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Claudin-2 knockdown decreases matrix metalloproteinase-9 activity and cell migration via suppression of nuclear Sp1 in A549 cells

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ABSTRACT

Aims: Claudin expression is altered in lung cancer, but the pathophysiological role of claudin is not well understood. We examined the effect of claudin-2 expression on cell migration using human adenocarcinoma A549 cells.
Main methods: The mRNA level was measured by real time polymerase chain reaction. To knockdown claudin-2 expression, we made the cells expressing doxycycline-inducible claudin-2 shRNA vector. The protein level was examined by Western blotting. Cell migration was measured by wound-healing assay. The enzymatic activity of MMP-9 was assessed by gelatin zymography.
Key findings: In A549 cells, claudin-2 expression was higher than in normal lung tissue. Claudin-2 knockdown did not affect the expression of other junctional proteins including claudin-1, occludin and E-cadherin. Claudin-2 knockdown decreased cell migration concomitant with a decrease in the mRNA level and enzymatic activity of MMP-9. The expression level of Sp1 in the nuclei was decreased by claudin-2 knockdown. In contrast, the expression levels of c-Fos, c-Jun and NF-kB p65 in the nuclei were not changed by claudin-2 knockdown. The knockdown of Sp1 expression by siRNA decreased cell migration, and the mRNA expression, enzymatic activity, and promoter activity of MMP-9.
Significance: Claudin-2 may increase the mRNA level and enzymatic activity of MMP-9 mediated by the elevation of nuclear distribution of Sp1, resulting in the up-regulation of A549 cell migration.

Introduction

Lung cancer is the major cause of cancer-related deaths in the world, and non-small cell lung cancer (NSCLC) accounts for more than 75% of cases. NSCLC comprises three subtypes, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Collins et al., 2007). Among them, adenocarcinoma is the most common case type in the United States and Asia, and generally develops resistance to radiation and chemotherapy. The strong invasive and metastatic characteristics of lung cancer cells are responsible for the relatively high malignancy. The cell–cell adhesion system is involved in the regulation of invasion and metastasis of cancer cells (Hirohashi, 1998).

At the apical pole of the intercellular junction of the lateral membrane, the tight junction (TJ) composes a large complex of proteins including transmembrane, scaffolding, and signaling proteins. The TJ separates the apical and basolateral epithelial compartments and restricts the flux of ions and solutes through the paracellular space (Anderson et al., 2004; Ikari et al., 2004; Powell, 1981). Claudin and occludin are tetraspanning proteins that extend their extracellular loops across neighboring cells. Claudins comprise a large family of 24 members that form homo- and heterotypic associations with each other (Turksen and Troy, 2004). Different combinations of claudins can confer different properties to epithelial cells in terms of permeability to ions and solutes. In addition, claudins regulate cell proliferation and differentiation (Matter et al., 2005).

The expression of certain claudins is altered in tumors including lung, breast, ovary, pancreas and colon (Dhawan et al., 2005; Jung et al., 2009; Morin, 2005; Nichols et al., 2004). The expression of certain claudins affects the development as well as invasive and migrative properties of cancer cells. Normal lung epithelia express claudin-1, -3, -4, -5, -7, and -18 (Coyne et al., 2003; Daugherty et al., 2004). In contrast, adenocarcinoma and squamous cell carcinoma shows immunopositivity for claudin-2 (Moldvay et al., 2007). Claudin-2 expression may be useful in differential diagnosis and as a target of chemotherapy. However, it has not been examined whether the alteration of claudin-2 expression affects tight junctional integrity and cell migration in lung cancer cells.

Cell migration is regulated by matrix metalloproteinases (MMPs), which belong to a family of zinc-dependent proteases (Nagase and Woessner, 1999). Over 25 members of MMPs have a variety of substrates including extracellular matrix components, cell surface

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receptors and adhesion proteins (Chang and Werb, 2001; Ra and Parks, 2007). MMP-9 expression is higher in NSCLC patients than in healthy subjects (Kaya et al., 2003; Yang et al., 2005). Tissue inhibitors of metalloproteinases (TIMPs) are the controlling factors in the actions of MMPs (Catania et al., 2007). Four distinct TIMP molecules, TIMP-1, TIMP-2, TIMP-3 and TIMP-4, have been cloned and characterized in vertebrates. Among them, TIMP-1 appears to be the major inhibitor and forms a complex with MMP-9 in a noncovalent fashion.

In the present study, we examined the effect of claudin-2 expression on cell migration in human lung adenocarcinoma A549 cells. Claudin-2 knockdown decreases the enzymatic activity of MMP-9 and cell migration. Sp1, a transcriptional factor of MMP-9, increases cell migration in lung cancer cells (Hung et al., 2010; Luo et al., 2009). We found that nuclear Sp1 level is decreased by claudin-2 knockdown. Our findings indicate that claudin-2 increases MMP-9 activity and cell migration mediated by the elevation of nuclear distribution of Sp1.

