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Tetraspanin CD9 induces MMP-2 expression by activating p38 MAPK, JNK and c-Jun pathways in human melanoma cells

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Abbreviations: AP-1, activating protein 1; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA

Abstract

Expression of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9), which correlates with tumor invasion and metastasis, has been known to be regulated by several intracellular signaling pathways. Since the CD9 membrane protein has been implicated in signal transduction and malignant progression of cancer cells, we examined the functional involvement of CD9 in the regulation of MMP-2 and MMP-9 expression by using stable CD9 transfectant clones of MelJuso human melanoma cells. The CD9 cDNA-transfected cells with elevated CD9 expression displayed increased MMP-2 and decreased MMP-9 expression when compared with the mock transfectant cells. Among several signal pathway inhibitors tested, SB203580 and SP600125, which inhibit p38 MAPK and JNK respectively, completely blocked the CD9-stimulated MMP-2 expression. Phosphorylation levels of p38 MAPK and c-Jun in MelJuso cells were also significantly increased by CD9 transfection. In addition, the down-regulation of p38 MAPK and JNK by siRNA transfection resulted in a decrease in MMP-2 expression by MelJuso cells. Promoter analysis and gel shift assay showed that the CD9-induced MMP-2 expression is mediated by a functional AP-1 site through interactions with AP-1 transcription factors including c-Jun. These results suggest that CD9 induces MMP-2 expression by activating c-Jun through p38 MAPK and JNK signaling pathways in human melanoma cells.

Keywords: AP-1; CD9; c-Jun; JNK; melanoma; MMP-2; p38 MAPK; tetraspanin

Introduction

The matrix metalloproteinases (MMPs), which are involved in the degradation of extracellular matrix (ECM), play a role in many physiological processes such as embryonic development, angiogenesis, ovulation, and repair of tissues. Since tumor invasion and metastasis also require controlled degradation of ECM, abnormal expression of MMPs is associated with malignant tumor progression (Chambers and Matrisian, 1997). Among members of the MMP family, MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) specifically degrade type IV collagen, the main component of the basement membrane, and appear to play a crucial role in tumor invasion and metastasis (Bernhard *et al.,* 1994; Liabakk *et al.,* 1996; Itoh *et al.,* 1998; Johnsen *et al.,* 1998). Increases in activity and expression of MMP-2 and/or MMP-9 have been frequently observed in many human cancers with invasive and metastatic capability (Bernhard *et al.,* 1994; Heppner *et al.,* 1996; Basset *et al.,* 1997; Johnsen *et al.,* 1998). Expression of both MMP-2 and MMP-9 is induced by growth factors, cytokines, phorbol esters, and other environmental factors such as binding to the extracellular matrix (ECM) (Segain *et al.,* 1996; Sehgal *et al.,* 1996; Basset *et al.,* 1997; Kondapaka *et al.,* 1997; Johnsen *et al.,* 1998; Westermarck and Kahari, 1999). In addition, some of the cellular proto-oncogenes have been shown to regulate the expression of MMPs; for example, transfection of MCF-10A breast cancer cells with *c-erbB-2* or *c-ras* led to increased production of MMP-2, whereas transfection of MCF-7 breast cancer cells with ets gene, *PEA-3*, resulted in increased expression of MMP-9 (Giunciuglio *et al.,* 1995; Kaya *et al.,* 1996). Apart from the proto-oncogenes, p53 tumor suppressor gene has also been shown to upregulate MMP-2 expression (Bian and Sun, 1997).

Tetraspanins, or transmembrane 4 superfamily (TM4SF) molecules, are characterized by the exis-

tence of four highly conserved transmembrane domains (Maecker *et al.,* 1997; Boucheix and Rubinstein, 2001; Yanez-Mo *et al.,* 2001) and several tetraspanin proteins such as CD9, CD63, CD82, and CD151 have been implicated in tumor invasion and metastasis (Ikeyama *et al.,* 1993; Dong *et al.,* 1995; Radford *et al.,* 1995; Kohno *et al.,* 2002). In particular, CD9, which has also been identified as motility related protein-1(MRP-1), has been suggested to be involved in the malignant progression of several types of human cancer; i.e. CD9/MRP-1 was found to inversely correlate with the stage of tumor progression or appearance of metastases for several malignant diseases including melanoma, breast, lung, colon, and ovarian cancer (Si and Hersey, 1993; Miyake *et al.,* 1995; Adachi *et al.,* 1998; Mori *et al.,* 1998; Houle *et al.,* 2002). Transfection with CD9 cDNA resulted in suppression of tumor cell motility and metastasis (Ikeyama *et al.,* 1993; Miyake *et al.,* 2000). Besides the functional involvement of CD9 in tumor cell malignancy, CD9 has also been implicated in various cellular and physiological processes, including cell adhesion, motility, proliferation, differentiation, development, fertilization, and wound healing (Maecker *et al.,* 1997; Aoyama *et al.,* 1999; Baudoux *et al.,* 2000; Miyado *et al.,* 2000; Penas *et al.,* 2000; Boucheix and Rubinstein, 2001; Clay *et al.,* 2001). However, the biological mechanisms of CD9 are still not clearly understood.

In the present study, we investigated the activity and expression of type IV collagenases in melanoma cells after transfection with CD9 cDNA, because most of the CD9-related biological processes including tumor metastasis are known to be regulated by the MMPs, particularly MMP-2 and MMP-9. Our results indicate that high CD9 expression in melanoma cells results in increased expression of MMP-2 through p38 MAPK- and JNK-dependent AP-1 activation, in contrast to its effect on MMP-9 expression.

