Induction of matrix metalloproteinase-9 by galectin-7 through p38 MAPK signaling in HeLa human cervical epithelial adenocarcinoma cells

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Abstract. The expression of matrix metalloproteinase-9 (MMP-9) has been reported in various cancers. Its expression is associated with tumorigenesis and tumor metastasis. Studies have shown that galectin-7, a ß-galactoside-binding animal lectin, is involved in various processes including the suppression of tumor growth. However, a recent study reported that the development of thymic lymphoma is accelerated by galectin-7 expression. In this report, we demonstrate that the expression of MMP-9 was increased by galectin-7 in human cervix epithelial cells (HeLa). When the galectin-7 gene was transfected to HeLa cells with the ECFP vector, the expression of MMP-9 mRNA increased, as compared to non-transfected cells. As a result, MMP-9 protein levels also increased, as indicated by Western blot and gelatin zymography. In addition, galectin-7-transfected cells exhibited increased invasion in the matrigel invasion system. Expression of MMP-9 is involved in several signaling pathways by various stimulation factors. Therefore, we investigated how the signaling pathway in galectin-7-transfected cells differs from that of non-transfected cells. Upon transfection of galectin-7, p38 MAPK was activated and SB203580, a chemical inhibitor of p38 MAPK, reversed the effects of galectin-7. These results indicate that galectin-7 increases the expression of MMP-9 through the p38 MAPK signaling pathway.

Introduction

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes. They have been implicated in normal physiological and pathological processes, such as progression and metastasis (1,2). MMPs are involved in the degradation of extracellular matrix (ECM) proteins, including basement membrane collagen, interstitial collagens and various accessory ECM proteins (3). Consequently, MMP expressions are reported to be higher with invasion and poor prognosis in all human cancers. Most MMPs are expressed at low levels or not at all in resting-state tissue. But their expressions increase when tissues undergo inflammation, physical cellular interactions by stimuli, and various cancers. A change in MMP levels can affect the invasion behavior of tumor cells and metastasis ability in animal models (2).

Matrix metalloproteinase-9 (MMP-9), which is a gelatinase, is the salient MMP responsible for ECM protein degradation that promotes metastasis of tumor cells (3). MMP-9 expression in malignant cancer is higher than that in benign tumors. Its level in malignant cancer is critical evidence for the role of MMP-9 (4). Several studies have shown that elevated expression of MMP-9 is associated with increased metastasis in various cancers, such as breast cancer, prostate cancer and lymphoma (5,6). MMP-9 is of central importance in catalyzing the cleavage of epithelial basement membrane components (7). The expression of MMP-9 can be stimulated by various factors, including cytokines and growth factors, through the activation of their gene promoters by signal transduction pathways (8-12).

Galectin-7 is a 15-kD galectin with a single CRD that was first cloned from human epidermis. This protein is normally expressed in stratified squamous epithelia. Galectin-7 has various functions. According to several studies, galectin-7 has the potential to mediate corneal epithelial cell migration and the re-epithelialization of wounds (13,14).

Moreover, galectin-7 can act as either a positive or negative regulator in tumor development (4). One study suggested that galectin-7 acts on a common point in the apoptosis signaling pathways (15). Another study reported that galectin-7 suppresses tumor growth in human colon carcinoma cells (16). These studies have demonstrated how galectin-7 can play a negative role in tumor cells. In contrast, recent studies have revealed that galectin-7 modulates the aggressive behavior of lymphoma cells by controlling the expression of MMP-9 (6). This positive effect of galectin-7 on tumor development has also been suggested in other studies. Studies have shown that

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Abbreviations: MMP-9, metalloproteinase-9; MAPK, mitogen-activated protein kinase; TGF-ß, transforming growth factor-ß

Key words: galectin-7, invasion, lectin, MMP-9, p38 MAPK, tumor metastasis

up-regulation of galectin-7 in murine lymphoma cells is associated with aggression, and galectin-7 is overexpressed in rat mammary carcinomas induced by DMBA (17,18).

To gain insight into the molecular roles of galectin-7 in MMP-9 expression and the signal pathway involved, we generated galectin-7-expressing HeLa cells. The results show that galectin-7 expression increases the invasion of HeLa cells in matrigel via the induction of MMP-9, and that MMP-9 induction by galectin-7 is mediated by activation of the p38 MAPK signaling pathway in human cervical epithelial cells.

Materials and methods

Cell lines and culture conditions. HeLa cells and galectin-7-transfected HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 unit/ml penicillin-streptomycin and 5% CO_2. The p38 inhibitor was applied at 20 μM for 2 h (SB203580; Calbiochem).

