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Dioscorea nipponica Makino inhibits migration and invasion of human oral cancer HSC-3 cells by transcriptional inhibition of matrix metalloproteinase-2 through modulation of CREB and AP-1 activity

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ABSTRACT

Oral cancer mortality has increased during the last decade due to the difficulties in treating related metastasis. *Dioscorea nipponica* Makino, a popular folk medicine, exerts anti-obesity and anti-inflammation properties. However, the effect of this folk medicine on metastasis of oral cancer has yet to be fully elucidated. The present study demonstrates that *D. nipponica* extracts (DNE), at a range of concentrations (0–50 μg/mL), concentration-dependently inhibited migration/invasion capacities of human oral cancer cells, HSC-3, without cytotoxic effects. The anti-migration effect of DNE was also observed in two other OSCC cell lines, Ca9-22 and Cal-27. Zymography, real time PCR, and Western blotting analyses revealed that DNE inhibited matrix metalloproteinase-2 (MMP-2) enzyme activity, and RNA and protein expression. The inhibitory effects of DNE on MMP-2 proceeded by up-regulating tissue inhibitor of metalloproteinase-2 (TIMP-2), as well as suppressing nuclear translocation and DNA binding activity of cAMP response element-binding (CREB) and activating protein-1 (AP-1) on the MMP-2 promoter in HSC-3 cells. In conclusion, DNE inhibited the invasion of oral cancer cells and may have potential use as a chemopreventive agent against oral cancer metastasis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer, with poor prognosis due to frequent lymph node metastasis and local invasion (Spiro et al., 1974). Cancer invasion and metastasis, the spread of cancer cells from the primary neoplasm to distant sites and their growth there, are the major causes of death in various cancer patients including OSCC (Weiss, 1990). Metastasis of cancer cells involves multiple processes and various cytophysiological changes, including changing adhesion capabilities between cells and the extracellular matrix (ECM) and damaging intercellular interaction. Thus, degradation of the ECM and components of the basement membrane caused by a concerted action of proteinases, such as matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA), play a critical role in tumor invasion and metastasis (Westermarck and Kahari, 1999; Yoon et al., 2003). MMPs are overexpressed in almost all human cancers (Cakarovski et al., 2004; Toda et al., 2006) including OSCC (Patel et al., 2007). Of the MMPs, MMP-2, MMP-9 and their upstream enzyme, urokinase-PA (u-PA), are the most vital enzymes for the degradation of the main constituent of the basement

Abbreviations: DNE, 50% ethanol extract of *Dioscorea nipponica*; ECM, extracellular matrix; MMP, matrix metalloproteinase; u-PA, urokinase plasminogen activator; TIMP, tissue inhibitor of metalloproteinase; CREB, cAMP response element-binding; AP-1, activating protein-1; OSCC, oral squamous cell carcinoma; ChIP, chromatin immunoprecipitation; HPLC, high-pressure liquid chromatography.

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membrane, type IV collagen, and deeply involved in cancer invasion and metastasis (Bjorklund and Koivunen, 2005; Mackay et al., 1990; Nelson et al., 2000). The inhibition of migration or invasion mediated by MMP-2, -9 or u-PA could, therefore, putatively provide a preventive measure against cancer metastasis (Bjorklund and Koivunen, 2005).

In recent years, naturally occurring plant products have gained increasing attention for potential use in intervention against malignant invasive progression in late stage neoplastic diseases (Ravindranath et al., 2009; Shankar et al., 2008). Previous research has demonstrated that certain foods, including many vegetables, fruits, and grains, as well as phytochemicals of diversified pharmacological efficacies, offer significant protection against various cancers (Chen et al., 2005; Huang et al., 2008; Vieira et al., 2007). There is increasing focus on providing a scientific basis for use of these agents as a preventive strategy for people with high risk of cancers. *Dioscorea* plants are important agricultural crops in tropical regions, grown for their large tubers in parts of Africa, Asia, and Oceania (Oke, 1972). The rhizome of *Dioscorea nipponica* Makino has high saponin content and use in folk medicine as treatment for coronary heart disease, asthma, rheumatoid arthritis, and hyperlipidemia, as well as a source of hormonal sterol synthesis precursors (Yoshikawa et al., 2007). Recently, studies revealed that *Dioscorea* plant extracts exert anti-obesity effects (Kwon et al., 2003; Xiao et al., 2010). However, limited studies exist concerning the anti-cancer effects of *D. nipponica* Makino. The present study aimed to investigate the effects of *D. nipponica* extracts (DNE) on cell migration and invasion in cultured OSCC and to study the possible underlying mechanisms.

2. Materials and methods

2.1. Materials

Cell culture materials and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). Antibodies specific for MMP-2, TIMP-2, CREB, SP1, c-Fos, β-actin and C23 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Unless otherwise specified, other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The primers used for amplification of the MMP-2 promoter gene region were as follows: the forward primer, 5′-GGTACCCAGATCGCGAGAGAGGCAAGTAA-3′ and the reverse primer, 5′-AAGCTTGGTTGGAGCCTGCTCCGCGGCG-3′. The PCR-amplified DNA was digested with KpnI and HindIII and cloned into the pGL3-Luc-Basic reporter vector (Promega Corp., Madison, MI).

