

Differential Roles of Matrix Metalloproteinase-9 and -2, Depending on Proliferation or Differentiation of Retinoblastoma Cells

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PURPOSE. To investigate the differential roles of matrix metalloproteinase (MMP)-9 and MMP-2 in the proliferation or differentiation of retinoblastoma cells.

METHODS. Cell proliferation assay with an MMP-9 inhibitor and cell viability assay with an MMP-2 inhibitor were performed in retinoblastoma cells with 5 ng/mL fibroblast growth factor 2 for proliferation, 0.1% bovine serum albumin for differentiation, or reverse transcriptase-polymerase chain reaction (RT-PCR) for MMP-9, MMP-2, and their tissue inhibitors TIMP-1 and TIMP-2. Immunohistochemistry for MMP-2 and nm23 was performed using an experimental model of retinoblastoma. With the use of an MMP-2 inhibitor, Western blot analysis was performed for neurofilament, extracellular signal-regulated kinases 1 and 2 (ERK 1/2), and phospho-ERK 1/2, and neurite length was measured in differentiated retinoblastoma cells.

RESULTS. With the proliferation of retinoblastoma cells, MMP-9 expression was upregulated without alteration of MMP-2, TIMP-1, or TIMP-2. However, proliferation was not affected by the inhibition of MMP-9 activity. Interestingly, only MMP-2 expression, colocalized with differentiated cells in retinoblastoma tissue, was significantly increased in the differentiation of retinoblastoma cells. Inhibition of MMP-2 activity did not affect cellular viability but attenuated neurite outgrowth and neurofilament expression of differentiated retinoblastoma cells, which was mediated through the suppression of ERK 1/2 activation.

CONCLUSIONS. The authors suggest that differential expression of MMP-9 and -2 could reflect biological features, such as proliferation and differentiation, of retinoblastoma cells. In particular, MMP-2 could be directly involved in the regulation of differentiation of retinoblastoma cells. Therefore, therapeutic targeting to MMP-2 may prove useful for reducing malignancy through the differentiation of retinoblastoma cells. (*In-*

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Retinoblastoma is the most common intraocular malignancy in children, the development of which is fundamentally caused by mutation of the retinoblastoma gene, located on 13q14.¹ On the basis of data from our reports and the reports of others^{2,3} showing that retinoblastoma is characterized by the proliferation of cells originating from nucleated retina, it is apparent that the progression of retinoblastoma is closely related to tumor growth, with invasion of the vitreous, optic nerve, choroid, and orbit.⁴⁻⁶ Tests for these well-known pathologic characteristics, however, lack both sensitivity and specificity for predicting disease progression.⁷ Therefore, further investigation to determine the prognostic variable that will best predict the clinicopathologic outcome of retinoblastoma is still needed.

Tumor formation in retinoblastoma results from the dysregulation of integral cellular processes such as cell proliferation and cell survival. With progression to late-stage retinoblastoma, retinoblastoma cells lose their ability to differentiate,⁸ which is followed by tumor progression, reflecting a changing ratio of highly differentiated and less differentiated cell types, with the most advanced tumors composed primarily of undifferentiated cells.^{9,10} During the multistep processes of tumor progression, the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a critical step in disrupting the barrier restricting tumor growth and invasion.¹¹ MMPs, a family of zinc ion-dependent endopeptidases, are capable of digesting a broad spectrum of substrates, including collagen types I, II, III, and IV and stromelylin, and are divided into subgroups that include collagenases, stromelysins, and stromelysin-like matrilysins, gelatinases, and membrane-type MMPs.¹² These protease activities of MMPs in the tissue are regulated by tissue inhibitors of metalloproteinases (TIMPs).¹³ In particular, of more than 20 different MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play key roles in the degradation of the main components of ECM, collagen type IV and gelatin. Their natural inhibitors are TIMP-2 and TIMP-1, respectively. Regardless, though it is well known that the expression of MMPs and TIMPs is closely related to tumor invasion,¹¹⁻¹³ the role of MMPs and TIMPs in retinoblastoma has not yet been elucidated. Recently, one report suggested that MMPs and TIMPs might be involved in the tumor invasion of retinoblastoma, which, however, has the limitation that its clinicopathologic study is based only on immunohistochemistry.¹⁴

In our study, we demonstrated that MMP-9 and -2 are differentially expressed in retinoblastoma cells, whose expression closely depends on the proliferation and differentiation of retinoblastoma cells. With the proliferation of retinoblastoma cells, MMP-9 expression was significantly increased, whereas MMP-2 expression was upregulated in the differentiation of