Transient transfection and generation of stable transfected cells. Transfection was performed on 20 μg DNA per $1x10^7$ cells on ice using a Gene Pulser II electroporator (Bio-Rad) at 500 μF and 0.3 kV. After 48 h incubation in complete medium, the culture was grown in complete medium containing 1 mg/ml G418 sulfate (Calbiochem). Colonies were selected and expanded. Controls were generated using HeLa cells transfected with the pECFP-endo vector.

Measurement of carbohydrate binding activity of galectin-7. The galectin-7 activity of the galectin-7-transfected HeLa cells was measured using a lactose column. The cells were lysed by the addition of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, pH 7.5). The cell lysate was passed through the lactose column (α-Lactose-Agarose; Sigma). Fractions were collected from the flow-through, then washed with PBS, and eluted with 0.1 M D-lactose (Sigma). The binding activity was confirmed by Western blot analysis.

RT-PCR and real-time PCR. Total RNA was isolated from HeLa cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA concentration was determined at 260 nm. For RT-PCR analysis, cDNA synthesis was performed using 1 μg of total RNA with the ImpromII™ reverse transcription system (Promega). The primer sequences for MMP-9 PCR were 5'-TTCATCTTCCAAGGCCAATC-3' (forward) and 5'-CTTGTCGCTGTCAAAGTTCG-3' (reverse). The primer sequences for GAPDH cDNA were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAA GATGGTGATGGGATTTC-3' (reverse). Final primer concentrations were 0.5 pM. The samples were initially denatured at 94˚C for 5 min, followed by 30 cycles of 94˚C for 1 min, 62˚C for 1 min and 72˚C for 1 min, followed by a final extension at 72˚C for 10 min (GeneAmp® PCR system 2700; Applied Biosystems).

Real-time RT-PCR was performed using a Bio-Rad iQ5 real-time PCR cycler with a DyNAmo™ HS SYBR (Green qPCR kit (Finnzymes). The final results were expressed as n-fold differences in MMP-9 gene expression, relative to GAPDH. Experiments were performed in triplicate for each data point.

Western blots. Protein concentrations were determined using the BCA™ protein assay reagent (Pierce). Equal amounts of extracted protein (10 μg) from cell lysates were solubilized in 5X SDS sample buffer, and subjected to 10% SDS-PAGE. The separated proteins were transferred to Immobilon-P PVDF (polyvinylidene fluoride) membrane (Millipore), then blocked with 0.1% Tween-20 and 5% non-fat dry milk for 2 h at room temperature before incubating with antibodies overnight at 4˚C. Anti-ERK, anti-JNK, anti-phosphorylated JNK, and anti-p38 antibodies were purchased from Cell Signaling Technology, Inc. Anti-phosphorylated ERK, anti-phosphorylated p38, and anti-MMP-9 antibodies were obtained from Santa Cruz Biotechnology, Inc. After three 10 min washes with TBS containing 0.2% Tween-20 (TTBS; Amresco), the membranes were incubated with peroxidase-labeled anti-rabbit or mouse IgG (Vector Laboratories, Inc.) at room temperature for 30 min. Membranes were washed, transferred to ECL solution (West-Zol® Plus; iNtRON) for 1 min and exposed to X-ray film.

In vitro invasion assay. The *in vitro* invasion assay was performed using a BD BioCoat™ Matrigel™ invasion chamber (BD Biosciences) containing an 8-μm pore size PET membrane with a thin layer of Matrigel basement membrane matrix. Chambers were rehydrated for 2 h in culture media at 37˚C in 5% CO_2 atm. After removal of the media, HeLa and HeLa-galectin-7 cell suspensions ($5x10^4$ cells/ml) in serum-free culture medium were placed in the upper chamber. A chemoattractant, such as 5% FBS culture medium, was placed in the plate, incubated for 8 h, and the chamber media was changed to that with or without 20 μM p38 inhibitor for 16 h in 37˚C, 5% CO_2 atm. After incubation, the non-invading cells were removed from the upper side of the membrane by scrubbing. The cells on the lower side of the membrane were fixed and stained with Wright's stain. Experiments were performed in triplicate.

Quantification of MMP-9 protein by ELISA. Cells were seeded in 6-well tissue culture plates in serum-free DMEM media with or without p38 inhibitor and cultured to 80% confluence; then the culture supernatant was used to determine the concentration of MMP-9, using the human MMP-9 ELISA kit (RayBiotech, Inc.). Experiments were performed in triplicate following the instructions of the manufacturer.

