Blockade of Tumor Growth Due to Matrix Metalloproteinase-9 Inhibition Is Mediated by Sequential Activation of β1-Integrin, ERK, and NF-κB*

Praveen Bhoopathi‡, Chandramu Chetty‡, Sateesh Kunigal‡, Sravan K. Vanamala‡, Jasti S. Rao‡§, and Sa Jani S. Lakka‡§

From the ‡Program of Cancer Biology, Department of Cancer Biology and Pharmacology, and the §Department of Neurosurgery, University of Illinois College of Medicine at Peoria, Peoria, Illinois 61605

We previously showed that matrix metalloproteinase (MMP)-9 inhibition using an adenovirus-mediated delivery of MMP-9 small interfering RNA (Ad-MMP-9), caused senescence in medulloblastoma cells. Regardless of whether or not Ad-MMP-9 would induce apoptosis, the possible signaling mechanism is still obscure. In this report, we demonstrate that Ad-MMP-9 induced apoptosis in DAOY cells as determined by propidium iodide and terminal deoxynucleotidyltransferase-mediated nick end labeling staining. Ad-MMP-9 infection induced the release of cytochrome c, activation of caspase-9 and -3, and cleavage of poly(ADP-ribose) polymerase. Ad-MMP-9 infection stimulated ERK, and electrophoretic mobility shift assay indicated an increase in NF-κB activation. ERK inhibition, using a kinase-dead mutant for ERK, ameliorated NF-κB activation and caspase-mediated apoptosis in Ad-MMP-9-infected cells. β1-Integrin expression in Ad-MMP-9-infected cells also increased, and this increase was reversed by the reintroduction of MMP-9. We found that the addition of β1 blocking antibodies inhibited Ad-MMP-9-induced ERK activation. Taken together, our results indicate that MMP-9 inhibition induces apoptosis due to altered β1-integrin expression in medulloblastoma. In addition, ERK activation plays an active role in this process and functions upstream of NF-κB activation to initiate the apoptotic signal.

Apoptosis is a programmed cell death involved in many physiological and pathological regulations (1). New understanding of the mechanisms underlying apoptosis has resulted in the development of new strategies for treating certain illnesses, and several clinical trials are under way. The apoptotic pathway consists of several triggers, modulators, and effectors. The mitogen-activated protein kinase (MAPK) family, which is composed of serine/threonine kinases, is one such modulator. MAPks are mediators of intracellular signals that respond to various stimuli. The importance of MAPK signaling pathways in regulating apoptosis during conditions of stress has been widely investigated. MAPks include extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase, and p38 MAPK. Each MAPK is activated through a specific phosphorylation cascade. The ERK pathway is activated by mitogenic stimuli, including growth factors, cytokines, and phorbol esters, and plays a major role in regulating cell growth and differentiation (2–3). Many such studies have supported the general view that activation of the ERK pathway delivers a survival signal that counteracts proapoptotic effects associated with c-Jun N-terminal protein kinase and p38 activation (4–7). However, the proapoptotic influence of ERK activation has also been demonstrated (8–11).

Matrix metalloproteinases (MMPs) are capable of digesting various components of the extracellular matrix and other molecules, such as growth factors, cell surface receptors, and cell adhesion molecules. MMPs play an important role in tissue repair, tumor invasion, and metastasis (12, 13). The generation and analysis of transgenic and knock-out mice for both MMPs and tissue inhibitors of MMPs have revealed that MMPs also play key roles in the process of carcinogenesis (14). As such, the inhibition of MMPs seems to be an ideal solution to control tumor growth. However, the enthusiasm generated by a large number of in vitro and in vivo studies has dramatically diminished in recent years due to the failure of MMP inhibitors to block tumor progression in clinical trials (15). To better target MMPs, an appreciation of their many extracellular and intracellular roles in cell death is required. To this effect, we have constructed an adenovirus capable of expressing siRNA targeting the human MMP-9 gene (Ad-MMP-9). We demonstrated that MMP-9 inhibition induced senescence in medulloblastoma cells in vitro and regressed pre-established tumor growth in an intracranial model (16). The aims of the present study were to further delineate the role of MMP-9 in medulloblastoma tumorigenesis and to evaluate the mechanisms underlying the apoptotic induction caused by MMP-9 inhibition. Molecular dissection of the signaling pathways that activate the

siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase; Ad, adenovirus; MOI, multiplicity of infection; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.
apoptotic cell death machinery is critical for both our understanding of cell death events and the development of novel cancer therapeutic agents. We show that MMP-9 inhibition induced apoptosis in medulloblastoma in vitro and in vivo. Our data also suggest that up-regulation of the ERK pathway is critical for NF-κB activation and caspase-mediated cell death.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The sources for antibodies were as follows: antibodies against ERK, phospho-ERK, and MMP-9 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); active caspase-3, NF-κB p65, and NF-κB p50 (Abcam Inc., Cambridge, MA); cytochrome c (BD Biosciences, San Diego, CA); caspase-3, -8, and -9 (Cell Signaling Technology, Beverly, MA); anti-β1-integrin (Chemicon, Temecula, CA; ab1952 for Western blots and mAb1959 for functional blocking); poly(ADP-ribose) polymerase (PARP), NF-κB inhibitor II (EMD Biosciences, San Diego, CA); and NF-κB oligonucleotide consensus sequences for shift assay (Promega Corp., Madison, WI). All other reagents were of analytical reagent grade or better.

**Daoy Cell Culture**—Daoy cells were cultured in advanced minimal essential medium supplemented with 5% fetal bovine serum, 2 mM/liter L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

**Adenoviral siRNA Constructs and Infection**—The adenoviral siRNA for MMP-9 (Ad-MMP-9) and scrambled vector (Ad-SV) were constructed and amplified as described previously (16). Viral titers were quantified as plaque-forming units/ml following infection of 293 cells. We obtained the following titers for the viruses: Ad-SV (7.6 × 10¹¹ plaque-forming units/ml) and Ad-MMP-9 (5.0 × 10¹¹ plaque-forming units/ml). The amount of infective adenoviral vector per cell (plaque-forming units/cell) in culture media was expressed as multiplicity of infection (MOI). Virus constructs were diluted in serum-free culture media to the desired concentration, added to cells, and incubated at 37 °C for 1 h. The necessary amount of complete medium was then added, and cells were incubated for the desired time periods.

**Transfection with Plasmids**—All transfection experiments were performed with fuGene HD transfection reagent according to the manufacturer’s protocol (Roche Applied Science). Daoy cells were transfected with plasmid constructs containing ERK dominant negative mutant (Dn-ERK) (17), MMP-9-expressing cDNA (pcMMP-9) construct, or commercial MMP-9 siRNA (25 and 50 µl of 10 nm). Briefly, plasmid containing either Dn-ERK or pcMMP-9 was mixed with fuGene HD reagent (1:3 ratio) in 500 µl of serum-free medium and left for 0.5 h for complex formation. The complex is then added to the plate, which had 2.5 ml of serum-free medium (2 µg of plasmid/ml of medium). After 6 h of transfection, complete medium was added and kept for 24 h and used for further experiments.

**Western Blotting**—Western blot analysis was performed as described previously (16). Briefly, 48 h after infection with mock, 100 MOI of Ad-SV, or various MOI of Ad-MMP-9, Daoy cells were collected and lysed in radioimmunoprecipitation assay buffer, and protein concentrations were measured using BCA protein assay reagents (Pierce). Equal amounts of proteins were resolved on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The blot was blocked and probed overnight with a 1:1000 dilution of primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. An ECL system was used to detect chemiluminescent signals. All blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase antibody for measuring equal loading.