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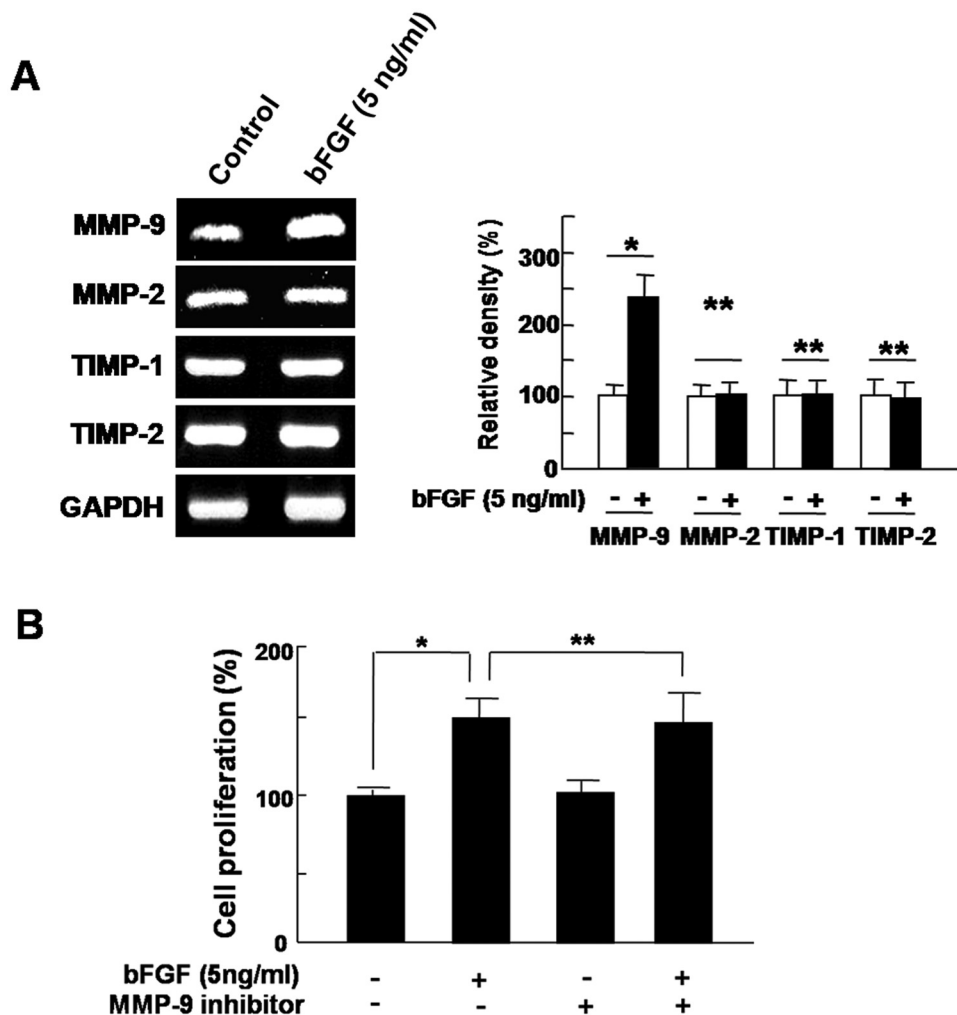


FIGURE 1. MMP-9 expression is up-regulated in the proliferation of retinoblastoma cells, yet inhibition of its activity does not affect cell proliferation. (A) Total mRNAs were isolated from retinoblastoma cells, and reverse transcriptase-polymerase chain reaction was performed with specific primers for MMP-9, MMP-2, tissue inhibitor of metalloproteinase 1 (TIMP-1), and TIMP-2. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as an internal control. The figures that appear here were selected as representative of data from three independent experiments. Quantitative analysis was performed by measuring mRNA expression relative to the control. Each value represents the mean \pm SE from three independent experiments (* P < 0.05, ** P > 0.05). (B) Retinoblastoma cells were treated with either 5 ng/mL fibroblast growth factor 2 or 100 nM MMP-9 inhibitor and then were incubated for 48 hours. Cell proliferation was measured by BrdU incorporation assay. Quantitative analysis was performed by measuring cells with BrdU incorporation relative to the control. Each value represents the mean \pm SE from three independent experiments (* P < 0.05, ** P > 0.05).

retinoblastoma cells. Interestingly, although the inhibition of MMP-9 activity did not affect the proliferation of retinoblastoma cells, the inhibition of MMP-2 activity effectively attenuated the neurite formation of differentiated retinoblastoma cells, which was accompanied by inhibition of neurofilament expression. Moreover, we showed that MMP-2 is involved in the differentiation of retinoblastoma cells through extracellular signal-regulated kinase (ERK) 1/2 activation. We suggest that taken together, the differential expression of MMP-9 and -2 could reflect biological features—proliferation and differentiation—of retinoblastoma cells. In particular, MMP-2 could be directly involved in the regulation of the differentiation of retinoblastoma cells.