Meterials and Methods

Cell culture and reagent

C8161 and MelJuso human melanoma cell lines were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco-BRL, Grand Island, NY) in 5% CO_2 at 37°C. Anti-CD9 mAb was purchased form PharMingen (San Diego, CA). Antibodies for phospho-Src (Tyr416), Src, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-c-Jun (Ser63/Ser73), and c-Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GF109203X, PD98059, SP600125, and PP1 were purchased from Biomol (Plymouth Metting, PA). LB42708 were obtained form LG Life Sciences (Daejeon, Korea). All other reagents were from Sigma (St. Louis, MO), unless indicated otherwise.

RT-PCR analysis

Total cellular RNA was purified from the cultured cells using Trizol reagent (Gibco-BRL) according to the manufacturer's protocol. First strand cDNA synthesis was performed with 1 μg of total RNA using a cDNA synthesis kit (Promega, Madison, WI). For PCR amplification, 5'-CAAGCTTGGGATGCCGGTCAAAGG-3' was used as the sense primer and 5'-CGA-ATTCCTAGACCATCTCGCGGTTCC-3' as the antisense primer. This primer pair amplifies a 680 bp fragment of CD9 cDNA. The reaction mixture was subjected to 25 PCR amplification cycles of 60 sec at 94°C, 90 sec at 55°C, and 90 sec at 72°C.

Transfection of CD9 cDNA and selection of stable clones

A full-length of CD9 cDNA was subcloned into the *Eco*RI/*Kpn*I sites of pcDNA3 vector (Invitrogen, San Diego, CA), downstream of CMV promoter. The CD9 cDNA expression construct was transfected into MelJuso human melanoma cells by using lipofectAMINE (Gibco-BRL) according to the manufacturer's instructions. pcDNA3 vector only was also transfected as a control. Neomycin-resistant clones were isolated by growing the cells in DMEM/F-12 containing 10% FBS and 0.5 mg/ml G418 (Gibco-BRL). Stable transfectant clones were characterized by Western blotting and flow-cytometric analyses for their expression levels of CD9 protein.

Western blotting analysis

Cell lysates were prepared and resolved on 10% SDS-PAGE as described (Jee *et al.,* 2003). After transferring to ECL Hybond membrane (Amersham, Piscataway, NJ), proteins were probed with antibodies against CD9, phospho-Src, Src, phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, p38 MAPK, phospho-c-Jun, and c-Jun. After washing, membrane filters were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. The specific proteins were visualized by the Amersham ECL reagent and exposure to Kodak Xomat film.

Flow-cytometric analysis

Cells were incubated with 10 μg/ml anti-CD9 mAb for 30 min, washed with cold PBS, and then incubated with saturating concentrations of FITC-conjugated goat anti-mouse IgG (Parmingen) for 30 min at 4°C. After washed with PBS, the cells were fixed with 2% formaldehyde in PBS. Cell surface immunofluorescence was analyzed by flow cytometry performed on a FACScan (Beckon Dickinson, San Diego, CA).

Gelatin zymography

Type IV collagenase activities present in conditioned medium were visualized by electrophoresis on gelatin-containing polyacrylamide gel as described (Jang and

Lee, 2003). Briefly, conditioned medium from cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer (40% (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% bromophenol blue) and loaded without boiling onto 10% SDS-polyacrylamide gel containing type 1 gelatin (1.5 mg/ml). After electrophoresis at 4°C, the gel was soaked in 2.5% Triton X-100 with gentle shaking for 30 min with one change of detergent solution. The gel was rinsed and incubated for 24 h at 37°C in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$ and 0.02% NaN_3). Following incubation, the gel was stained with 0.05% Coomassie brilliant blue G-250 and destained in 10% acetic acid and 20% methanol.

Promoter assay

A 1716 bp DNA fragment (-1659 to +57), corresponding to the promoter of the human MMP-2 gene, was generously gifted by Dr. Seung-Taek Lee (Yonsei Univ., Korea). The MMP-2 promoter DNA region was used as a template for PCR reaction to generate a series of promoter deletion mutants for AP-1 (-1270 to -1263), c-myc (-1162 to -1157), and CREB (-300 to -295) binding sites. The PCR products were subcloned into a promoterless luciferase expression vector, pGL3 (Promega). The pGL3 vector containing wild-type (WT) or mutant MMP-2 promoters were transfected into MelJuso cells by using LipofectAMINE (Gibco-BRL). Luciferase activity in cell lysate was measured using Promega luciferase assay system according to the instructions of the manufacturer. To normalize the luciferase activity, each of the pGL3 vectors was co-transfected with a pRL-SV40ΔEnh, which express *Renilla* luciferase by an enhancerless SV40 promoter (Hah and Lee, 2003).

Electrophoretic mobility shift assay (EMSA)

Cells were incubated with serum-free medium for 4 h and nuclear extracts were prepared as described (Na *et al.,* 2004). A double-stranded AP-1-specific probe (5'-CGCTTGATGAGTCAGCCGGAA-3'; the AP-1 recognition sequence is underlined) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on a G-50 Sephadex column. The ^{32}P-labeled probe (~40,000 cpm) was then incubated with the nuclear extracts (10 μg of protein) for 20 min at room temperature. Samples were resolved on native 5% polyacrylamide gel, and the gel was dried and subjected to autoradiography.