**Isolation of Cytosol and Mitochondrial Fractions**—Cells were infected as described above. 48 h later, cells were collected and resuspended in 1 ml of lysis buffer A containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 250 mM sucrose. The cells were homogenized with a 26-gauge needle syringe 4–6 times and centrifuged at 750 × g for 10 min at 4 °C to remove nuclei and unbroken cells. Then the supernatant was centrifuged at 10,000 × g for 15 min at 4 °C, and the resulting supernatant was collected (i.e. the cytosolic extract). The pellet with mitochondria was lysed in lysis buffer B containing 50 µl of 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin and centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was collected for the mitochondrial fraction. The protein content of the fractions was determined by the BCA method. Equal amounts of lysates were subjected to Western blot analysis as described above and probed for cytochrome c.

**FACS Analysis**—FACS analysis was performed as described earlier (16). Briefly, cells were infected as described above for 48 h and collected. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), stained with propidium iodide (2 mg/ml) in 4 mM/liter sodium citrate containing 3% (w/v) Triton X-100 and RNase A (0.1 mg/ml) (Sigma) and were analyzed with the FACSCalibur system (BD Biosciences). The percentages of cells undergoing apoptosis were assessed using Cell Quest software (BD Biosciences).

**Treatment with NF-κB Inhibitor II (JSH-23)**—Daoy cells were infected with Ad-MMP-9 as described above. After 36 h of infection, the cells were treated with 50 µM JSH-23 (NF-κB inhibitor) for 6 h. After the treatment, the cells were collected, and nuclear extractions were prepared and immunoblotted.

**Electrophoretic Mobility Shift Assay**—The electrophoretic mobility shift assay was performed as described by Chaturvedi et al. (18). Briefly, cells were infected for 48 h, collected, lysed in cell lysis buffer (10 mM HEPES, 10 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 10 µM dithiothreitol, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 500 µg/ml benzamidine), and centrifuged for 1 min. The pellet was lysed in nuclear extract buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 µM dithiothreitol, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 500 µg/ml benzamidine) and centrifuged at 13,000 × g for 5 min at 4 °C. The supernatant was estimated for protein concentration, aliquoted, and stored at −80 °C.

**Binding reaction** was performed with 5 µg of nuclear protein in a total volume of 20 µl containing 40 ng of poly(dI-dC), 4 µl of 5× binding buffer (1 × binding buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol). For supershift, protein extracts were incubated with 6 µg of p65 or p50 monoclonal antibody or isotype control before the addition of the ³²P-labeled probe. DNA–protein complexes were resolved on 5% PAGE in Tris/glycine buffer at 4 °C. The
double-stranded oligonucleotides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) used in this study for NF-κB were as follows: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-GCC TGG GAA AGT CCC CTC AAC T-3′. Oligonucleotides were end-labeled with 40 μCi (1480 MBq) of [γ-32P]ATP using T4 polynucleotide kinase and were purified on NAP-5 Sephadex G-25 DNA grade columns.

**Intracranial Tumor Model and Immunohistochemistry**—Daoy cells were stereotactically implanted as described previously (16). Two weeks after tumor cell implantation, treatments were given as described earlier (16). Animals losing >20% of body weight or having trouble ambulating, feeding, or grooming were sacrificed. Animals were monitored for 180 days, the designated termination point of the experiment. Excised brains were fixed in 10% formalin and embedded in paraffin. Tissue sections (5 mm thick) were subjected to immunostaining with antibodies for either active caspase-3 or pERK. Protein expression was detected using 3,3-diaminobenzidine solution (Sigma). Sections were counterstained with hematoxylin, and negative control slides were obtained by nonspecific IgG. Sections were washed and mounted with mounting solution and analyzed with an inverted microscope.