MMP-9 gelatin zymography. Cells were cultured in serum-free media with or without p38 inhibitor. The culture media was collected and centrifuged at 1300 rpm for 10 min at 4˚C to remove cell debris. The supernatant was mixed with 5X non-reducing sample buffer and electrophoresed on 10% SDS-PAGE containing 1% gelatin at 150 V for 1.5 h. After electrophoresis, the gel was washed in 2.5% Triton X-100 (Amresco) for 1 h, and then incubated for 16 h at 37˚C in gelatinase substrate buffer (1 M Tris, 1 M $CaCl_2$, 5 M NaCl, 0.2 mM $ZnCl_2$, 25% TX-100, 0.2% NaN_3). The gel was stained with 0.5% Coomassie Blue for 1 h, followed by destaining.

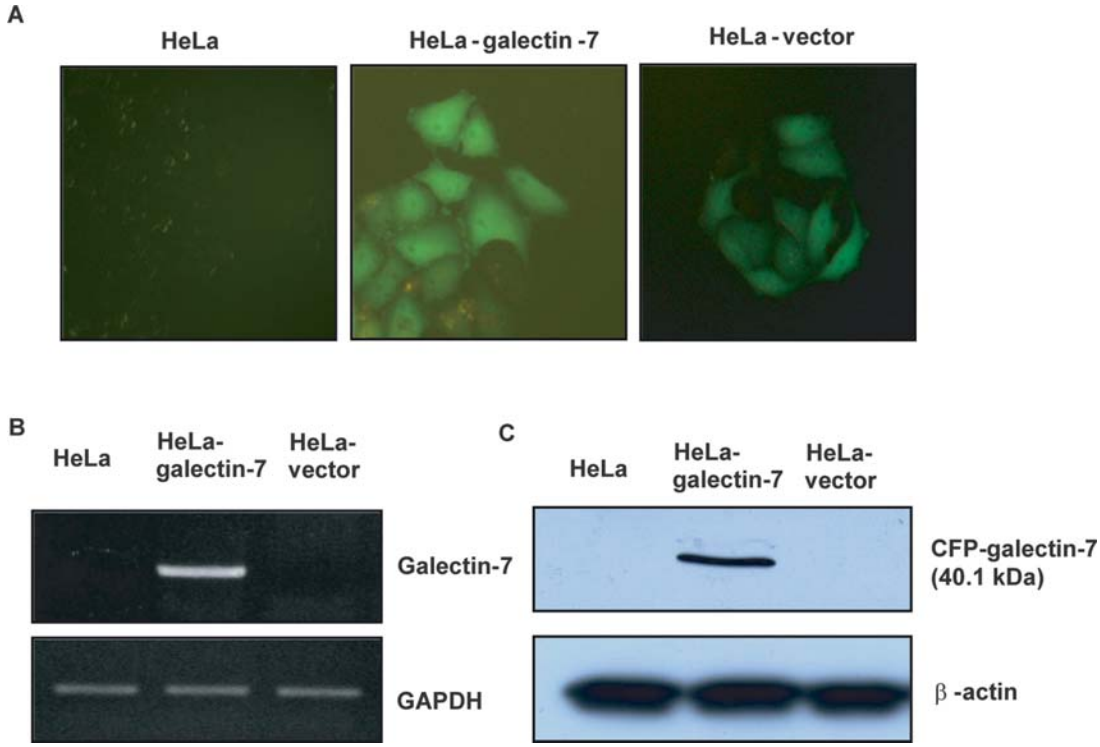

Figure 1. Expression of galectin-7 in HeLa cells. (A) ECFP-galectin-7 and ECFP-endo vector control were each transfected to HeLa cells by electroporation. Transfected cells were selected using G418, and antibiotics. The fluorescence of ECFP-galectin-7- and ECFP-endo-transfected cells was visualized by fluorescence microscopy at x400. (B) Total RNAs were isolated as described. cDNA synthesis was performed using 1 μg of total RNA. PCR using galectin-7-specific primers was performed. (C) The levels of expressed galectin-7 protein determined by Western blotting. ß-actin was used as a control.

Statistical analysis. All experiments were repeated a minimum of three times. All data collected from real-time PCR and ELISA were expressed as mean ± SD. Statistical significance was determined using the Student's t-test.

Results

Expression of intact galectin-7 in HeLa cells. To obtain a stable transfectant of HeLa-galectin-7 cells, the ECFP-galectin-7 vector was transfected into HeLa cells by electroporation. The ECFP-endo vector was also transfected as a control. Because the vector encodes cyan fluorescent protein, both ECFP-galectin-7-transfected HeLa (HeLa-galectin-7) cells and ECFP-endo-transfected HeLa (HeLa-endo) cells could be visualized by fluorescence microscopy (Fig. 1A). To determine whether galectin-7 was expressed in galectin-7-transfected cells, HeLa cells and HeLa-galectin-7 cells were assessed by RT-PCR and Western blot analyses. Results confirmed the presence of both the expressed cDNA and the galectin-7 protein in ECFP-galectin-7-transfected HeLa cells (Fig. 1B and C).