MATERIALS AND METHODS

Retinoblastoma Cells

A human retinoblastoma cell line, SNUOT-Rb1, established by our group,¹⁵ was maintained in RPMI 1640 medium (Welgene Inc., Seoul, Korea) supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) at 37°C in a moist atmosphere of 95% air and 5% CO₂. The medium was changed every third day. Cultured tumor cells were observed daily under a phase-contrast microscope (Carl Zeiss, Chester, VA).

Animals: BALB/c Nude Mice

Mice were purchased from Samtako (Osan, Korea). Care, use, and treatment of all animals in this study were in strict agreement with the

ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were exposed to standard 12-hour dark/12-hour light cycles in a room with a temperature of approximately 23°C. As in our previous report regarding the inducement of orthotopic retinoblastoma,¹⁶ SNUOT-Rb1 cells (1×10^7) were suspended in phosphate-buffered saline and intravitreally inoculated into the right eyes of the mice. Tumor development was checked by indirect ophthalmoscopic examination twice a week for 4 weeks.

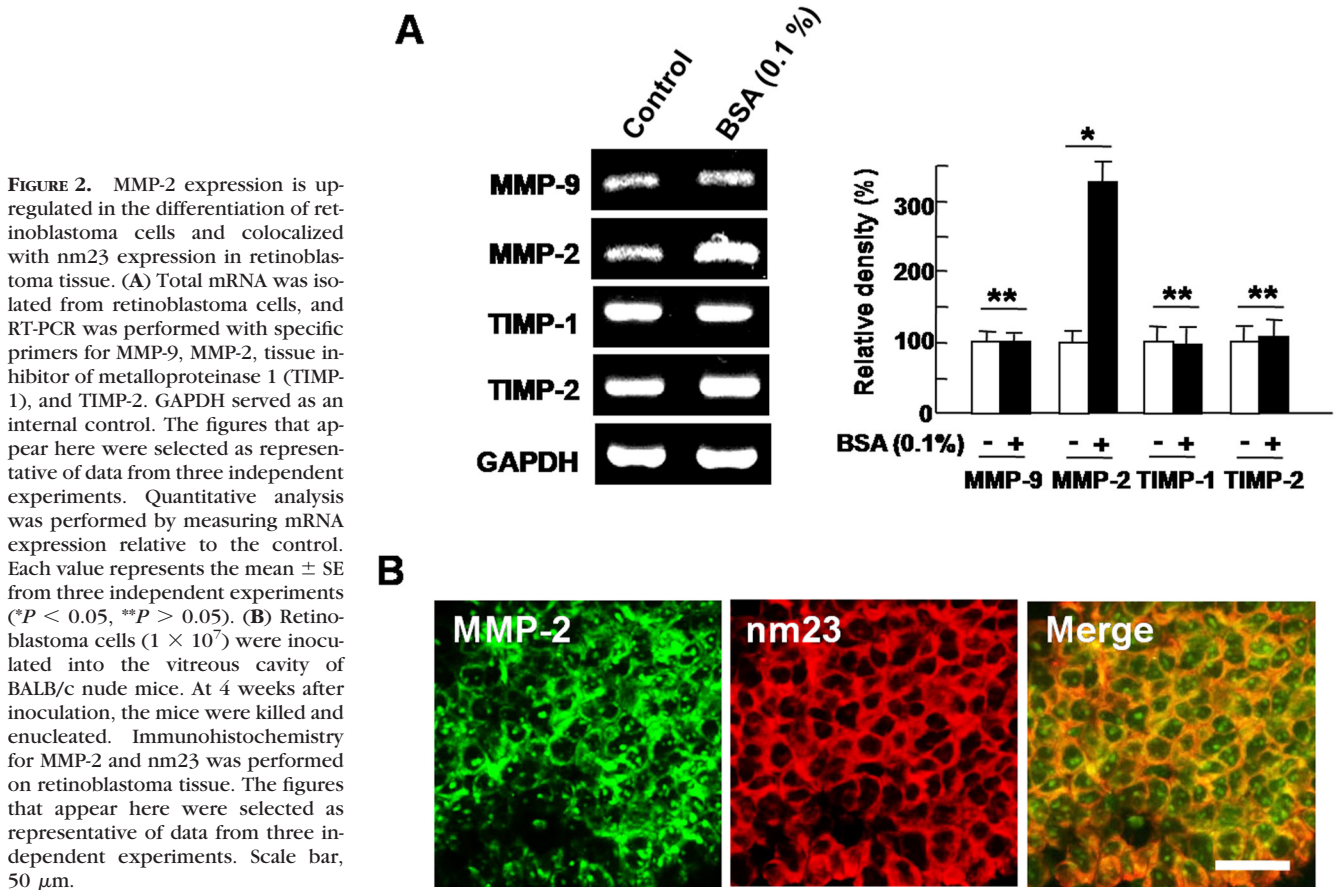
At 4 weeks after inoculation, the mice were killed and both eyes in each mouse were enucleated for determining, by histologic examination, whether there was tumor formation.