**FIGURE 1. Ad-MMP-9 induces apoptosis in Daoy cells.** Daoy cells were infected with mock, 100 MOI of Ad-SV, and the indicated MOI of Ad-MMP-9 for 48 h. A, FACS analysis was performed to demonstrate and quantify cell death by propidium iodide (PI) staining. B, the percentage of apoptotic cells (TUNEL-positive) was calculated (means ± S.E.) (p < 0.01). C, cells were harvested at 60-h time points, and caspase-9 and -3 and PARP cleavage was assessed by Western blot analysis using anti-PARP and anti-caspase-3 antibodies. Results are representative of three independent experiments. D, the band intensities of cleaved subunits of caspase-9 and -3 and cleaved PARP were quantified by densitometry and normalized with the intensity of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band as shown in the corresponding bar graph (means ± S.E.) (p < 0.01).
**MMP-9 Inhibition Induces ERK and NF-κB Activation**

**FIGURE 2.** Ad-MMP-9 activates the ERK signaling pathway and induces NF-κB activation. A, dose-dependent activation of ERK by Ad-MMP-9. Daoy cells were treated with mock, 100 MOI of Ad-SV, and the indicated MOI of Ad-MMP-9 for 48 h, after which cell lysates were assessed for total ERK and active ERK levels by Western blot analysis using anti-ERK antibody and anti-phospho-ERK antibodies. B, densitometric analyses of Western blots described in A. Results are expressed as fold increase in phosphorylation compared with the mock control. Phosphorylation is calculated as ratios of the phosphorylated versus nonphosphorylated forms (means ± S.D.; n = 3). Data are representative of three independent experiments (p < 0.05). C, Daoy cells were infected with mock, 100 MOI of Ad-SV, and the indicated MOI of Ad-MMP-9 for 48 h. An electrophoretic mobility shift assay was performed with 32P-labeled NF-κB oligonucleotide using 5 μg of protein from nuclear extracts as described under “Experimental Procedures.” A solid arrow indicates the specific NF-κB complexes, and open arrows indicate antibody supershift. D, nuclear and cytoplasmic proteins were prepared as described under “Experimental Procedures.” Equal amounts of proteins were resolved on an SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane. Signals were detected by using antibodies specific for IκBα, p65, and p50. The data represent a typical experiment conducted three times with similar results.

**TUNEL Assay**—To evaluate the apoptotic response of Ad-MMP-9, we have performed the terminal deoxynucleotidyl transferase (TdT)-mediated biotin-dUTP nick end labeling technique using the commercially available in situ cell death detection kit fluorescence (Roche Applied Science). Briefly, 20,000 cells were seeded onto the 8-well chamber slides and infected with mock, 100 MOI of Ad-SV, or various MOIs of Ad-MMP-9. After 60 h of infection, the cells were washed and fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution. These cells were then incubated with TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The slides were washed three times with PBS, and the incorporated biotin-dUTP was detected under a fluorescence microscope. For the paraffin-embedded tissue sections, slides were dewaxed and fixed according to standard protocols and then were treated as described above.

**Statistical Analysis**—The significance of differences between experimental conditions was determined using the two-tailed Student’s t test.

**RESULTS**

**MMP-9 Inhibition Induces Apoptosis in Medulloblastoma Cells**—We have previously shown that MMP-9 inhibition mediated by adenoviral delivery of siRNA against the human MMP-9 gene caused specific inhibition of MMP-9 and induced senescence in medulloblastoma cells and decreased medulloblastoma tumor growth in vivo (16). To examine the ability of Ad-MMP-9 to induce apoptosis in medulloblastoma cells, Daoy cells were infected with various doses of Ad-MMP-9 and subjected to FACS analysis after propidium iodide staining and TUNEL staining. Ad-MMP-9 caused apoptosis in Daoy cells in a dose-dependent manner, with a concentration of 100 MOI resulting in the death of more than 75% of the cell population compared with mock and Ad-SV. There was no major difference in the number of apoptotic cells in cells infected with mock (PBS control) and scrambled vector (Ad-SV). MMP-9 inhibition significantly increased the number of apoptotic cells (fraction of subdiploid) as determined by FACS analysis (Fig. 1A). Also, the number of apoptotic cells increased from ~4% (cells infected with mock and Ad-SV) to 56.45% in cells infected with 50 MOI and 78.05% in cells infected with 100 MOI of Ad-MMP-9 as determined by TUNEL analysis (Fig. 1B). It is known that the translocation of cytochrome c from the mitochondria to the cytosol is an important step in the apoptotic signaling pathway, linking mitochondrial changes to the activation of caspases (19). Once located in the cytosol, cytochrome c, together with Apaf-1 and procaspase-9, forms a multiprotein complex, which initiates the activation of caspase-3, leading to cell apoptosis (20). Cytochrome c levels in the cytoplasm increased in response to Ad-MMP-9 treatment, and this finding correlated with the cleavage of both caspase-9 and -3. Consistent with the activation of caspase-3, the degradation of PARP, which is a caspase-3 substrate, was also observed (Fig. 1, C and D).

