Induction of Matrix Metalloproteinase-9 Requires a Polymerized Actin Cytoskeleton in Human Malignant Glioma Cells*

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Alterations in cytoskeleton and subsequent cell shape changes exert specific effects on the expression of various genes. Our previous results suggested that malignant human gliomas express elevated levels of matrix metalloproteinases compared with normal brain tissue and low grade gliomas. To understand the role of cell shape changes on matrix metalloproteinase expression in human glioma cells, we treated SNB19 cells with cytochalasin-D, an inhibitor of actin polymerization, and colchicine-B, a tubulin inhibitor, in the presence of phorbol 12-myristate 13-acetate. Cytochalasin-D treatment of SNB19 cells resulted in the loss of phorbol 12-myristate 13-acetate-induced matrix metalloproteinase-9 (also known as gelatinase-B) expression and coincided with inhibition of actin polymerization, resulting in cell rounding. Moreover, compared with monolayers, cells grown as spheroids or cell aggregates failed to express matrix metalloproteinase-9 in the presence of phorbol 12-myristate 13-acetate. Matrix metalloproteinase-9 expression was also inhibited by calphostin-C, a protein kinase inhibitor, suggesting the involvement of protein kinase C in matrix metalloproteinase-9 expression. Phorbol 12-myristate 13-acetate-induced invasion of SNB19 cells through Matrigel was inhibited by cytochalasin-D and calphostin-C. These results suggest that the actin polymerization transduces signals that modulate the expression of matrix metalloproteinase-9 expression and the subsequent invasion of human glioma cells.

Basement membrane and extracellular matrix degradation by proteolytic enzymes is the critical event that takes place during tumor cell invasion and metastasis (1). Several proteases are secreted by invading tumor cells, such as serine proteases, plasminogen activators, and matrix metalloproteinases (MMPs)1 (2, 3). Among the proteases, elevated levels of MMPs are secreted by invading tumor cells, such as serine proteases (MMPs)1 (2, 3). Among the proteases, elevated levels of MMPs are secreted by invading tumor cells, such as serine proteases (MMPs)1 (2, 3).

The production of MMPs is influenced by many factors. Physiologically relevant cytokines such as interleukin-1 and tumor necrosis factor-α have been shown to induce the expression of MMPs (14, 15). Earlier studies have shown that a number of pharmacological agents including 12-O-tetradecanoyl-13-phorbol acetate induce procollagenase, MMP, and stromelysin expression (16–18) and the expression of various biological markers associated with tumorigenesis (19). Alteration of cell shape by growing cells on biological matrices also results in dramatic changes in the phenotypes of a number of cell types (20), and a strong correlation has been shown with a change in cell morphology; it has been speculated that cell shape plays a major role in chondrogenesis (21, 22) and adipogenesis (23, 24). Moreover, growth of cells in three-dimensional collagen matrices alters the cytoskeleton organization (25–27). Recent studies reported that changes in the cytoskeleton activate MMP-2 (28) and inhibit MMP-9 (29).

Although the above studies have shown that cell shape changes result in modulation of various phenotypic changes and MMP expression, studies on extracellular matrix and cytoskeleton organization and the associated changes in gliomas are of special importance because these tumors are more highly infiltrative than all other tumor types. Despite a number of studies on the role of MMPs and invasive phenotypes, the activation of MMPs which contributes significantly to glioma invasiveness is not clearly understood. In this study, we show that cell shape changes, particularly those resulting from inhibition of actin polymerization, suppress the activation of MMP-9 and the invasion properties of human gliomas.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium was obtained from Life Technologies, Inc. Cytochalasin-D, colchicine-B, dexamethasone, phorbol 12-myristate 13-acetate (PMA), and TRITC-phalloidin were obtained from Sigma. Calphostin-C, genistein, HA1004, and 8-bromo-cAMP were purchased from Calbiochem. PolyHema (poly[2-hydroxyethylmethacrylate], also called CellForm Polymers) was obtained from ICI biochemicals (Aurora, OH). Agar was obtained from Difco Laboratories. Tissue culture plates were purchased from Becton Dickinson (Franklin Lakes, NJ). Focal adhesion kinase (FAK) antibody was obtained from Transduction Laboratories (Lexington, KY).