Cell Proliferation Assay

Cell proliferation assay was carried out using bromodeoxyuridine (BrdU) incorporation assay. Retinoblastoma cells (1×10^4) were seeded in 12-well culture plates and then treated with 5 ng/mL fibroblast growth factor 2 (FGF-2; Millipore, Bedford, MA) or 100 nM MMP-9 inhibitor (Calbiochem, La Jolla, CA) for 48 hours. In accordance with the manufacturer's instructions for the BrdU enzyme-linked immunosorbent assay kit (Roche, Indianapolis, IN), the cells were labeled with BrdU and incubated with anti-BrdU antibody. BrdU incorporation was detected using a colorimetric substrate solution, and absorbance was measured at 370 nm.

Cell Viability Assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Retinoblastoma cells (1×10^4) were seeded in 12-well culture plates, and then treated with 0.1%



bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) or 50 nM MMP-2 inhibitor (Calbiochem) for 48 hours. The medium was replaced, after treatment with BSA or MMP-2 inhibitor for 4 hours, with fresh medium containing 0.5 mg/mL MTT. After incubation, the medium was carefully removed from the plate, and dimethyl sulfoxide was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Neurite Length in Differentiated Retinoblastoma Cells

Making a minor modification to the method described in a recent report,¹⁷ we measured neurite length in differentiated retinoblastoma cells by manual tracing of neurite outgrowths in each retinoblastoma cell. Cells were evaluated in randomly selected fields at a magnification of $\times 400$ and photographed with a digital camera (DXC 930 P; Sony, Tokyo, Japan) under an inverted microscope (Axiovert 200M, Carl Zeiss). For analysis, differentiated retinoblastoma cells were selected randomly, and neurites were traced manually. Neurite length was measured from an arbitrary round line connecting edges of non-spiny cell membrane to the distal tip of the neurite. Every neurite was traced by a series of straight lines; each neurite can be mathematically described as a series of straight lines leading from the edge to the neurite tip. The length of each of these lines was determined by the curvatures of the neurite such that the line always overlaid the neurite. Accumulation of the straight line segments resulted in a polygon equivalent to the neurite length.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA from cells was isolated using reagent (TRIzol; Invitrogen) according to the manufacturer's instructions. First-strand cDNA was

synthesized with 3 μ g each of DNA-free total RNA and oligo-(dT) 16 primer by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Equal amounts of cDNA were subsequently amplified by PCR in a 50- μ L reaction volume containing 1 \times PCR buffer; 200 μ M dNTPs; 10 μ M specific primer for *MMP-2* (5'-GTGACGAAAGATGTGGTG-3' and 5'-GGTGTAGGTGTAATGGGTG-3'), *MMP-9* (5'-TTGACAGCGACAAGAAGTGG-3' and 5'-CAGTGAAGCGGTACATAGGG-3'), *TIMP-1* (5'-ACTTCCACAGGTCCCACAAC-3' and 5'-GCATTCCTCACAGCCAACAG-3'), *TIMP-2* (5'-GAAGCATTGACCCAGAGTG-3' and 5'-CCTTTCAGACCGAACCTACT-3'), and GAPDH (5'-TCCCTCAAGATTGTCAGCAA-3' and 5'-AGATCCACAACGGATACATT-3'); and 1.25 U *Taq* DNA polymerase (TaKaRa, Tokyo, Japan). Amplification was performed at the following temperatures: 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, for a total of 30 cycles. PCR products were separated on agarose gels and visualized using ethidium bromide staining under ultraviolet transillumination.

Western Blot Analysis

Cells were harvested, washed with ice-cold phosphate buffer solution, and lysed with buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P40, 2 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche). An equal amount (15 μ g) of the samples was separated on sodium dodecylsulfate-polyacrylamide gel and then transferred onto nitrocellulose filters (Bio-Rad Laboratories, Hercules, CA). The membranes were immunoblotted with primary antibodies against neurofilament (Chemicon, Temecula, CA), phospho-ERK 1/2 (Cell Signaling Technology, Beverly, MA), ERK 1/2 (Cell Signaling Technology), phospho-Akt (Cell Signaling Technology), or Akt (Cell Signaling Technology). To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β -actin.