**Ad-MMP-9 Infection Activates the MEK/ERK Signaling Pathway**—It is well known that the MAPK cascade plays an essential role in controlling cellular proliferation, differentiation, and apoptosis (21, 22). To elucidate the mechanism mediating the apoptotic effect, cell lysates of Ad-MMP-9-infected Daoy cells were subjected to Western blot analysis using antibodies against phospho-ERK and total ERK. Ad-MMP-9 infec-
Densitometric analysis indicated that phospho-ERK levels increased nuclear translocation of p50 and p65 proteins and p65. Western blot analysis revealed that Ad-MMP-9 transfected Daoy medulloblastoma cells with a dominant negative mutant of ERK (Dn-ERK; kinase-dead mutants of ERK-1) transfection suppressed Ad-MMP-9-induced NF-κB activation (Fig. 4A) and blocked p65 translocation to the nucleus (Fig. 4B). These data confirmed the requirement of activation of ERK for NF-κB activation.

Because cytochrome c release into the cytosol precedes the activation of caspase-3, we examined the role of ERK signal transduction in Ad-MMP-9-induced cytochrome c release. Dn-ERK transfection drastically reduced the release of cytochrome c into the cytosol by Ad-MMP-9 infection (Fig. 4C). These data suggest that cytochrome c is a key factor in Ad-MMP-9-induced apoptosis in Daoy cells and that its release may relate to the activation of caspase-9 and -3, which subsequently triggers the activation of caspase-3 and PARP, which is a biochemical feature of apoptosis (Fig. 3C). Furthermore, Dn-ERK transfection suppressed Ad-MMP-9-induced NF-κB activation (Fig. 4A) and blocked p65 translocation to the nucleus (Fig. 4B). These data confirmed the requirement of activation of ERK for NF-κB activation.

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FIGURE 4. ERK suppression blocks Ad-MMP-9-induced NF-κB activation. Daoy cells were transfected with Dn-ERK plasmid prior to infection with mock, 100 MOI of Ad-SV, and the indicated MOI of Ad-MMP-9. A, nuclear extracts were added to DNA binding mixtures containing a 32p-labeled NF-κB probe. The major inducible complexes are indicated with the arrows. B, Inhibition of p65 and p50 nuclear translocation by Dn-ERK. Nuclear extracts were resolved on SDS-PAGE, and p65 and p50 NF-κB subunits were detected by Western blotting. C, Western blotting for cytochrome c in the cytoplasmic fraction and phospho-ERK in total cell lysates. D, Western blot analysis for phospho-ERK levels, caspase-3, and PARP in Daoy cells treated with NF-κB inhibitor prior to infection with mock, Ad-SV, and Ad-MMP-9 infection. The data represent a typical experiment conducted three times with similar results.

reversed upon the reintroduction of MMP-9 expression (Fig. 5, C and D). These results suggest that MMP-9-mediated alteration in β1-integrin expression induces ERK phosphorylation.

Characterization of Tumors from Mice Treated with Ad-MMP-9—We have previously shown that Ad-MMP-9 inhibits medulloblastoma tumor growth in vivo in an intracranial model (16). A TUNEL assay was performed in established tumors from mice implanted with Daoy medulloblastoma cells and treated with mock, Ad-SV or Ad-MMP-9. The results show a clear increase in apoptosis in tumor sections from Ad-MMP-9-treated mice as compared with sections from mock- and Ad-SV-treated animals (Fig. 6A). To determine whether ERK phosphorylation mediates Ad-MMP-9-mediated apoptosis in vivo, phosphorylation of ERK1/2 (pERK) and cleaved caspase-3 were measured by immunohistochemical analysis. Consistent with the TUNEL results, an increase in cleaved caspase-3 was found in Ad-MMP-9-treated tumors (Fig. 6B). Extensive phosphorylation of ERK in tumors from mice treated with Ad-MMP-9 was observed compared with tumors from mice that received mock and Ad-SV treatments (Fig. 6C).

