Roles of Calcium-Binding Proteins, S100A8 and S100A9, in Invasive Phenotype of Human Gastric Cancer Cells

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INTRODUCTION

S100 calcium-binding proteins are involved in various calcium-mediated cellular functions including cell growth, differentiation, cell migration, cell adhesion, and signal transduction (Li et al., 2004; Arai et al., 2004; Kerkhoff et al., 1999; Li and Bresnick, 2006). Among the S100 proteins, S100A8 (myeloid-related protein-8, MRP8, or calgranulin A) and S100A9 (MRP14 or calgranulin B) have been shown to be implicated in tumor development or progression (Gebhart et al., 2002; Ott et al., 2003; Arai et al., 2004). S100A8 and S100A9 proteins are highly homologous and secreted as a S100A8/A9 heterocomplex (reviewed by Donato et al., 2001). S100A8 and S100A9 are overexpressed in various cancers including gastric cancer (El-Rifai et al., 2002), prostate cancer (Hermani et al., 2005), breast cancer (Cross et al., 2005), lung adenocarcinomas (Arai et al., 2001), pulmonary adenocarcinoma (Arai et al., 2001), and hepatocellular carcinoma (Arai et al., 2000).

Tumor cell invasion and metastasis are complex processes involving extracellular matrix (ECM)-degrading proteinase activity and migration through the ECMs (Fidler, 1990). Mounting evidence suggests a role for members of the matrix metalloproteinase (MMP) family on tumor invasion and metastasis formation, especially, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B) which degrade type IV collagen, the major structural collagen of the basement membrane (Tryggvason, 1993; Stetler-Stevenson, 1990; Liotta et al., 1991). Numerous studies show a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotypes of cancer cells (Ura et al., 1989). Our previous studies suggested the involvement of MMP-2 in H-ras-induced invasive phenotype of MCF10A human breast epithelial cells (Moon et al., 2000; Kim et al., 2003), while H-ras-mediated transformation and invasiveness were associated with enhanced expression of MMP-9 mRNA and protein in rat and human embryonic fibroblasts (Bernhard et al., 1994).
Gastric cancer is one of the most common malignancies and is a frequent cause of cancer-related death in Korea. Cure rate of gastric cancer is quite low because of local invasion and metastasis (Ji et al., 2005). Most patients who are diagnosed with gastric cancer exhibit advanced disease which is often associated with the submucosal invasion of tumor cells (Tsuchiya et al., 1995; Yasuda et al., 1999). It has been suggested that MMP-2 expression is involved in tumor invasion and metastasis of gastric carcinoma (Nomura et al., 1996; Sundlad et al., 1998; Monig et al., 2001; Takahashi et al., 2002). In the present study, we investigated the possible association of S100A8 and S100A9 in the invasive phenotype and MMP-2/9 expression of a gastric cancer cell line. We provide evidences that S100A8 and S100A9 play crucial roles in invasive phenotype and MMP-2 expression in SNU484 human gastric cancer cells.

MATERIALS AND METHODS

Cell lines
Hs683 human glioma cell line (KCLB No. 30138), SK-Hep-1 hepatocellular carcinoma cell line (KCLB No. 30052), MCF7 breast cancer cell line (KCLB No. 30022), and HeLa cervix cancer cell line (KCLB No. 10002) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). T47D breast cancer cell line, LNCaP human prostate cancer cell line and SNU484 gastric cancer cell line were provided by Dr. Y.-J. Surh (Seoul National University, Seoul, Korea), Dr. M.-S. Dong (Korea University, Seoul, Korea), and Dr. H.-D. Um (Korea Institute of Radiological and Medical Science, Seoul, Korea), respectively.

Hs683, SK-Hep-1, MCF7, T47D and HeLa cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin-streptomycin. LNCaP cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin-streptomycin. SNU484 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 50 units/ml gentamicin. Cells were maintained in humidified atmosphere with 95% air and 5% CO₂ at 37°C.

Immunoblot analysis
Equal amounts of protein extracts in SDS-lysis buffer were subjected to 12% SDS-PAGE analysis and electrophoretically transferred to nitrocellulose membrane. Anti-S100A8, anti-S100A9 and anti-MMP-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA). MMP-2 antibody was from R&D system (Minneapolis, U.S.A.). β-actin antibody was from Sigma-Aldrich (St. Louis, MO). Enhanced chemiluminescence (Amersham-Pharmacia, Buckinghamshire, U.K.) system was used for detection. Relative band intensities were determined by quantitation of each band with an Image Analyzer (Vilber Lourmnat, France).

Synthesis of small-interfering RNA (siRNA) and transfection
The siRNA molecules targeting S100A8 (5'-CCAUCA-UCAACACCUCUCCACCAAUA-3') and S100A9 (5'-CCU-UGAACUCUAGCAGCUCUA-3') were purchased from Invitrogen (Carlsbad, CA). Cells were plated in six-well plates at 2×10⁵ cells/well, grown for 24 h then transfected with each 25 pmole siRNA for 6 h using lipofectamine and OPTI-MEMI reduced serum medium (Invitrogen, Carlsbad, CA). Control cell were treated with Stealth™ RNAi negative control duplex (Invitrogen, Carlsbad, CA).

Gelatin zymogram assay
Cells were cultured in serum-free medium for 48 h. Gelatinolytic activity of the conditioned media was determined by gelatin zymogram assay as previously described (Moon et al., 2000). Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

In vitro invasion assay
In vitro invasion assay was performed using 24-well transwell as previously described (Kim et al., 2003). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Lower compartment was filled with serum-free media containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, incubated for 18 h, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400. 13 fields were counted for each filter and each sample was assayed in triplicate.

Transwell migration assay
In vitro migration assay was performed using a 24 well transwell unit with polycarbonate filters as previously described (Kim et al., 2003). Experimental procedures were the same as the in vitro invasion assay described above except that the filter was not coated with Matrigel for the migration assay.

Reverse transcription (RT)-PCR detection
Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the Manufacturer's Instruction. Incubated RNA/Oligo dTdTNP mix was reverse transcribed by RT-Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Primers were designed according to the