Transfection of siRNA

Small interfering RNAs (siRNAs) for JNK and p38 MAPK were designed and synthesized using the software and Silencer™ siRNA construction kit from Ambion (Austin, TX) according to the manufacturer's instructions. Specific oligonucleotide sequences for each target gene were as follows: 5'-UGUCUGGUAUGAUCCUUCUdTdT-3' (sense) and 5'-AGAAGGAUCAUACCAGACAdTdT-3' (antisense) targeting JNK; 5'-AGCAGGGACCUCCUUAUAGdTdT-3' (sense) and 5'-CUAUAAGGAGGUCCCUGCUdTdT-3' (antisense) targeting p38 MAPK. The siRNA control was 5'-AGGAGAUAUUUCGAGGCUUdTdT-3' (sense) and 5'-AAGCCUCGAAAUAUCUCCUdTdT-3' (antisense), which bears no homology with relevant human genes (Duxbury *et al.,* 2004). For siRNA transfection, cells (5×10^5) were seeded in 35 mm 6-well plates and grown for 24 h to reach 60-70% confluency. The different amounts of the siRNA and the lipofectAMINE reagent (5 μl) were diluted in 200 μl of DMEM/F-12 medium. The diluted siRNA-liposome complex was added into the wells containing 800 μl DMEM/F-12. Following 6 h incubation, cells were rinsed with fresh medium and grown for 24 h in normal growth medium containing FBS before analysis.

Results

CD9 expression in human melanoma cell lines and generation of CD9 transfectant clones

We performed RT-PCR analysis using primers specific for CD9 cDNA to examine the expression of CD9 in two human melanoma cell lines, C8161 and MelJuso. A 680 bp PCR product was detected in both melanoma cell lines, indicating that both C8161 and MelJuso are CD9-positive cell lines (Figure 1A). To examine the functional effect of CD9 expression on the basement membrane-degrading ability of melanoma cells, MelJuso cells which show lower CD9 expression level than C8161 cells were transfected with either a control pcDNA3 empty vector or a CD9 cDNA expression vector. Expression of CD9 protein in resultant transfectant clones was analyzed by Western-blotting and flow-cytometric analyses using anti-CD9 mAb (Figure 1B and 1C). Several stable transfectant clones obtained with the CD9 cDNA expression vector displayed increased levels of CD9 protein when compared with parental Meljuso cells. Two of these clones, CD9/C19 and CD9/C37, which exhibit much higher levels of CD9 protein than the mock transfectant generated with an empty vector, were selected for further analyses.

Modulation of the activities and expression levels of MMP-2 and MMP-9 by CD9 transfection

To investigate how increased CD9 expression affects activity and/or expression of the type IV collagenases, MMP-2 and MMP-9, gelatinase activity was determined by gelatin zymography from culture supernatants of the CD9-transfected MelJuso cells. As a result, both MMP-2 and MMP-9 activities were detected in the culture of MelJuso cells (Figure 2A). The activity of proMMP-9 was significantly decreased by CD9 transfection, although active MMP-9 was detected in one of the CD9 transfectant clones. On the contrary, the activity of proMMP-2 in the CD9 transfectant clones was much higher than that in the mock transfectant, and the activity corresponding to active

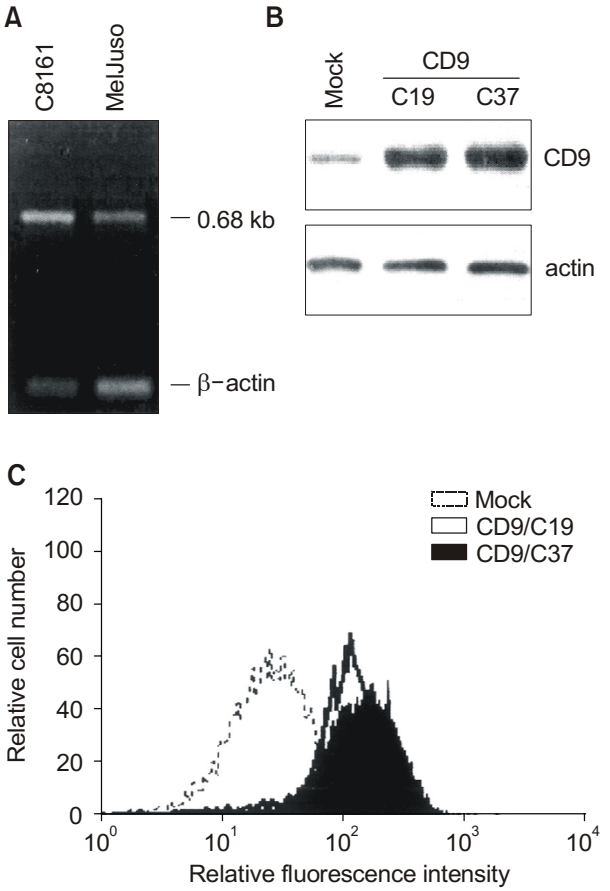

Figure 1. CD9 expression in the parental lines and transfectant clones of human melanoma cells. (A) CD9 mRNAs in total RNA (1 μg) obtained from two human melanoma cell lines, C8161, MelJuso, were amplified by RT-PCR, then electrophoresed on a 1.5% agarose gel. β-actin mRNA from each cell line was also analyzed to control for equal RNA amounts. (B) CD9 protein levels in the stable clones of CD9 cDNA-transfected MelJuso cells were analyzed by Western blotting using anti-CD9 mAb. (C) Cell surface expression levels of CD9 protein in the CD9 transfectant clones were analyzed by flow cytometry using anti-CD9 mAb.