DISCUSSION

Several previous studies have shown the ability of MMP inhibitors to block tumor growth and induce apoptosis. However, no studies have shown that apoptosis is directly related to demonstrate that the apoptosis-inducing ability of Ad-MMP-9 depends on NF-κB activation. Recent studies have focused on the proapoptotic or antiapoptotic role of NF-κB that mediates cell survival. Regulation of apoptotic behavior by NF-κB either in a proapoptotic or antiapoptotic manner is determined by the nature of the apoptotic stimuli. Inhibition of inducible or constitutive NF-κB activation confers sensitivity to apoptosis-inducing therapies, such as tumor necrosis factor α, in some cancer cell types (23). However, although less frequently observed than prosurvival functions, NF-κB activation triggers apoptosis (24, 25). Several examples of NF-κB-induced death have been reported in neuronal cell types. For example, dopamine-induced death of pheochromocytoma cells and neuronal death in response to ischemia require NF-κB (26, 27). Similarly, doxorubicin-induced cell death in neuroblastoma cells is mediated by IκBα degradation and an increase in the DNA binding of NF-κB p65/p50 heterodimer, and specific inhibition of NF-κB renders cells resistant to Dox (28). Aspirin prevents neuronal cell death via inhibition of NF-κB activation that indicates a proapoptotic role for NF-κB in neuronal cells (29).

Two major distinct apoptosis pathways have been described for mammalian cells. One involves caspase-8, which is recruited by the adapter molecule Fas/APO-1-associated death domain protein to death receptors upon extracellular ligand

MMP inhibition or whether or not it is a property of a given inhibitor or the cell system used. Moreover, the functional mechanism by which MMP inhibition induces apoptotic cell death is unclear. We have previously reported that MMP-9 inhibition caused senescence in medulloblastoma cells (16). In this study, we demonstrate that MMP-9 inhibition mediated by adenoviral delivery of MMP-9 siRNA (Ad-MMP-9) caused programmed cell death in medulloblastoma in vitro and in vivo. Further investigations of the signaling mechanism indicated that Ad-MMP-9-induced apoptosis in medulloblastoma cells was preceded by activation of the ERK pathway. We also determined the effect of altered integrin expression in Ad-MMP-9-infected cells on ERK activation. Thus, our data identify a novel mechanism whereby MMP inhibition-mediated alterations of integrin expression induced apoptosis following the activation of the ERK pathway.

MMP-9 inhibition caused apoptosis in medulloblastoma in vitro and in vivo as demonstrated by TUNEL staining, FACS analysis, and caspase-3 activation. We
were infected with mock, Ad-SV, and Ad-MMP-9 for 48 h. We measured the levels of MMP-9 in media and cytosol after 16 h and infected with mock, Ad-SV, and Ad-MMP-9. Shown are results from gelatin zymography for MMP-9, indicating that Ad-MMP-9 inhibited rather than activated caspase-3; this inhibition has been reported to diminish cytochrome c release, which plays a role in mediating Ad-MMP-9-induced apoptosis. The enhanced cytochrome c release into cytosol and the activation of caspase-9 and -3 in Daoy cells suggest that Ad-MMP-9 induces apoptosis by mitochondrial dysfunction mechanisms.