Since ECFP was fused at the N-terminus of galectin-7, the carbohydrate binding activity was confirmed. A cell lysate of galectin-7-transfected HeLa cells was used for lactose affinity chromatography. As shown in Fig. 2, ECFP-galectin-7 bound to the lactose column, indicating that the carbohydrate binding activity was intact.

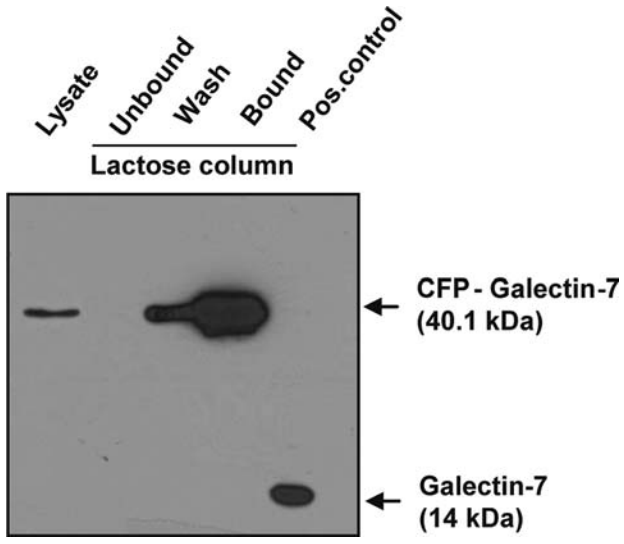

Figure 2. Confirmation of galectin-7 carbohydrate binding activity from galectin-7-transfected HeLa cells measured by lactose column affinity chromatography. Cell lysate was passed through the lactose column (0.6x3.0 cm). Samples were collected from the unbound fraction (flow-through), PBS wash, and bound fraction (0.1 M lactose elution). Each fraction was analyzed by Western blotting with the anti-galectin-7 antibody.

Induction of MMP-9 by galectin-7 in HeLa cells. To determine whether the expression of galectin-7 is associated with the level of MMP-9 expression, RNA levels of MMP-9 in HeLa-galectin-7 and HeLa-endo cells were compared to non-transfected HeLa cells. In RT-PCR, the expressions of MMP-9 mRNA were higher in HeLa-galectin-7 cells (Fig. 3A). Also, the relative levels of MMP-9 expression were measured by