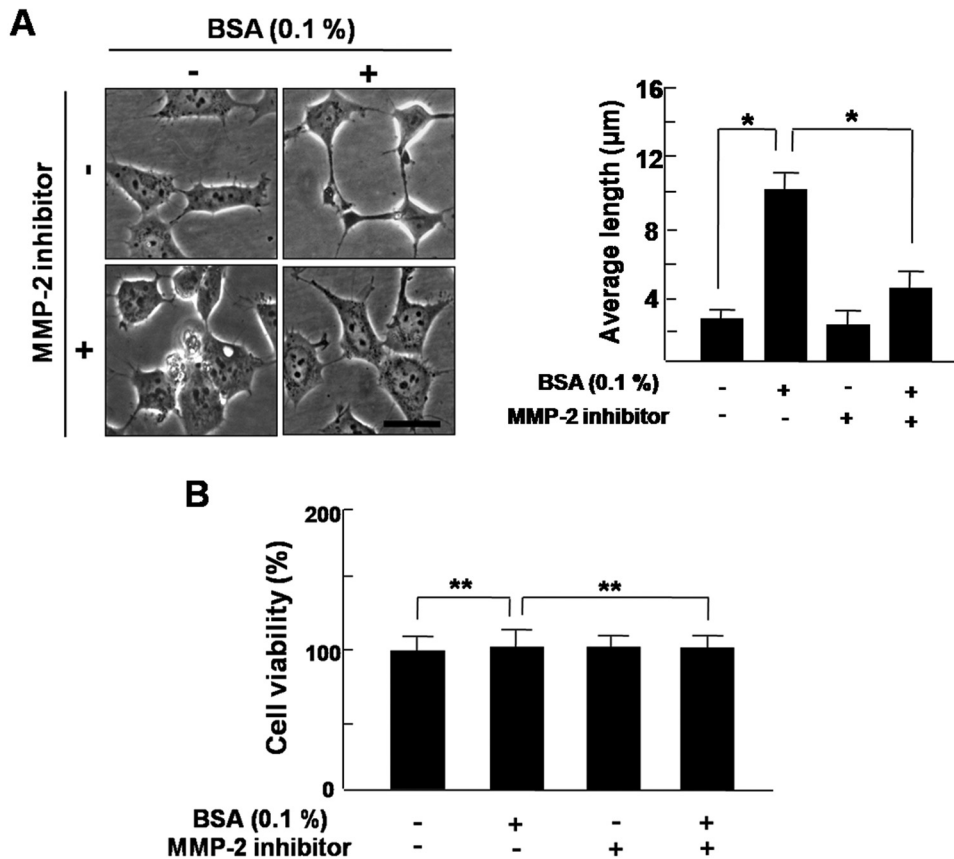


FIGURE 3. Inhibition of MMP-2 activity attenuates neurite outgrowth of differentiated retinoblastoma cells without cellular toxicity. **(A)** Neurite length in differentiated retinoblastoma cells was measured by manual tracing of neurite outgrowths in each cell. The figures that appear here were selected as representative of data from three independent experiments. Quantitative analysis was performed using average length from total neurites measured. Each value represents the mean \pm SE from three independent experiments ($*P < 0.05$). **(B)** Retinoblastoma cells were treated with either 0.1% BSA or 50 nM MMP-2 inhibitor and then incubated for 48 hours. Cell viability was measured by MTT assay. Quantitative analysis was performed by measuring viable cells relative to the controls. Each value represents the mean \pm SE from three independent experiments ($**P > 0.05$).

Immunohistochemistry

The enucleated mouse eyes used for immunohistochemistry were immersion fixed in 10% formaldehyde for 12 hours at room temperature, subsequently dehydrated through a series of graded ethanol solutions, and embedded in paraffin using standard techniques. Serial sections 4-mm thick were prepared from paraffin blocks and then deparaffinized and hydrated by sequential immersion in xylene and graded alcohol solutions. Next, they were treated with proteinase K for 5 minutes at 37°C and then treated for 10 minutes with normal serum obtained from the same species in which the secondary antibody was developed to block nonspecific staining. Slides were incubated overnight at 4°C with primary antibodies against MMP-2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and nm23 (1:100; Santa Cruz Biotechnology). Fluor 546 donkey anti-goat IgG (1:400; Molecular Probes) and Alexa Fluor 488 donkey anti-rabbit IgG (1:400; Molecular Probes) was used as secondary antibodies. The slides were mounted and observed under a fluorescence microscope (Axio Observer; Carl Zeiss).