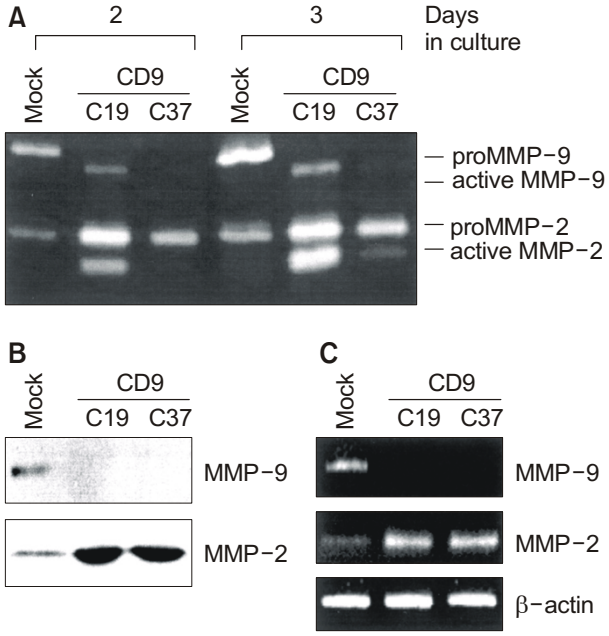

Figure 2. Activities and expression levels of type IV collagenases in the CD9-transfected MelJuso clones. Subconfluent cells were cultured in serum-free medium for the time period indicated. (A) Activities of MMP-2 and MMP-9 in conditioned medium of the cell culture were evaluated by gelatin zymography. (B) Protein levels of MMP-2 and MMP-9 in the conditioned medium of the 3-day cell culture were determined by Western blotting analysis using anti-MMP-2 and anti-MMP-9 mAb. In zymography and Western blotting analysis, the conditioned medium of each cell culture was normalized by cell counting after 3 day culture. (C) Total RNA was isolated from cells that were cultured in serum-free medium for 2 days and RT-PCR analysis was performed.

MMP-2 was also prominent after CD9 transfection. When the expression levels of MMP-2 and MMP-9 were examined, the CD9 transfectants exhibited significantly increased protein and mRNA levels of MMP-2 compared with the mock transfectants, whereas protein and mRNA levels of MMP-9 detected in the mock transfectant were completely knock-downed after CD9 transfection (Figure 2B and 2C). This result indicates that CD9 plays a differential role in modulating expression of type IV collagenases in MelJuso melanoma cells; that is, up-regulating MMP-2 expression versus down-regulating MMP-9 expression.

AP-1 involvement in the CD9-induced MMP-2 expression

To examine whether CD9 activates the MMP-2 promoter and determine which transcriptional factor binding sites in the MMP-2 promoter are important for the CD9-induced MMP-2 expression, wild-type (WT) and several deletion mutants (Δ) of the MMP-2 promoter were generated and subcloned into a pGL3-luciferase reporter vector (Figure 3A). When the WT-MMP-2 promoter reportor vector was transiently transfected into MelJuso cells, the CD9 transfectant cells showed about 7-fold higher luciferase activity than the mock transfectant cells (Figure 3B). In contrast to the wild-type promoter, a deletion mutation of the AP-1 binding site (ΔAP-1-MMP-2) abrogated the CD9-induced activation of the MMP-2 promoter. However, mutations of the c-myc (Δc-myc-MMP-2) and CREB (ΔCREB-MMP-2) binding sites did not abolish the stimulating effect of CD9 on the MMP-2 promoter activity. To determine whether CD9 expression increases DNA binding activity of AP-1 transcriptional factors, we compared the binding of AP-1 factors to a putative AP-1 binding site of MMP-2 promoter between the mock and CD9 transfectant cells (Figure 3C). DNA binding activity of AP-1 factors in the CD9 transfectant was higher than that in the mock transfectant. In particular, incubation of the AP-1/DNA complex with anti-c-Jun antibody resulted