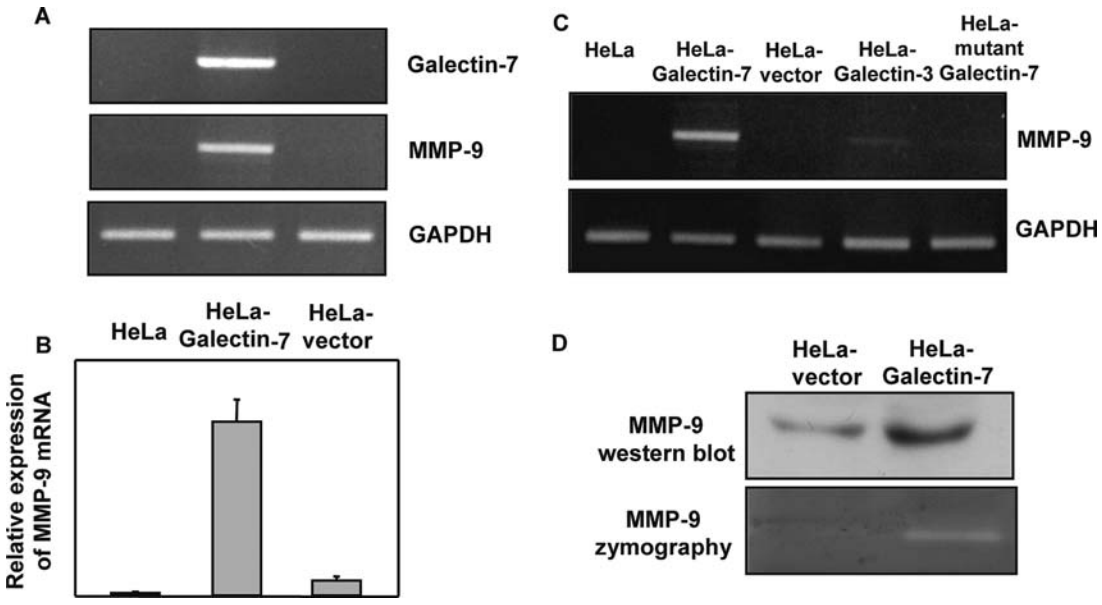

Figure 3. Induction of MMP-9 by galectin-7 in HeLa cells. (A) Expressions of galectin-7 and MMP-9 mRNA determined by RT-PCR using specific primers. Total RNAs were isolated as described. cDNA synthesis was performed using 1 μg of total RNA. GAPDH was used as a loading control. (B) The level of MMP-9 was confirmed by real-time PCR in the HeLa-galectin-7 cells. Bars represent the mean ± SD of at least three independent experiments for each condition. (C) MMP-9 mRNA levels from different galectin-transfected cells determined by RT-PCR. (D) Western blot analysis of MMP-9 protein expression in conditioned medium. The conditioned media were collected and analyzed by Western blotting as described. Gelatinolytic activity of secreted MMP-9 was determined by gelatin zymography assay. The conditioned media were collected and analyzed by zymography as described.

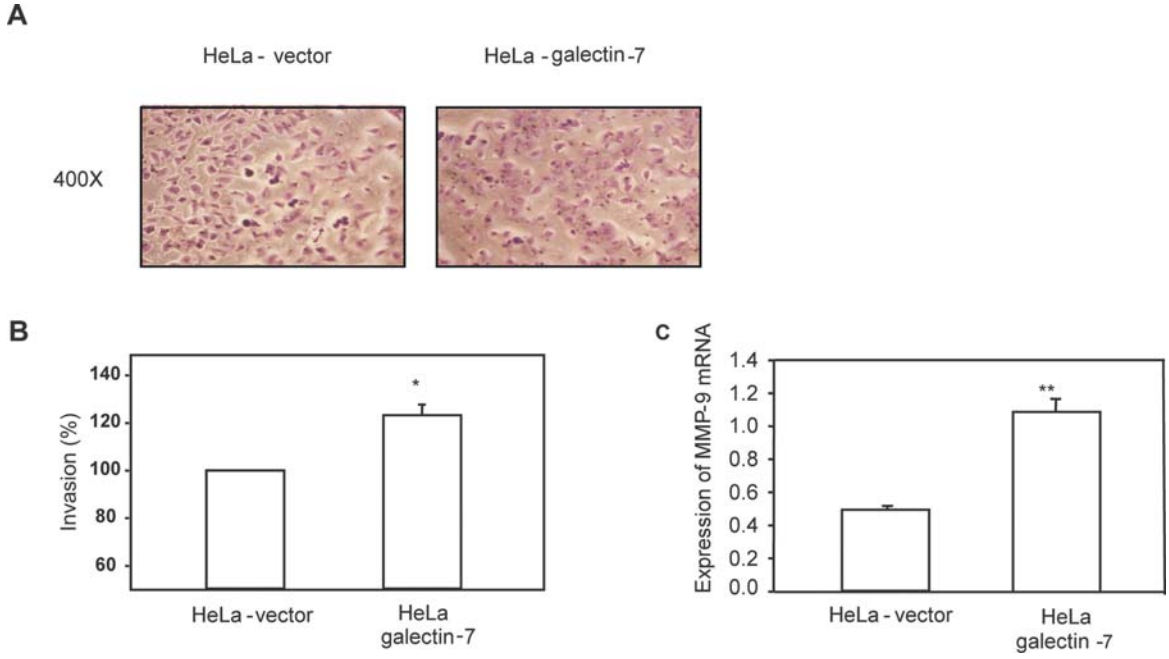

Figure 4. Galectin-7-transfected HeLa cells exhibit more aggressive invasion than non-transfected cells. (A) Visualization of the invasion of cells by Wright stain. Invasion was measured using an *in vitro* matrigel invasion chamber as described. (B) The numbers of invaded cells were counted in five fields (x400) after staining and are expressed as a percentage. Experiments were performed three times with different cell preparations. The bars represent the mean ± SD. (C) Effects of matrigel on expression of MMP-9 measured by real-time PCR. RNA isolation was performed after culturing on the matrigel as described. cDNA synthesis was performed using 1 μg of total RNA. $^{*}P<0.05$ and $^{**}P<0.01$ compared with vector control, respectively.

real-time PCR. As shown in Fig. 3, the level of MMP-9 mRNA was higher in HeLa-galectin-7 cells than in the HeLa-endo control. This result indicated that the MMP-9 expression was not increased by the ECFP vector. Next, by Western blot and gelatin zymography, the expression levels of MMP-9 protein in HeLa and HeLa-galectin-7 cells were tested. In both analyses the MMP-9 protein level was higher in the galectin-7-transfected cells, compared to HeLa cells (Fig. 3D). The different carbohydrate-binding specificities among the galectin family members are still under investigation, and the ligand