Statistical Analysis

Statistical differences between groups were evaluated with the Student's unpaired *t*-test (two-tailed). Data were recorded as mean \pm SD. $P \leq 0.05$ was considered significant.

RESULTS

Upregulation of MMP-9 in Proliferation of Retinoblastoma Cells without Contributing to the Proliferation Process

To determine whether expression of MMP-2, MMP-9, and their tissue inhibitors TIMP-1 and TIMP-2 is changed in the proliferation of retinoblastoma cells, we measured mRNA expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the

FGF-2-induced proliferation of retinoblastoma cells by RT-PCR. As shown in Figure 1A, mRNA of MMP-9 was significantly increased with FGF-2 treatment, whereas there was no change in expression of MMP-2, TIMP-1, and TIMP-2. We next investigated whether MMP-9 activity contributes to the proliferation of retinoblastoma cells. Treatment with 5 ng/mL FGF-2 significantly (1.5 times) increased BrdU incorporation in retinoblastoma cells compared with control, which did not change with inhibition of MMP-9 activity by 100 nM MMP-9 inhibitor (Fig. 1B). Even with an escalated dose of MMP-9 inhibitor (1 nM–1 mM), there was no change in proliferation of retinoblastoma cells (data not shown). These data suggested that although increased expression of MMP-9 reflects the proliferation of retinoblastoma cells, MMP-9 activity does not contribute to the proliferation process of retinoblastoma cells.

Upregulation of MMP-2 in Differentiation of Retinoblastoma Cells and Its Colocalization with nm23 Expression in Retinoblastoma Tissue

To determine whether expression of MMP-2, MMP-9, and their tissue inhibitors TIMP-1 and TIMP-2 changes in the differentiation of retinoblastoma cells, we used RT-PCR to measure mRNA expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the differentiation of retinoblastoma cells induced by 0.1% BSA. Interestingly, mRNA levels in MMP-2 were significantly increased with treatment with 0.1% BSA, whereas there was no change in expression of MMP-9, TIMP-1, or TIMP-2 (Fig. 2A). Next, to investigate whether MMP-2 expression is colocalized with differentiated cells in retinoblastoma tissue, we analyzed immunoreactivity for MMP-2 with nm23 in an experimental animal model of retinoblastoma. The decreased expression of nm23 was re-

