of numerous diseases, and high levels of proinflammatory and protease activity have been observed owing to its non-antibiotic properties.²⁴ Dox has been re-

ported to exert an anti-tumor effect by reducing the activity of gelatinase in different kinds of cancer cell lines.^{13,15,25-27} Mechanism of down-regulation of MMP expression by Dox in OSCCs has not yet been described. It has been reported that Dox causes conformational changes by chelation of the zinc atom at the enzyme binding site,²⁷ blocking the enzymatic activity of MMPs¹⁰ and inhibiting mRNA synthesis.²⁸ Liu et al.²⁹ reported that Dox altered the MMP expression at transcriptional level in part by reducing the stability of MMP-2 mRNA. In this study, it was observed that Dox decreased expression of pro-MMP-2 and pro-MMP-9 proteins and altered the expression level of MMP-9 mRNA but not MMP-2 mRNA, indicating the effect of Dox on MMP-9 expression at transcriptional level but on MMP-2 expression at post-transcriptional level. These findings are consistent with the observed effect of Dox on a human endometrial surface epithelial cell line but differ from the effect noted in endometrial glandular epithelial cells, stromal cells²⁸ and melanoma cells.²⁷ Hence, the effect of Dox on MMP-2/MMP-9 leading to modulation of the invasiveness of tumor cells may be exerted through different mechanisms. In this study, significant dose-dependent inhibitory effects on cell viability were noted at a Dox concentration of 10 µg/ml (reduction in MTS assay, Fig. 2A). However, there was no significant alteration of SCC-15 cells number at 2.5-5 µg/ml (Fig. 2A). Decrease in pro-MMP-2 and pro-MMP-9 production from SCC-15 cells culture treated with 2.5-5 µg/ml of Dox was quantified, and production of pro-MMP-2 was significantly reduced at 5 µg/ml and pro-MMP-9 was at 2.5 µg/ml (Fig. 1D and E). Hence, the extent of this cell growth

cm B G 600 Control Doxycycline (3mg/D) 500 Tumor volume (mm³) 100 0 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 Time after agent treatment (days)

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inhibitory effect was not identical to that of the decrease in MMP-2 and MMP-9 production in SCC-15 cells. In other words, SCC-15 cells are partially sensitive to Dox and reduction in the level of MMPs synthesis is not wholly caused by reduction in cell numbers. Therefore, reduction of expression level of MMP-2 and MMP-9 but not the decrease of cells number of SCC-15 seemed to be of primary significance in considering the possible applications of Dox in the treatment of OSCCs. Evidence of the anti-invasive activity of Dox on oral SCC cells was demonstrated by invasion/migration experiments, which showed that Dox decreased the invasive activity of OSCC cells. The process of invasion is multifaceted and involves the motility of tumor cells and capacity to degrade basement membrane. Our data indicated that anti-invasive activity of Dox on oral SCC cells may arise chiefly due to reduced production of MMPs (Fig. 1C-F, Fig. 4). Interestingly, we also found that Dox did not eliminate the invasive activity of SCC cells completely. This phenomenon may be attributed to the effects of other proteinases secreted by cancer cells that are not inactivated by Dox; for instance, urokinase-like plasminogen activator (uPA) has also been shown to be involved in neoplastic progression of esophageous SCC,³⁰ and Chang et al.³¹ noted that levels of secretion and activity of uPA are not inhibited by Dox. In this study, Dox exhibited an in vivo suppressive effect on the growth of SCC-15 tumors, and the inhibition rate was calculated to be 85.6% after 12-day of Dox administration. Reduction in expression of MMP-9 mRNA and larger areas of tumor necrosis were also evident in the Doxtreated group. These phenomena may contribute to the reduction in tumor size.

Sun et al.³² reported that Dox at a dose of 200 mg/person/day resulted in little adverse reaction. In the xenograft model, we used a dose of 3 mg/mice/day, which is equivalent to about 180 mg/day for an adult via oral administration.³³ In our experiment, neither weight loss nor poor appetite was noted for the animals in the Dox-treated group. A number of synthetic MMP inhibitors have recently been developed as targets for anti-invasive and anti-metastasis intervention.⁹ Dox is comparatively less noxious than other agents and is a widely-accepted treatment that has been reported to exert no obvious side effects when used as an anticancer agent.

In conclusion, our study showed that Dox down-regulated expression of MMP-9 and MMP-2 in SCC-15 cells and inhibited xenograft tumor growth in nude mice. These data may provide evidence of the suitability of Dox as an adjuvant anti-invasive treatment of oral SCCs.

Conflict of Interest Statement

None declared.

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