Cell Culture—An established human glioma cell line SNB19 (30) was

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used in the current study. Cells were routinely grown in high glucose Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 20 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Cells were passaged every 3–5 days. Cells were being tested in preliminary experiments, reagents were used at concentrations that have maximal inhibitory or stimulatory effect on MMP-9 expression without affecting cell viability (by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay): 50 ng/ml PMA, 1 μM demethasone, 40 μM genistein, 1 μM 8-bromo-cAMP, 5 μM cytochalasin-D, 5 μM colchicine-B, 10 μM HA-10004, and 300 μM calphostin-C.

**Immunofluorescent Staining—** SNB19 cells grown in chamber slides (Nunc Inc., Naperville, IL) for 24 h at 37°C were washed with PBS, fixed by adding 3% paraformaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. Where indicated, TRITC-phalloidin (200 nM/ml in PBS) was applied for 30 min to Triton X-100-permeabilized cells to stain for F-actin.

**Gelatin Zymography—** Analysis of MMP-2/MMP-9 was performed on SDS-polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polycrylamide (w/v) as described elsewhere (30). Cells were grown in 100-mm2 tissue culture plates in Dulbecco’s modified Eagle’s medium/Ham’s F-12. Cells were treated with trypsin, and 200 μl of cell suspension (1 × 105 cells/ml) from each treatment was added to triplicate wells. After 48 h of incubation, the cells that passed through the filter into the lower wells were stained with Hema-3 (CMS Inc., Houston) and photographed under a microscope.

**Expression of FAK—** Western blotting for p125FAK was performed by lysing cells with RIPA buffer (1% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 1 mM NaN3, 5 mM EDTA, 0.1 mg/liter aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cells grown in 100-mm tissue culture plates were washed with cold PBS before the addition of RIPA buffer and incubated for 10 min. Cells were scraped and pipetted into Eppendorf tubes and centrifuged for 20 min at 12,000 × g. Supernatants were transferred to fresh Eppendorf tubes, and protein was determined by BCA assays (Pierce Chemical Co.). 20 μg of this initial lysate was loaded onto gels for electrophoresis and Western blotting. After reduction in the sample buffer, samples were loaded onto a 10% resolving SDS-polyacrylamide gel with a 4.5% stacking gel. Samples were electrophoresed at a constant current (40 mA) for 2–4 h at 4°C, and then electroblotted onto a nitrocellulose membrane overnight at 4°C at a constant current of 80 mA. Western blotting was performed with a 1:2500 dilution anti-FAK monoclonal antibody (Transduction Laboratories). Immunoreactive bands for FAK were visualized using horseradish peroxidase-conjugated anti-mouse IgG secondary antibody and ECL reagents (Amer sham Pharmacia Biotech). Film was analyzed with a scanning densitometer, and the images were reproduced by UMAX scanner with an Adobe Photoshop imaging system on a Macintosh computer.

**Electrophoretic Mobility Shift Assay—** Electrophoretic mobility shift assay was performed according to the procedures described previously (32, 33). Briefly, 1 × 106 cells were washed with cold Dulbecco’s phosphate-buffered saline and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 μg/ml benzamidine). Cells were allowed to swell on ice for 20 min followed by the addition of 12.5 μl of 10% Nonidet P-40. The tubes were then vortexed vigorously for 10 s, and the homogenate was centrifuged for 30 s. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 μg/ml benzamidine). Cells were allowed to swell on ice for 20 min followed by the addition of 12.5 μl of 10% Nonidet P-40. The tubes were then vortexed vigorously for 10 s, and the homogenate was centrifuged for 30 s. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 μg/ml benzamidine) and incubated on ice for 30 min with intermittent vortexing. Samples were centrifuged for 5 min at 4°C, and the supernatant (nuclear extract) was either used immediately or stored at −70°C. The protein content was measured by the method of Bradford (34).

**RESULTS**

**Phorbol Ester Induces MMP-9 Expression in SNB19 Cells—** SNB19 cells were treated with trypsin, and a single-cell suspension was obtained. Cell suspensions containing 1 × 105 cells/ml were plated in six-well tissue culture plates and treated with 50 ng/ml PMA. PMA was added during the plating, after plating, or during and after plating. Fig. 1 shows that treatment of SNB19 cells with PMA resulted in the induction of MMP-9, which is normally absent in this cell line, irrespective of the time of addition of PMA, although long term treatment resulted in reduced expression of MMP-9 (results not shown). MMP-2 expression was largely unaltered, although activation into the 66-kDa form resulted occasionally.