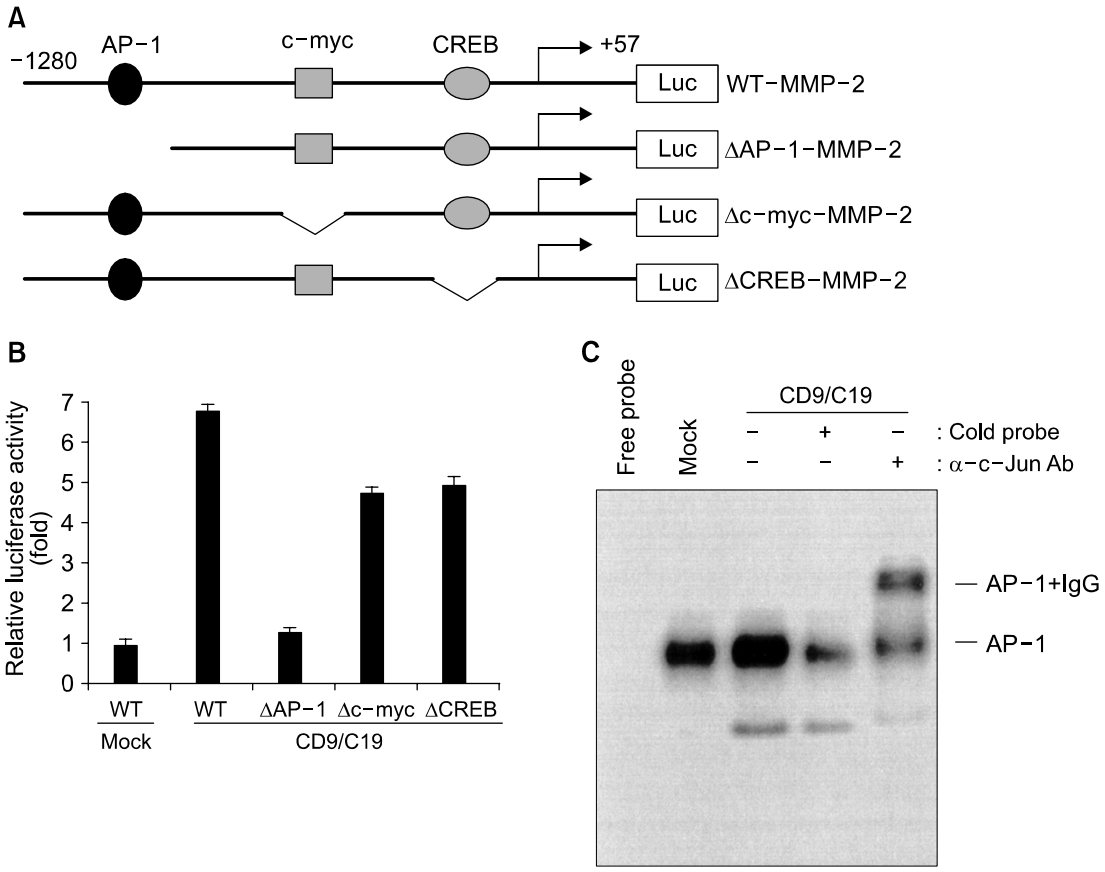

Figure 3. MMP-2 promoter activity and nuclear AP-1 activity in CD9-transfected MelJuso cells. (A) Human wild-type MMP-2 promoter (WT-MMP-2) and the MMP-2 promoter deletion mutants for an AP-1 (ΔAP-1-MMP-2), c-myc (Δc-myc-MMP-2), or CREB (Δ CREB-MMP-2) binding site were fused to a luciferase reporter gene of pGL3 vector. Cells were transiently transfected with the MMP-2-luciferase constructs by using LipofectAMINE. 48 h after transfection, luciferase activity was measured in cell extracts by a luminometer. Each sample was assayed in triplicate and the experiment was repeated two independent times. (C) DNA binding activity of AP-1 factors in nuclear extract of the CD9 transfectant cells was analyzed by EMSA. Specific binding activity of AP-1 factors was determined with 3-fold excess of cold wild-type probe or anti-c-Jun mAb.

in partial supershift of the complex band in the gel shift assay, indicating that c-Jun participates in the formation of the AP-1/DNA complex. Therefore, it seems likely that CD9 induces MMP-2 gene expression by increasing MMP-2 gene promoter binding activity of AP-1 transcription factors including c-Jun.

Signaling pathways for the CD9-stimulated MMP-2 expression

To identify signaling pathway(s) taken downstream of CD9 for the induction of MMP-2 expression, we examined the activity and expression level of MMP-2 in the CD9-transfected MelJuso cells in the presence of several inhibitors of signaling molecules with the highest concentration that did not induce MelJuso cell death for the 2-day culture period. Some of the inhibitors such as GF109203X (a protein kinase C inhibitor), wortmanin (a phosphatidylinositol 3-kinase inhibitor), PD98059 (a MEK inhibitor), and PP1 (a Src kinase family inhibitor) exerted little inhibition effect on

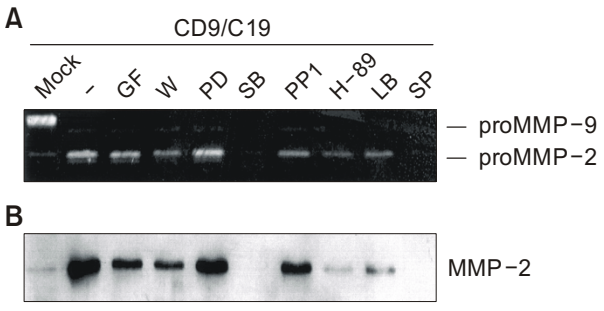

Figure 4. Effects of signaling molecule inhibitors on the CD9-induced MMP-2 activity/expression in the CD9 transtantant cells. The CD9 transfectant clone, CD9/C19 was cultured in serum-free medium containing GF109203X (10 μM), wortmanin (0.1 μM), PD98059 (30 μM), SB203580 (30 μM), PP1 (0.25 μM), H-89 (0.12 μM), LB42708 (10 μM), or SP600125 (20 μM). Following 2 day incubation, the culture supernatant was collected and analyzed with (A) gelatin zymography and (B) Western blotting using anti-MMP-2 mAb. Before the analyses, the each culture supernatant was normalized by viable cell counting with trypan blue staining after 2-day culture.