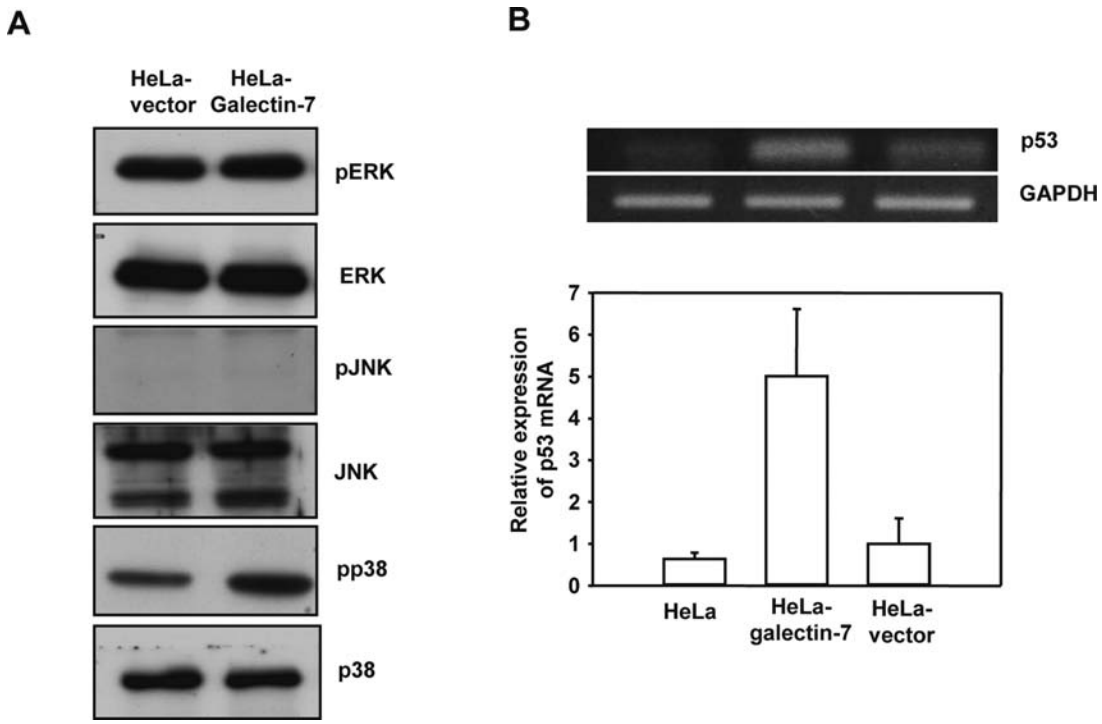

Figure 5. p38 MAPK signaling pathway regulates galectin-7-induced MMP-9 expression. (A) Cellular protein was isolated from non-transfected HeLa and HeLa-galectin-7 cells. The levels of ERK, JNK and p38 MAPK were determined by Western blotting using phosphor-ERK, JNK and p38 antibodies. (B) Total RNAs were isolated as described. cDNA synthesis was performed using 1 μg of total RNA. The p53 expression levels were measured by RT-PCR and real-time PCR. GAPDH served as internal control for normalization. The bars represent the mean ± SD.

for each galectin family has not been elucidated. To examine whether a different galectin could induce the same effect, genes for galectin-3 and a mutant galectin-7, in which carbohydrate binding activity is abolished, were transfected to HeLa cells. The mutated galectin-7 did not increase the transcription of MMP-9 mRNA. Galectin-3-transfected HeLa cells showed little increase in MMP-9 mRNA (Fig. 3C).

Galectin-7-transfected HeLa cells exhibit more aggressive invasion than non-transfected cells. Since the expression of the galectin-7 caused an increase in MMP-9 expression, we examined whether the HeLa-galectin-7 cells have any effect on invasion. The *in vitro* matrigel invasion assay was performed with HeLa and HeLa-galectin-7 cells. The results revealed that the HeLa-galectin-7 cells were 25% more invasive than HeLa cells (Fig. 4A and B). The matrigel components affected the expression of MMP-9, as evidenced by the observation that the MMP-9 mRNA level in HeLa cells cultured on the matrigel coated plate (Fig. 4C) was higher than the HeLa cells on the regular plate (Fig. 3B). The real-time PCR results demonstrated that the matrigel might increase MMP-9 expression in HeLa cells, but still, the HeLa-galectin-7 expressed more MMP-9 (Fig. 4C).