2.2. Preparation of D. nipponica extracts (DNE)

D. nipponica Makino was purchased from herb stores and the *D.* nipponica extracts (DNE) were prepared by initial condensation followed by lyophilization as described previously (Ho et al., 2011). Hundred grams of air-dried *D. nipponica* Makino was boiled at 50 °C for 12 h with 500 mL of 50% ethanol. The extraction procedure was repeated twice. Solvent was removed from the combined extract using a vacuum rotary evaporator and the filtrate was, then, lyophilized and stored at −20 °C. The chemical profile of DNE was analyzed using high-pressure liquid chromatography (HPLC)–mass spectrometry as described previously (Ho et al., 2011).

2.3. Cell culture

HSC-3, CAL-27 and CA9-22 were squamous cell carcinoma (SCC) and originated from the tumor of mid-internal jugular lymph node (metastasis of tongue cancer) (HSC-3, poorly differentiated cell), middle of the tongue (CAL-27, poorly differentiated cell), and mandibular gingiva (CA9-22, well differentiated cell), respectively. Previously study has shown that these three OSCC cell lines exhibited higher migration ability than other OSCC cell lines (e.g. SAS, FaDu, and SCC-9) (Tseng et al., 2009) and suitable for using in migration/invasion assay. All the OSCC cell lines and normal gingival fibroblast were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES (pH 7.4) in a humidified 37 °C incubator.

2.4. Cell viability assay (MTT assay)

HSC-3 and normal gingival fibroblast cells were grown to 80% confluence, treated with DNE (0–50 μg/mL) for 24 h, and then subjected to cell viability assay (MTT assay). Data were collected from three replicates.

2.5. Flow cytometric analysis

As previously describe (Lin et al., 2001), HSC-3 cells were grown in DMEM supplemented with 10% FBS. After the cells had grown to 80% confluence, treated with DNE (0–50 μg/mL) for 24 h. They were harvested, washed twice with PBS/0.1% dextrose, and then fixed in 70% ethanol at −20 °C. Nuclear DNA was stained with a reagent containing PI (50 mg mL^{-1}) and DNase-free RNase (2 U mL^{-1}) and measured using a fluorescence-activated cell sorter (FACS). The proportion of nuclei in each phase of the cell cycle was determined using established WinMDI 2.9 DNA analysis software.

2.6. In vitro wound closure

HSC-3 cells (1×10^5 cells/well) or CA9-22 and CAL-27 cells (5×10^5 cells/well) were plated in 6-well plates for 24 h, wounded by scratching with a pipette tip, then incubated with DMEM medium containing 0.5% FBS and treated with or without DNE (10–50 μg/mL) for 0, 6, 12, and 24 h. Cells were photographed using a phase-contrast microscope (100×) as previously described (Ho et al., 2011).

2.7. Cell invasion and migration assays

Cell invasion and migration were assayed according to the methods described by Yang et al. (2010b). After treatment with DNE (0–50 μg/mL) for 24 h, surviving cells were harvested and seeded in a Boyden chamber (Neuro Probe, Cabin John, MD, USA) at a density of 10^4 cells/well in serum free medium, and then incubated for 24 h. For invasion assay, 10 μl Matrigel (25 mg/50 mL; BD Biosciences, MA, USA) was applied to 8 μm pore size polycarbonate membrane filters and the bottom chamber contained standard medium. The invaded cells were fixed and stained with 5% Giemsa. Cell numbers were counted under a light microscope. Migration assay was carried out as described in the invasion assay but with no Matrigel coating.

2.8. Gelatin zymography

The activities of MMP-2 and MMP-9 in conditional medium were measured using gelatin zymography protease assays as described previously (Yang et al., 2008). Collected media of an appropriate volume were subjected to 0.1% gelatin–8% SDS–PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in reaction buffer (40 mM Tris–HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) for 12 h at 37 °C. The gel was, then, stained with Coomassie brilliant blue R-250.

2.9. RNA preparation and TaqMan quantitative real-time PCR

Total RNA was isolated from oral cancer cells using Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Quantitative real-time PCR analysis was carried out using TaqMan one-step PCR Master Mix (Applied Biosystems). Hundred nanograms of total cDNA was added per 25 μl reaction with MMP-2 or GAPDH primers and TaqMan probes. The MMP-2, TIMP-2 and GAPDH primers and probes were designed using commercial software (ABI PRISM Sequence Detection System; Applied Biosystems). The oligonucleotide sequences of TaqMan probes and primers were described in Supporting Table S1. Quantitative real-time PCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected.

2.10. Western blot analysis

The total cell lysates or nuclear extracts were prepared as previously described (Yang et al., 2010b). Western blot analysis was performed using primary antibodies against MMP-2, TIMP-2, CREB, SP-1 or c-Fos. The relative photographic densities were quantitated by scanning the photographic negatives using a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

2.11. Transfection and MMP-2 promoter-driven luciferase assays

HSC-3 cells were seeded at a concentration of 5×10^4 cells per well in 6-well cell culture plates. After 24 h of incubation, pGL3-basic (vector) and pMMP-2-luciferase (Luc) or CREB binding site mutant (mCREB) MMP-2-Luc were co-transfected with a β-galactosidase expression vector (pCH110) into cells using Turbofect