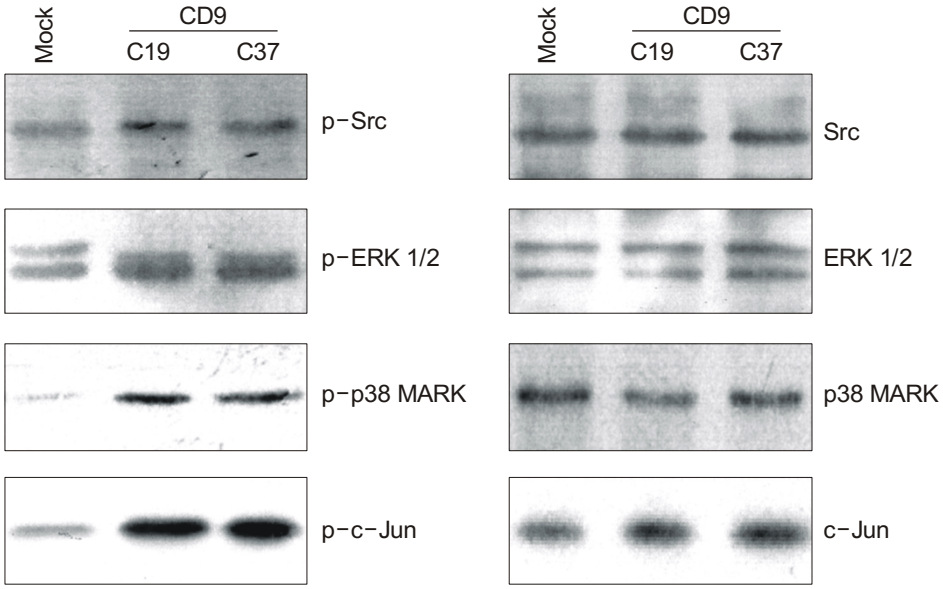

Figure 5. Activation status of intracellular signal transduction mediators in the CD9 transfectant clones. Total cellular proteins were extracted from subconfluent cells that were starved for 2 h and analyzed by Western blotting analysis. Antibodies specific for phospho-Src$^{(Tyr416)}$, Src, phospho-ERK1/2$^{(Thr202/Tyr204)}$, ERK1/2, phospho-p38 MAPK$^{(Thr180/Tyr182)}$, p38 MAPK, phospho-c-Jun$^{(Ser63/Ser73)}$ and c-Jun were used for immunodetection.

the CD9-stimulated MMP-2 activity and expression level (Figure 4). However, the activity and expression of MMP-2 in the CD9 transfectant cells were significantly inhibited by H-89 (a protein kinase A inhibitor) and LB42708 (a $p21^{ras}$ signaling blocker). In particular, SB203580 (a p38 MAPK inhibitor) and SP600125 (a JNK inhibitor) completely abolished the MMP-2 activity and expression in MelJuso cells. This result suggests that protein kinase A, $p21^{ras}$, p38 MAPK, and JNK may participate in the CD9 signaling pathway for the induction of MMP-2 expression. We next examined the activation status of p38 MAPK and c-Jun in the CD9 transfectant cells by monitering the phosphorylation levels of those proteins. As expected from the inhibition effects of SB203580 and SP600125, the phosphorylation levels of both p38 MAPK and c-Jun in the CD9 transfectants were much higher than those in the mock transfectant (Figure 5). However, little significant differences in the phosphorylation levels of Src and ERK-1/2 were observed between the mock and CD9 transfectants, in accordance with little effects of PP1 and PD98059 on the CD9-stimulated MMP-2 expression. It therefore appeared likely that p38 MAPK and JNK mediate CD9 signaling pathway(s) leading to increased MMP-2 gene expression.

Blocking effects of siRNAs specific for p38 MAPK and JNK on the CD9-induced MMP-2 expression

To verify the participation of p38 MAPK and JNK in the CD9 signaling pathway(s) for the induction of MMP-2 expression, siRNAs targeted to p38 MAPK and JNK were transfected into the CD9-transfected MelJuso cells. As a result, mRNA levels of p38 MAPK and JNK in MelJuso cells were completely knockdowned by each specific siRNA with an amount of 80 and 20 pmole, respectively (Figure 6A). In gelatin zymography, MMP-2 activity in the CD9 transfectant cells was shown to be decreased by transfections of both siRNAs targeted to p38 MAPK and JNK (Figure 6B). Also, both of the p38 MAPK- and JNK-knockdowned cells exhibited much lower expression levels of MMP-2 compared with control siRNA-transfected cells retaining endogenous levels of p38 MAPK and JNK (Figure 6C). These data demonstrate that induction of MMP-2 expression by CD9 is dependent on the activation of p38 MAPK and JNK signaling pathways.

Discussion

It was reported that activation of some cell surface molecules such as $\alpha v\beta 3$ integrin and CD44 resulted in the up-regulation of MMP-2 production in human melanoma cells (Seftor *et al.,* 1992; Takahashi *et al.,* 1999). In this study, we found that high expression of CD9 membrane protein up-regulates expression of MMP-2 in MelJuso human melanoma cells, in contrast to its effect on MMP-9 expression (Figure 2). In spite of high homology in amino acid sequence between MMP-2 and MMP-9, the promoters of MMP-2 and MMP-9 are composed of different *cis*-elements (Westermarck and Kahari, 1999; Vincenti, 2001), hence they not only respond to different stimuli, but also respond differently to the same stimulus. Although the mechanisms of MMP-2 gene activation in human cancer cells are not well defined, the MMP-2 promoter has been shown to contain many common transcription factor binding sites, such as those for p53, AP-1, Ets-1, c-myc, PEA3, CREB, GCN4, and AP-2 (Bian and Sun, 1997). We found here that the AP-1 binding site in MMP-2 promoter is a key cis-element for the induction of MMP-2 gene expression by CD9 (Figure 3). In addition, the CD9 transfectant cells were shown