Materials and methods

Materials

Rabbit anti-claudin-2, claudin-1, and goat anti-occludin antibodies were obtained from Zymed Laboratories (South San Francisco, CA, USA). Mouse anti-E-cadherin and nucleoporin p62 antibodies were from Becton Dickinson Biosciences (San Jose, CA). Mouse anti-Sp1, goat anti-c-Fos, c-Jun, β-actin, and rabbit anti-NF-kB p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Three independent mRNAs of normal lung tissue were from Clontech laboratories (Mountain View, CA), Agilent Technologies (Santa Clara, CA), and BioChain (Hayward, CA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade of purity available.

Cell culture and transfection of inducible claudin-2 shRNA vector

A549 cell line was obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Saint Louis, MO) supplemented with 5% fetal calf serum (HyClone, Logan, UT), 0.07 mg/ml penicillin-G potassium, and 0.14 mg/ml streptomycin sulfate in a 5% CO_2 atmosphere at 37 °C. For the inducible knockdown of endogenous claudin-2 expression, double-stranded oligonucleotides (5′-TCGAGGGAAGTTCGAGATCGGAGAATTCAAGAGATTCTCCGATCTCGAACTTCCTTTTTTACGCGTA-3′) were cloned into the vector pSingle-tTS-shRNA (Clontech). Claudin-2/pSingle-tTS-shRNA was then introduced into A549 cells using Lipofectamine 2000 as recommended by the manufacturer. Stable clones were screened in the presence of 500 μg/ml nG418. The knockdown of claudin-2 was determined by immunoblotting using the anti-claudin-2 antibody.

Preparation of cell lysate and nuclear extraction

Cells were scraped into cold PBS and precipitated by centrifugation. They were lysed in a RIPA buffer containing 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl (pH 8.0), a protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate, and sonicated for 20 s. After centrifugation at 6000×*g* for 5 min, the supernatant was collected (cell lysate). Nuclear and cytoplasmic fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents as recommended by the manufacturer (Pierce, Rockford, IL). Protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was carried out as described previously (Ikari et al., 2001). In brief, the sample (30–60 μg) was applied to the SDS-polyacrylamide gel. Proteins were blotted onto a PVDF membrane and incubated with each primary antibody for 16 h at 4 °C followed by a peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, the blots were stained with an ECL Western blotting kit from GE Healthcare UK Ltd. (Amersham Place, England).

Immunofluorescence measurement

Cells were cultured on cover glasses. The cells were fixated with methanol for 10 min at −20 °C, then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 2% block ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, the cells were incubated with anti-Sp1, c-Fos, c-Jun, NF-kB p65 antibodies for 16 h at 4 °C. They were then incubated with AlexaFluoro 488 or 543 antibody and DAPI for 1 h at room temperature. Immunolabeled cells were visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany).

Wound-healing migration assay

Before wounding, cells were allowed to form a confluent monolayer in a 35-mm dish. Cells were wounded with a 1000-μl pipette tip and incubated in new DMEM supplemented with 1% FCS for the periods indicated. Photographs were taken using an Olympus IX70 microscope with the acquisition software QCapture Pro. The cell-free area was measured using Scion Image software (Scion Corporation, Frederick, Maryland). Cell migration was calculated as the percentage of the remaining cell-free area compared with the area of the initial wound.

RNA isolation and RT-PCR

Total RNA was isolated from A549 cells using TRI reagent (Sigma-Aldrich). Reverse transcription was carried out with M-MLV reverse transcriptase (Promega, Madison, WI) and random primers. Single strand cDNA was amplified by PCR using GoTaq DNA polymerase under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min; these steps were repeated 30 cycles. The primers used in PCR are listed in Table 1. Quantitative real time PCR was performed using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan). The threshold cycle (ct) for each PCR product was calculated with the instrument's software, and ct values obtained for claudin-1–7, -9, -12, and -18, MMP-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 were normalized by subtracting the ct values obtained for β-actin. The resulting Δct values were then used to calculate the relative change in mRNA expression as a ratio (R) according to the equation $R = 2^{-(\Delta ct(\text{treatment}) - \Delta ct(\text{control}))}$.

Gelatin zymography assay

The proteolytic activity of MMP-9 was assessed by gelatin zymography as described elsewhere (Llorens et al., 1998). Samples were mixed with SDS sample buffer without a reducing agent and separated on 7.5% SDS-PAGE gels containing 0.1% gelatin at 4 °C. The gels were washed with 2.5% Triton X-100 for 1 h at 25 °C and incubated in a development buffer (200 mM NaCl, 5 mM $CaCl_2$, and 50 mM Tris–HCl, pH 7.5) overnight at 37 °C. Bands were visualized by staining with Coomassie Brilliant Blue R250.