**MMP-9 Induction Is Inhibited by Cytochalasin-D—** To understand the role of actin polymerization, we treated SNB19 cells

![Fig. 1. Induction of MMP-9 expression in SNB19 cells. SNB19 cells were plated (1 × 10^5 cells/well) in a six-well culture plate in the presence or absence of PMA (50 ng/ml). Medium was replaced after overnight incubation at 37°C, and fresh serum-free medium was added after two washes including a 4-h incubation between washes. Finally, fresh serum-free medium was added and allowed to condition for further 48 h. Conditioned medium containing an equal amount of protein (20 μg) was mixed with Laemmli loading buffer (without reducing agent) and run on 10% SDS-polyacrylamide gels containing gelatin for comparison, as shown by the Experimental Procedures). After the gels were washed with Triton X-100 and incubated in buffer containing CaCl2, they were stained with Coomassie Brilliant Blue and then destained. The zymographic assay for MMPs was shown after the cells were treated with PMA during plating and after washes with serum-free medium (+/+), at the time of plating but not after washes with serum-free medium (+/-), only after washes with serum-free medium (-/+), and for untreated cells (-/-). MMP-9 (92 kDa) expression was induced by PMA treatment, irrespective of the time of PMA addition.**
with PMA during the initial plating and then treated them with cytochalasin-D (actin polymerization inhibitor) during plating, after plating, and during and after plating. SNB19 cells treated with PMA expressed MMP-9, and the expression of this enzyme was lost by the addition of cytochalasin-D, provided it was added during or during and after plating (Fig. 2). However, there was no change in the expression of MMP-9 when cytochalasin-D was added after spreading (Fig. 2). Similar results were obtained in another malignant glioma cell line, U5 (results not shown). Treatment of the cells with colchicine-B (tubulin inhibitor) had no effect on the expression of MMP-9, induced by PMA treatment (Fig. 3). To rule out a nonspecific effect of actin polymerization inhibition on MMP-9 expression, similar experiments were conducted using the HT1080 fibrosarcoma cell line, which constitutively expresses both MMP-2 and MMP-9. HT1080 cells treated with cytochalasin-D lost the ability to express MMP-9 provided the agent was added during or during and after spreading (Fig. 4); the ability to express proMMP-2 was unaltered by the addition of cytochalasin-D. On the other hand, treatment of HT1080 cells with colchicine-B had no effect on either MMP-2 or MMP-9 expression (Fig. 4). These results show that the induced expression of MMP-9 in SNB19 cells and constitutive expression of MMP-9 in HT1080 cells are lost by alterations in the actin cytoskeleton.

Inhibition of MMP-9 Is Caused by Alteration in Actin Polymerization—Because inhibitors of actin polymerization resulted in MMP-9 inhibition, we performed cell spreading assays where changes in cell shape and actin polymerization were observed during spreading by staining the cells with TRITC-phalloidin. Fig. 5 shows that treatment of cells with cytochalasin-D resulted in changes in the cell shapes as observed both by light microscopy and fluorescence microscopy. Treatment with cytochalasin-D resulted in the loss of actin cytoskeleton, provided the agent was added during spreading or during and after spreading. Although treatment with cytochalasin-D after spreading resulted in some changes in cell shape, most of the cells were spread and retained stress-fiber formation. However, colchicine-B had no effect on actin polymerization in SNB19 cells. We treated SNB19 cells further with dexamethasone, which induces a rapid change of nonpolymerized actin to polymerized actin. Cells treated with PMA followed by dexamethasone showed induction of MMP-9 as expected (Fig. 9). Interestingly, cells treated with PMA and dexamethasone followed by cytochalasin-D retained the ability to express MMP-9, whereas cells treated with cytochalasin-D in the presence of PMA invariably lost the ability to express MMP-9, as observed in earlier experiments (see Fig. 2). Treatment with dexamethasone alone did not result in the induction of MMP-9 and had no effect on the constitutive expression of MMP-2 in SNB19 cells. These results suggest that polymerization of the actin cytoskeleton induced by dexamethasone is resistant to disruption by cytochalasin-D treatment.