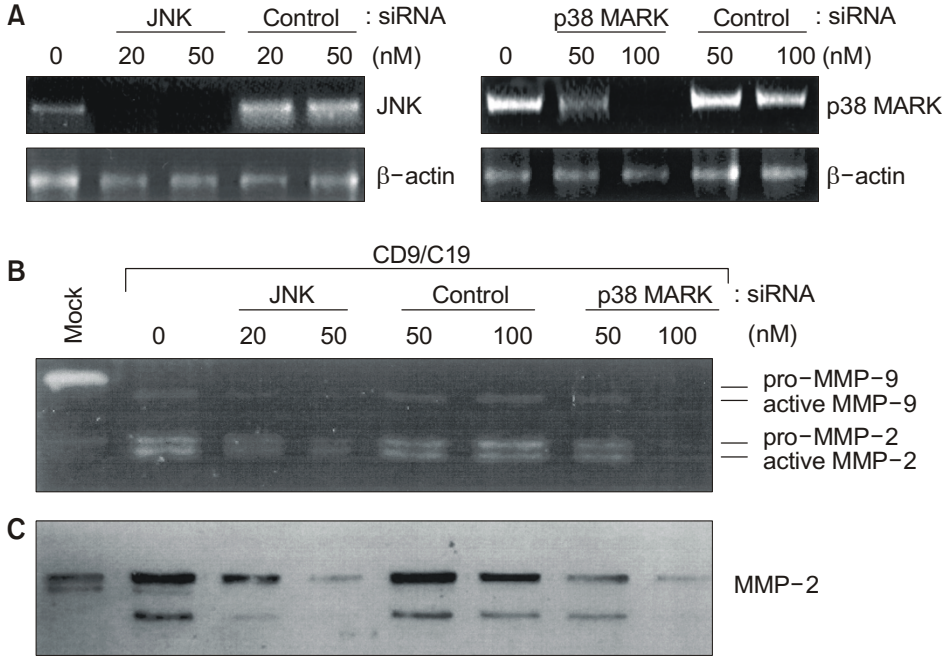

Figure 6. Effect of knock-down of p38 MAPK and JNK by siRNA on CD9-induced MMP-2 activity/expression in the CD9 transfectant cells. Cells at 60-70% confluency were transfected with the indicated amounts of control, p38 MAPK, or JNK siRNAs as described in Materials and Methods. After transfection, the cells were stabilized in normal growth medium containing FBS for 24 h. (A) mRNA levels of JNK and p38 MAPK in the siRNA-transfected cells were then analyzed by RT-PCR. (B, C) Conditioned medium from the siRNA-transfected cells cultured in serum-free medium for 2 days was collected and analyzed with (B) gelatin zymography and (C) Western blotting using anti-MMP-2 mAb.

to possess more AP-1 transcription factors capable of binding to the AP-1 site than the mock transfectant cells. It was also shown that c-Jun is a predominant member of AP-1 factors to be activated by CD9. Although MMP-2 expression has been widely considered to be independent of the AP-1 transcriptional complex, these data demonstrated the functional involvement of AP-1 transcription factors including c-Jun in up-regulating expression of the MMP-2 gene by CD9. In accordance with our results, the positive role of AP-1 factors in MMP-2 synthesis was also observed in cardiac cells, where a functional AP-1 site mediates MMP-2 transcription through interactions with Jun-Fra and Jun-Fos heterodimers (Bergman *et al.,* 2003).

Recently, CD9 was re-discovered to be a receptor for one of the pregnancy-specific glycoproteins and plays an essential role in sperm-egg fusion (Le Naour *et al.,* 2000; Kaji *et al.,* 2002; Waterhouse *et al.,* 2002; Ellerman *et al.,* 2003). However, the possibility of existence of other ligand(s) for CD9 in cells other than gametes can not be excluded. Like other tetraspanin proteins, CD9 has been known to be involved in signal transduction by forming complexes with integrins and other cell surface molecules including tetraspanin members (Maecker *et al.,* 1997; Boucheix and Rubinstein, 2001; Yanez-Mo *et al.,* 2001). In platelets, CD9 was shown to associate with some small GTP-binding proteins (Seehafer and Shaw, 1991) and CD9 ligation with anti-CD9 mAb increased p72syk-associated tyrosine kinase activity, along with cell aggregation (Ozaki *et al.,* 1995). A recent report showed that the CD9 gene transduction down-regulates Wnt signaling pathways in fibrosarcoma and lung cancer cells (Huang *et al.,* 2004). However, intracellular signaling pathways of CD9 have not been thoroughly investigated. In this study, we found that several inhibitors specific to signal transducing molecules suppressed the induction of MMP-2 expression by CD9 in MelJuso melanoma cells (Figure 4). In particular, SB203580, a p38 MAPK inhibitor, and SP600125, a JNK inhibitor, completely abolished the positive effect of CD9 on MMP-2 expression. The participation of p38 MAPK and JNK in CD9 signaling pathway(s) became apparent when phosphorylation levels of p38 MAPK and c-Jun, which are assumed to reflect the activation of the proteins, were compared between the CD9 and mock tranfectant cells; that is, higher phosphorylation levels of both proteins were observed in the CD9 transfectant cells than those in the mock transfectant cells (Figure 5). Knock-down of either p38 MAPK or JNK by siRNA transfection abrogated the stimulating effect of CD9 on MMP-2 expression, indicating the absolute role of p38 MAPK and JNK in the CD9 signaling pathway(s) for the up-regulation of MMP-2 expression (Figure 6). The data in the present study demonstrate that CD9 activates p38 MAPK/JNK-c-Jun signaling pathways, leading to an increase in MMP-2 gene expression. The functional involvement of p38 MAPK in MMP-2 expression of melanoma cells was also suggested by a recent report, in which inhibition of p38 MAPK by the specific inhibitor SB203580 down-regulated MMP-2 mRNA and protein levels, whereas the specific MEK-1 inhibitor PD98059 did not change expression of MMP-2 (Denkert *et al.,* 2002). We also found here that the CD9-stimulated MMP-2 expression is mediated by p38 MAPK and JNK pathways, but not by MEK-ERK signaling pathway. Therefore, MMP-2 expression seems to be up-regulated by signaling pathways utilizing p38 MAPK and JNK, instead of the classical MAP kinase, ERK-1/2.