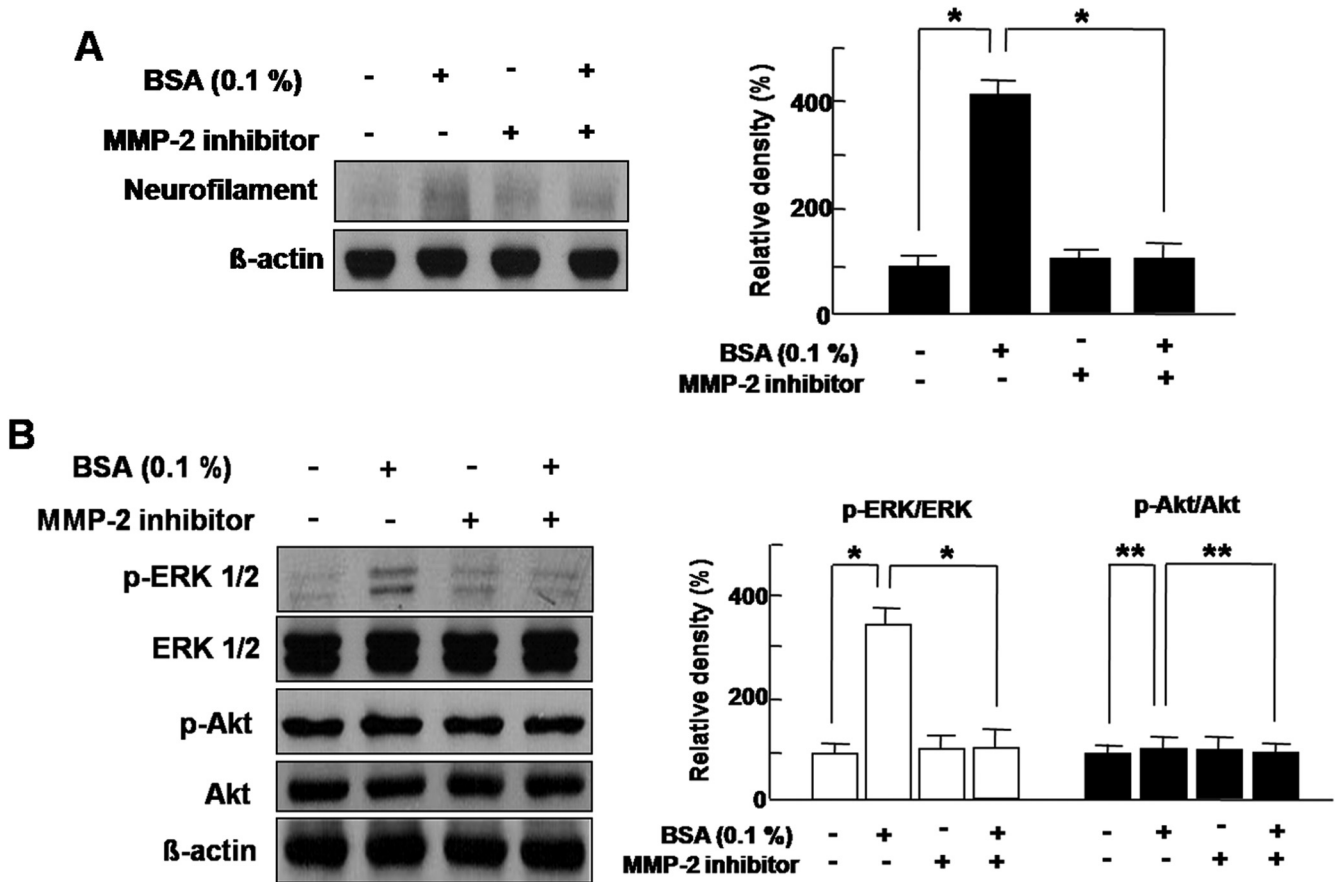


FIGURE 4. Inhibition of MMP-2 activity blocks the differentiation of retinoblastoma cells through the suppression of ERK 1/2 activation. Retinoblastoma cells were treated with 0.1% BSA or 50 nM MMP-2 inhibitor. Neurofilament (A) or phospho-ERK 1/2, ERK 1/2, phospho-Akt, and Akt (B) were detected by Western blot analysis. β -Actin served as a loading control. The figures that appear here were selected as representative of data from three independent experiments. Quantitative analysis was performed by measuring protein expression relative to the controls. Each value represents the mean \pm SE from three independent experiments (* $P < 0.05$, ** $P > 0.05$).

ported to be closely related to poorly differentiated retinoblastoma and retinoblastoma with invasiveness.¹⁸ As shown in Figure 2B, MMP-2 expression was primarily detected in more differentiated areas with high nm23 immunostaining, where MMP-2 expression was colocalized with nm23 expression.

Attenuation of Neurite Outgrowth of Differentiated Retinoblastoma Cells by Inhibition of MMP-2 Activity

To determine whether MMP-2 activity is involved in the differentiation of retinoblastoma cells, we investigated whether inhibition of MMP-2 activity could attenuate neurite outgrowth in differentiated retinoblastoma cells that were incubated for 48 hours after treatment with 0.1% BSA or 50 nM MMP-2 inhibitor. Neurite length was measured by manual tracing of neurite outgrowths from differentiated retinoblastoma cells. As demonstrated in Figure 3A, neurite outgrowth significantly increased with 0.1% BSA, which was, however, nearly blocked by inhibition of MMP-2 activity (MMP-2 inhibitor, 50 nM). To exclude the results from the inhibition of neurite outgrowth from cytotoxicity findings for retinoblastoma cells, cell viability was evaluated after treatment with 50 nM MMP-2 inhibitor. Differentiation induced by 0.1% BSA and inhibition of MMP-2 activity by administration of 50 nM MMP-2 inhibitor did not affect cellular viability of retinoblastoma cells compared with controls (Fig. 3B).

Blocking of Differentiation of Retinoblastoma Cells through Suppression of ERK 1/2 Activation by Inhibition of MMP-2 Activity

Next, to confirm the contribution of MMP-2 activity to the differentiation of retinoblastoma cells, we measured neurofilament expression in differentiated retinoblastoma cells after treatment with 50 nM MMP-2 inhibitor. As shown in Figure 4A, with the differentiation of retinoblastoma cells by 0.1% BSA, neurofilament expression was increased but was blocked by the inhibition of MMP-2 activity. To determine whether MMP-2 activity is involved in the differentiation of retinoblastoma cells via ERK 1/2 activation, we investigated whether ERK 1/2 activation is changed in differentiated retinoblastoma cells by the inhibition of MMP-2 activity (MMP-2 inhibitor, 50 nM). As demonstrated in Figure 4B, when retinoblastoma cells were treated with 0.1% BSA, phospho-ERK 1/2 was significantly upregulated, though there was no change in ERK expression. However, inhibition of MMP-2 activity by 50 nM MMP-2 inhibitor completely blocked ERK 1/2 activation. Interestingly, expression of Akt and phospho-Akt in retinoblastoma cells was not changed by BSA-induced differentiation and inhibition of MMP-2 activity (Fig. 4B).