Cell Shape Alteration by Cytochalasin-D Alters the Expression of FAK—As the cells treated with cytochalasin-D lost their cytoskeletal organization, we were interested to see the changes in the expression of FAK where stress fibers terminate. Cells were plated as described above and treated with cytochalasin-D or colchicine-B; after the treatments, cells were extracted in RIPA buffer, and FAK protein was examined by Western blotting. The Western blot for FAK in Fig. 6 shows.

Fig. 2. Addition of cytochalasin-D inhibits MMP-9 induction. SNB19 cells were plated (1 x 10^5 cells/well) in a six-well tissue culture plate in the presence of PMA (50 ng/ml) and incubated overnight at 37 °C. Where indicated, cytochalasin-D was added during the plating and after the washes (+/+); added during the plating (+/-), or cells were not treated with cytochalasin-D (-/ -). Conditioned medium was collected after a 48-h incubation and run on SDS-polyacrylamide gels containing gelatin. Zymographic analysis shows that PMA-induced MMP-9 expression was inhibited when cytochalasin-D was added during spreading but not after spreading.

Fig. 3. Colchicine-B treatment does not alter MMP-9 induction. SNB19 cells were plated as described in Fig. 2 and treated with PMA (50 ng/ml). Cells were also treated with colchicine-B during plating and after washes (+/+-), added during plating (+/-/ ), added after plating (+/+), or not treated (-/+). Conditioned medium containing an equal amount of protein was run on SDS-polyacrylamide gels containing gelatin. No alteration in PMA-induced MMP-9 expression was observed, and cytochalasin-B had no effect on MMP-2 expression.

Fig. 4. Cytochalasin-D inhibits constitutive expression of MMP-9 in HT1080 cells. HT1080 cells were plated (1 x 10^5 cells/well) in six-well tissue culture plates and treated with cytochalasin-D or colchicine-B during and after plating (+/+-), added during plating (+/-), added after plating (+/-), or not treated (-/-), in the absence of PMA. Additionally, in a separate set of experiments HT1080 cells were also treated with PMA alone. Conditioned medium was collected and run on SDS-polyacrylamide gels containing gelatin. HT1080 cells constitutively expressed both MMP-2 and MMP-9; however, expression of MMP-9 but not of MMP-2 is inhibited significantly in the presence of cytochalasin-D. The addition of PMA has no significant effect on the expression of both MMP-2 and MMP-9.
Actin Cytoskeleton Modulates MMP-9 Expression

that SNB19 cells without any treatment or with PMA treatment expressed FAK protein. However, treatment of cells with cytochalasin-D during or during and after treatment resulted in the loss of FAK expression. On the other hand, similar levels of FAK were expressed in colchicine-B-treated cells compared with levels expressed by control and PMA-treated cells. These observations show that both the actin cytoskeleton and the expression of FAK are lost when cells are treated with cytochalasin-D. This in turn results in altered cell shape and consequent loss of ability to express MMP-9.

Cells Cultured as Three-dimensional Spheroids Do Not Express MMP-9—Because cell “rounding” in the presence of cytochalasin-D resulted in the loss of MMP-9 expression, we grew cells on PolyHema or on agar-coated plates; this prevents the cells from attaching to the tissue culture plastic and results in cell aggregation or spheroid formation. Cells are then treated with 50 ng/ml PMA. Gelatin zymography of the conditioned medium from cells grown as spheroids (Fig. 7, left panel, lane B) and cell aggregates (Fig. 7, lane C) failed to express MMP-9 in the presence of PMA, whereas cells grown as monolayers expressed MMP-9 in response to PMA (Fig. 7, lane A), indicating that cell spreading and subsequent actin polymerization are necessary for MMP-9 expression. MMP-2 expression was unaltered by growing the cells on PolyHema or on agar-coated plates.

Expression of NF-κB Is Lost by Cytochalasin-D Treatment—It is speculated that cell shape changes exert specific effects on gene expression by modulating the activity of transcriptional factors that reside in the cytoplasm of the unstimulated cells in an inactive form and migrate to the nucleus in response to various stimuli. One such factor is NF-κB, a dimeric complex that activates transcription of a variety of genes, including MMP-9 expression, activation of cell surface receptors, and activation of cell adhesion molecules. SNB19 cells were treated with PMA in the presence and absence of cell shape modulators, cytochalasin-D and colchicine-B as described above. As shown in Fig. 8, untreated cells have no DNA binding activity of NF-κB. Stimulation of NF-κB was observed when the cells were treated with PMA. Subsequently, PMA-induced NF-κB binding was decreased in the presence of cytochalasin-D, whereas significant binding of NF-κB was observed in colchicine-B-treated cells. These observations suggest that PMA-induced NF-κB binding was inhibited by disruption of the actin cytoskeleton.