To gain further insight into the mechanisms by which Ad-MMP-9 promotes apoptosis in medulloblastoma cells, we looked at the early signaling events. Integrins act as important regulators of cell function through their ability to mediate adhesion to extracellular matrices, to induce cytoskeletal rearrangements, and to activate intracellular signaling pathways (31, 32). Our previous studies indicated that Ad-MMP-9 infection caused increased expression of integrins (16). In this study, we show that ectopic expression of MMP-9 in Ad-MMP-9-treated cells reversed the increase in β1-integrin level, which was originally caused by Ad-MMP-9 infection, indicating that the β1 expression is regulated by MMP-9. Many intracellular signaling molecules are activated by integrin engagement, including components of the Ras/Raf/MAPK/ERK pathway (33). We show that blocking β1-integrin, using blocking antibodies, decreased Ad-MMP-9-induced ERK phosphorylation. We have presented much evidence suggesting that ERK activation plays a key role in Ad-MMP-9-mediated induction of apoptosis. We demonstrate that ERK activation plays a central role in Ad-MMP-9-mediated apoptosis using Dn-ERK plasmid. Daoy cells transfected with Dn-ERK prior to Ad-MMP-9 infection indicated that the ERK signaling pathway is involved in the activation of NF-κB and downstream caspase activation. The ability of ERK inhibition to diminish cytochrome c release suggests that the ERK signaling pathway functions upstream of cytochrome c release in the induction of caspase-mediated cell death. Furthermore, the inhibition of ERK also inhibited caspase-3 cleavage. A link between ERK and caspase-3 activation has been described in leptin-induced apoptosis in bone marrow cells, although the mechanism of this link is not fully understood (34). It was reported that PARP cleavage was suppressed by a MEK inhibitor, demonstrating that the caspase cascade is downstream of the MEK/ERK pathway (35). Consistent with this observation, our studies also indicate that ERK inhibition decreased PARP cleavage, suggesting that ERK activation is necessary for the caspase cascade. Our results clearly indicate ERK inhibition in Ad-MMP-9-treated cells, decreased cytochrome c release, NF-κB activation, and caspase-3 cleavage. Generally, ERK inhibits rather than activates caspase-3; this inhibition has been associated with NF-κB activation (36) or with direct phospho-

binding (30). The other involves cytochrome c release-dependently activate caspase-9 (20). We did not observe any change in either Fas or FasL expression in Ad-MMP-9-infected Daoy cells (data not shown). We did, however, observe increased levels of cytochrome c in the cytoplasm of Ad-MMP-9-treated cells relative to control and Ad-SV-treated cells, suggesting that...
MMP-9 Inhibition Induces ERK and NF-kB Activation

![Flow diagram](Image)

**FIGURE 7. Flow diagram.** Shown is a schematic representation of the sequence of events leading to apoptosis due to inhibition of MMP-9.

rylation of caspase-9 and subsequent inhibition of caspase-3 activation (37). Determining whether NF-kB activation or inhibition of phosphorylation of caspase-9 is responsible for the opposing effects of ERK activation in cell survival in Ad-MMP-9-infected cells will be an area for future attention.

In summary, our study suggests that MMP-9 takes considerable part in the regulation of apoptosis in human medulloblastoma cells. We propose a model in which Ad-MMP-9-mediated alterations in integrin expression potentiate ERK-induced apoptosis in medulloblastoma cells. We propose a model in which Ad-MMP-9-infected cells will be an area for future attention.