The level of CD9 expression was demonstrated to inversely correlate with the stage of tumor malignant progression or appearance of metastases in several types of human cancers including melanoma, breast, lung, colon, and ovarian cancer (Si and Hersey, 1993; Miyake *et al.,* 1995; Adachi *et al.,* 1998; Mori *et al.,* 1998; Houle *et al.,* 2002). Overexpression of CD9 through gene transfection or adenovirally gene delivery resulted in the suppression of cell motility and pulmonary metastasis (Ikeyama *et al.,* 1993; Miyake *et al.,* 2000), suggesting that a decrease in CD9 expression may be associated with or causes highly metastatic behavior of cancer cells. However, high expression of CD9 was more frequently found in high-grade astrocytic tumors than in low-grade tumors (Kawashima *et al.,* 2002). Moreover, routine immunohistochemical staining of CD9 in malignant breast tumors did not provide useful prognostic information on breast cancer, suggesting the need for close examination of tumor tissue (Jamil *et al.,* 2001). A recent study raised the possibility that during tumor progression, CD9 is not only downregulated to promote the expansion of malignant cells, but also locally re-expressed to adjust to microenvironmental requirements (Sauer *et al.,* 2003). Immunohistochemical staining of CD9 in cervical tumors showed that overall CD9 expression is downregulated in most invasive cervical carcinomas as compared to normal squamous epithelium of the cervix, but apparently re-expressed at sites of invasion into blood or lymphatic vessels. These observations suggest that constant high or even elevated expression of CD9 at contact sites between invasive tumor cells and vascular endothelial cells may contribute to the transmigration of tumor cells into blood or lymphatic vessels through the basement membrane. The role of CD9 in heterotypic interactions between tumor and endothelial cells during transendothelial invasion was also demonstrated by an *in vitro* study of transendothelial migration of A375 melanoma cells, in which CD9 provided by endothelial rather than cancer cells was suggested to play a crucial role in the transendothelial migration (Longo *et al.,* 2001). In addition to the CD9-mediated interactions between tumor and endothelial cells, MMP-2 induced by CD9 may contribute to transendothelial invasion of cancer cells by degrading ECM around endothelial cells. It therefore can be postulated that motile cells having low CD9 level drive local invasion of tumor cells into surrounding tissue at an early stage of tumor invasion, but only cells able to upregulate CD9 can enter blood or lymphatic vessels during further invasive progression.

Although the expression of type IV collagenases in malignancies is widely believed to promote tumor metastasis, the specific role of MMP-2 and MMP-9 in the progression of cancer may be more complex than has previously been assumed. In contrast to the essential role for MMP-9 in tumor cell invasion, it has been shown that MMP-9 as well as MMP-7 can generate angiostatin by cleaving plasminogen, indicating that MMP-9 expression in peritumoral area may inhibit angiogenesis and thereby suppress tumor growth and metastasis *in vivo* (Patterson and Sang, 1997). We found here that increased expression of CD9 does not only upregulate MMP-2 expression, but also downregulates MMP-9 expression in highly invasive MelJuso cells (Figure 2). It can be postulated from previous reports and our current study that the differential regulation of MMP-2 and MMP-9 synthesis by CD9 may be needed for the vascular dissemination of malignant melanoma cells by transendothelial invasion and the formation of new blood vessels. The precise expression pattern of MMP-2 and MMP-9 in CD9-positive cell clusters associated with transendothelial invasion will have to be investigated by future immunohistochemical studies with human melanoma tissues.

In conclusion, we have demonstrated that CD9 tetraspanin protein stimulates MMP-2 expression of human melanoma cells by activating c-Jun *via* the p38 MAPK/JNK pathways. CD9 expression has been linked to a variety of cellular and physiological processes, such as cell motility, adhesion, proliferation, activation, differentiation, development, invasion, metastasis, and wound healing, most of which are also known to be modulated by MMPs. The p38 MAPK and JNK signaling pathways have also frequently reported to play an important role in those biological processes. If this overlap is not merely coincidental, the results in the present study could introduce a new viewpoint to understanding the mechanism underlying the biological function of the CD9 tetraspanin protein.

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