DISCUSSION

Cellular interactions with ECM play a crucial role in tumor processes, including proliferation, migration, adhesion, and

invasion of tumor cells.¹¹⁻¹³ Accordingly, tumor development requires remodeling of a complex structural entity surrounding tumor cells, in both the breakdown and the resynthesis of ECM components, that must be regulated by proteolytic degradation of ECM.^{12,13} Such degradation of ECM is primarily mediated by MMPs, which may be definitely inhibited by TIMPs. In particular, among 23 different MMPs, MMP-2 or MMP-9 expression and activity are more crucial in tumor invasion.¹³ The enzymatic activity of MMP-2 and MMP-9 is blocked specifically by TIMP-2 and TIMP-1, respectively.^{12,13} The expression and activity of MMPs and TIMPs in retinoblastoma cells have not been investigated, though they have been in other cancer cells. Recently, on the basis of immunohistochemical analysis of retinoblastoma tissues, it was reported that MMP-2, MMP-9, TIMP-1, and TIMP-2 are expressed in more than 50% of retinoblastoma tissues that have been examined.¹⁴ Interestingly, MMP-2, MMP-9, TIMP-1, and TIMP-2 all seem to be frequently expressed in invasive retinoblastoma with optic nerve or choroidal infiltration.¹⁴ However, it remains to be elucidated how MMPs and TIMPs contribute to the tumor biology of retinoblastoma, whether proliferation or differentiation.

In our study, we clearly demonstrated that MMP-9 and MMP-2 are differentially expressed in retinoblastoma cells, depending on the proliferation and differentiation, respectively, of retinoblastoma cells. In proliferation, MMP-9 expression was upregulated, whereas MMP-2, TIMP-1, and TIMP-2 expression were unchanged. Transcriptional activity of MMPs in tumors can be induced by variable growth factors, cytokines, and oncogenic products.^{12,13} Once activated, MMP activity is regulated by TIMPs. Therefore, MMP activity is determined by the balance of MMP and TIMP expression. However, as our data show, MMP-9 activity may not be directly involved in the proliferation of retinoblastoma cells mediated by FGF-2. This means that MMP-9 expression seems to reflect the proliferation of retinoblastoma cells even though MMP-9 activity never contributes to proliferation itself.

Interestingly, in differentiation, MMP-2 expression, which is colocalized with differentiated retinoblastoma cells with high nm23 immunostaining, was upregulated in the differentiation of retinoblastoma cells without alteration of MMP-9, TIMP-1, or TIMP-2 expression. In contrast to MMP-9, MMP-2 activity is directly involved in the differentiation of retinoblastoma via the ERK 1/2 signaling pathway. With differentiation of a model neuronal cell system of neuroblastoma cells, neurofilament expression and neurite outgrowth could be easily observed,¹⁹ by which the mitogen-activated protein kinase pathway seems to be the major signaling pathway for mediating differentiation.²⁰ In the same context, as in our recent reports,^{16,21} retinoblastoma cells could be differentiated through ERK 1/2 activation as well. In addition, inhibition of MMP-2 activity does not compromise the Akt survival pathway in retinoblastoma cells,²² which was demonstrated as inhibition of differentiation by MMP-2 inhibitor without alterations of cellular viability and Akt activation. This means that MMP-2 activity is specifically engaged in the differentiation of retinoblastoma cells.

The differential expression of MMPs—as MMP-9 or MMP-2—shows different phases of retinoblastoma cells: proliferation and differentiation. Although MMP-9 appears to be related to the proliferation of retinoblastoma without direct contribution to the cellular proliferative processes, MMP-2 is directly related to the differentiation of retinoblastoma cells. MMP-2 regulates the differentiation of retinoblastoma cells through the ERK 1/2 signaling pathway. Therefore, in view of the association of differential expression of MMPs with the proliferation and differentiation of retinoblastoma cells, we suggest that differential expression of MMP-9 and MMP-2 could be a

significant pathologic factor that reflects biological features of retinoblastoma cells and, furthermore, that therapeutic targeting of MMP-2 may prove useful for reducing malignancy through the differentiation of retinoblastoma cells.

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