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REFERENCES

1. Gerschenson, L. E., and Rotello, R. J. (1992) FEBS Lett. 6, 2450–2455
2. Gate, L., Lunk, A., and Tew, K. D. (2003) Biochem. Pharmacol. 65, 1611–1622
3. Stork, P. J., and Schmitt, J. M. (2002) Trends Cell Biol. 12, 258–266
4. Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Klejvitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C. F., Kitsis, R. N., and Molkentin, J. D. (2000) EMBO J. 19, 6341–6350
5. Kurokawa, H., Lenferink, A. E., Simpson, J. F., Piscanc, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. (2000) Cancer Res. 60, 5887–5894
6. Shelton, J. G., Steelman, L. S., Lee, J. T., Knapp, S. L., Blalock, W. L., Moyle, P. W., Franklin, R. A., Pohnert, S. C., Mirza, A. M., McManus, M., and McCubrey, J. A. (2003) Oncogene 22, 2478–2492
7. Yan, C. Y., and Greene, L. A. (1998) J. Neurosci. 18, 4042–4049
8. Bacus, S. S., Gudkov, A. V., Lowe, M., Lyass, L., Lyng, Y., Komarov, A. P., Keyomarsi, K., Yarden, Y., and Seger, R. (2001) Oncogene 20, 147–155
9. Dempke, W., Voigt, W., Grothey, A., Hill, B. T., and Schmoll, H. J. (2000) Anticancer Drugs 11, 225–236
10. Wang, X., Martin, J. L., and Holbrook, N. J. (2000) J. Biol. Chem. 275, 39435–39443
11. Woessmann, W., Zwanzger, D., and Borkhardt, A. (2004) Cell Biol. Int. 28, 403–410
12. Egeblad, M., and Werb, Z. (2002) Nat. Rev. Cancer 2, 161–174
13. Sternlicht, M. D., and Werb, Z. (2001) Annu. Rev. Cell Dev. Biol. 17, 463–516
14. Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000) Cell 103, 481–490
15. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) Science 295, 2387–2392
16. Rao, J. S., Bhoopathi, P., Chetty, C., Guijrat, M., and Lakka, S. S. (2007) Cancer Res. 67, 4956–4964
17. Lakka, S. S., Jasti, S. L., Gondi, C. S., Boyd, D., Chandrasekar, N., Dinh, D. H., Olivero, W. C., Guijrat, M., and Rao, J. S. (2002) Oncogene 21, 5601–5608
18. Chatuvvedi, M. M., Mukhopadhyay, A., and Aggarwal, B. B. (2000) Methods Enzymol. 319, 585–602
19. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
20. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
21. Hagemann, C., and Blank, J. L. (2001) J. Biol. Chem. 276, 2387–2392
22. Madhani, H. D., Styles, C. A., and Fink, G. R. (1997) Cell 86, 743–753
23. Rayet, B., and Gelinas, C. (1999) Oncogene 18, 6938–6947
24. Dumont, A., Hehner, S. P., Hofmann, T. G., Ueffing, M., Drogue, W., and Schmitz, M. L. (1999) Oncogene 18, 747–757
25. Kimura, K., and Gelmann, E. P. (2002) Cell Death Differ. 9, 972–980
26. Panet, H., Barzilai, A., Daily, D., Melamed, E., and Offen, D. (2001) J. Neurochem. 77, 391–398
27. Schneider, A., Martin-Villalta, A., Weih, F., Vogel, I., Wirth, T., and Schwangier, M. (1999) Nat. Med. 5, 554–559
28. Bian, X., Lister-Lucas, L. M., Shao, F., Schumacher, K. R., Feng, Z., Porter, A. G., Castle, V. P., and Opipari, A. W., Jr. (2001) J. Biol. Chem. 276, 48921–48929
29. Grilli, M., Pizzi, M., Memo, M., and Spano, P. (1996) Science 274, 1383–1385
30. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
31. Hynes, R. O. (1992) Cell 69, 11–25
32. Renshaw, M. W., Price, L. S., and Schwartz, M. A. (1999) J. Cell Biol. 147, 611–618
33. Schoenwaelder, S. M., and Burridge, K. (1999) Curr. Opin. Cell Biol. 11, 274–286
34. Kim, G. S., Hong, J. S., Kim, S. W., Koh, J. M., An, C. S., Choi, J. Y., and Cheng, S. L. (2003) J. Biol. Chem. 278, 21920–21929
35. Mohr, S., McCormick, T. S., and Latopera, E. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5045–5050
36. Shimada, K., Nakamura, M., Ishida, E., Kishi, M., Yonehara, S., and Konishi, N. (2002) Mol. Carcinog. 35, 127–137
37. Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) Nat. Cell Biol. 5, 647–654
38. Roberts, P. J., and Der, C. J. (2007) Oncogene 26, 3291–3310