p38 MAPK signaling pathway regulates the galectin-7-induced MMP-9 expression. The above results demonstrated that galectin-7 caused an increase in MMP-9 expression and a more invasive character for the cells. To understand how galectin-7 is involved in MMP-9 expression, we investigated the MMP-9 signal pathway involved. Various studies have demonstrated that the activation of MMP-9 could be involved in MAPK signaling pathways (11,10). The Western blot analysis presented in Fig. 5 shows that the level of phosphorylated p38 increased with transfected galectin-7; but neither the total protein nor phosphorylated ERK or JNK increased. The level of p53 mRNA, a downstream signal of p38, was higher (Fig. 5B). Inhibition of the p38 pathway may revert the galectin-7-induced MMP-9 induction. Thus, SB203580, an inhibitor of p38 kinase, was added to HeLa cells and HeLa-galectin-7 cells. As shown in Fig. 6A, a significant reduction in MMP-9 mRNA expression was observed upon p38 inhibitor treatment. The MMP-9 protein level was also reduced (Fig. 6B). The *in vitro* matrigel invasion assay was performed with HeLa cells and HeLa-galectin-7 cells with p38 inhibitor. The p38 inhibitor treatment of HeLa-galectin-7 cells caused a reduction in the number of invaded cells (Fig. 6C). These results strongly suggest that the transformation of HeLa cells to more aggressive cells via MMP-9 induction by galectin-7 expression may be related to the p38 signaling pathway.

Discussion

This study provides evidence that MMP-9 expression increases due to galectin-7 in human cervix epithelial cells (HeLa). When the galectin-7 gene was transfected to HeLa cells, the expression of MMP-9 mRNA increased, compared to non-transfected HeLa cells. As a result, the MMP-9 protein level also increased. Increased MMP-9 expression may result in more invasive HeLa cells in a matrigel invasion system. Upon transfection of galectin-7, p38 MAPK is activated, and a chemical inhibitor of p38 MAPK, SB203580, reverses the effects of galectin-7.

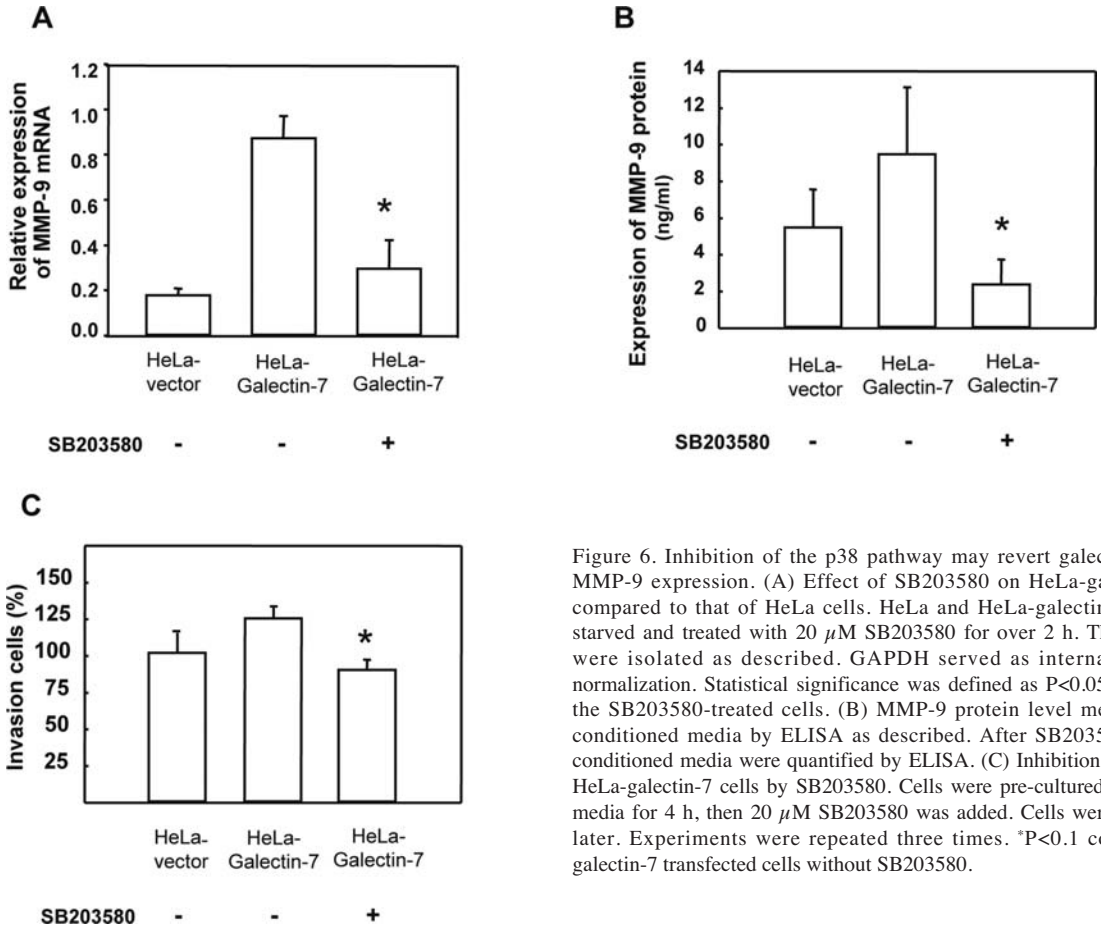

Figure 6. Inhibition of the p38 pathway may revert galectin-7-induced MMP-9 expression. (A) Effect of SB203580 on HeLa-galectin-7 cells compared to that of HeLa cells. HeLa and HeLa-galectin-7 cells were starved and treated with 20 μM SB203580 for over 2 h. The total RNAs were isolated as described. GAPDH served as internal control for normalization. Statistical significance was defined as $P<0.05$, compared to the SB203580-treated cells. (B) MMP-9 protein level measured in the conditioned media by ELISA as described. After SB203580 treatment, conditioned media were quantified by ELISA. (C) Inhibition of invasion of HeLa-galectin-7 cells by SB203580. Cells were pre-cultured in serum-free media for 4 h, then 20 μM SB203580 was added. Cells were stained 24 h later. Experiments were repeated three times. *$P<0.1$ compared with galectin-7 transfected cells without SB203580.

The role of galectin-7 has been suggested as operating in many different ways. Termed as PIG1 (p53 induced gene 1) (19), functions related to proapoptotic properties were reported (15,16). In those experiments, when apoptosis-inducing agents were used to treat cells, galectin-7 overexpressing cells were more prone to apoptosis. However, the overexpression of galectin-7 itself did not cause apoptosis. In contrast, recent studies have shown new aspects of galectin-7. The up-regulation of galectin-7 is reported in many different cancers. In thyroid cancer, carcinomas expressed higher levels of galectin-7, compared to adenomas, including the normo-macrofollicular, microfollicular, and trabecular variants (20). IGF-1 transgenic mice developed skin tumors by DMBA, and both the mitogenic and anti-apoptotic pathways were up-regulated (21). In the epidermis of IGF-1 transgenic mice, galectin-7 expression was reported to be 3-fold higher, compared to non-transgenic mice (22). Moreover, recently it was reported that galectin-7 is the most up-regulated molecule when benign lymphoma cells progress to the metastatic variant (17). It has been reported that the development of T cell lymphoma is accelerated when induced by lymphoma cells overexpressing galectin-7. These experiments suggest that galectin-7 modulates the aggressive behavior of lymphoma cells by controlling the expression of the metastatic gene, MMP-9 (6). Those results indicated that galectin-7 might work as an intermediate in the signaling pathway inside the cells. Accumulating evidence suggests this possibility. When galectin-7 was transfected in HeLa cells, it was found both cytosol and nucleus and when galectin-7 added in culture extracellularly it did not enhance apoptosis in the presence of actinomycin D (15). In hepatocytes, the hepatocyte growth factor accelerates the nuclear export of Smad3 by enhancing its interaction with galectin-7, and galectin-7 siRNA abolished the suppressive effect of HGF (23). TGF-ß1 induced MMP-9 mRNA and protein in human head and neck squamous cell carcinoma cell lines. The application of Smad2/3 siRNA attenuated the inductive effect of activation of TGF-ß on MMP-9 expression. These findings indicate the significance of the TßRI-Smad pathway in TGF-ß1-induced MMP-9 expression (24). Overexpression of galectin-7 may trigger the activation of Smad3 signaling by translocation of Smad3. Interestingly, a specific inhibitor of p38 MAPK, SB202190, abolished the TGF-inducible activation of the Smad-dependent promoter and decreased Smad2 phosphorylation (25). In ovarian cancer cells, a p38 MAPK inhibitor, PD169316, inhibited TGF-ß-induced activation of Smad signaling by reducing Smad2 and Smad3 phosphorylations (26). These data reveal that there is cross-talk between Smad and p38 MAPK signaling.

In conclusion, the expression of galectin-7 in HeLa cells may activate MMP-9 transcription by affecting cross-talk between Smad and p38 MAPK signaling.

Acknowledgements

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