Inhibition of MMP-9 Expression by Protein Kinase C Inhibitors—It has been shown that MMP induction in glioma cells is dependent on protein kinase C (PKC) expression. To under-

FIG. 5. Cell morphology and cytoskeleton organization are altered by cytochalasin-D. SNB19 cells were plated (1 × 10⁵ cells/well) in chamber slides, treated with various agents, and photographed under phase-contrast microscopy; or cells were fixed with 4% paraformaldehyde, treated with Triton X-100, and stained with TRITC-phalloidin to display actin filaments. Panel A shows the actin cytoskeleton in cells treated with PMA (50 ng/ml) as described in Fig. 1. Panel B shows the cell morphology under a phase-contrast microscope, and panel C shows the actin cytoskeleton in cells treated with cytochalasin-D as described in Fig. 2. Panel D shows the cell morphology under phase-contrast microscopy, and panel E shows the actin cytoskeleton in cells treated with colchicine-B as described in Fig. 3. Cell rounding and loss of actin polymerization were observed only in cells treated with cytochalasin-D during spreading and during and after spreading.

FIG. 6. Loss of FAK expression in SNB19 cells. Cells were plated (1 × 10⁵ cells/well) in six-well tissue culture plates that were precoated with fibronectin (5 μg/ml) in the presence of PMA (50 ng/ml). Where indicated, cells were treated with cytochalasin-D as described in Fig. 2 and colchicine-B as described in Fig. 3. Medium was aspirated, and the cells were washed with cold PBS followed by scraping the cells in RIPA buffer (for details, see “Experimental Procedures”). Samples containing equal amounts of protein (20 μg), including a positive control, were run on 10% SDS-polyacrylamide gels under reducing conditions. Proteins were transferred onto nitrocellulose membranes and incubated with blocking buffer. Nitrocellulose membranes were incubated with anti-FAK antibody, followed by a secondary antibody, and the bands were visualized on x-ray film using ECL reagent according to the manufacturer’s instructions. The figure shows that 125-kDa FAK protein expression was lost by treating the cells with cytochalasin-D during and during and after spreading.
Actin Cytoskeleton Modulates MMP-9 Expression

**DISCUSSION**

Changes in cytoskeletal architecture reflected in cell shape changes are well known to accompany changes in gene expression in a number of cell types such as mammary epithelium (38), chondrocytes (22), adipocyte precursors (24), and cells from synovial tissues (39). Unemori and Werb (40) reported that disruption of the actin cytoskeleton stimulated procollagenase and stromelysin secretion in rabbit synovial fibroblasts, which led to the speculation that perturbation of the actin microfilaments might be linked to the expression of genes involved in the initiation of extracellular matrix degradation. In addition, two recent reports demonstrating that MMP-9 was suppressed by an alteration in cell shape in melanoma cells (29) and that MMP-2 activation was regulated by organization of the polymerized actin in human palmar fascial fibroblasts stand the role of actin cytoskeleton in PKC-dependent MMP expression, cells were treated with PMA and incubated with various PKC and tyrosine kinase inhibitors. MMP-9 expression was induced by PMA as expected (Fig. 9), and the PKC-induced MMP-9 expression (and to some extent MMP-2 expression) was inhibited by calphostin-C, a specific inhibitor of PKC, but not by the tyrosine kinase inhibitors genistein and HA1004 (Fig. 9). This showed that induction of both MMP-2 and MMP-9 is dependent on PKC expression in SNB19 human glioma cells.

**Fig. 7.** Expression of MMPs in cells grown in three-dimensional spheroids. SNB19 cells were grown as aggregates on Poly-Hema or as spheroids on agar-coated tissue culture plates. Cell aggregates or spheroids were treated with PMA and incubated for 48 h at 37 °C. Conditioned medium was collected and run on 10% SDS-polyacrylamide gels containing gelatin. The left panel shows cells grown as monolayers and treated with PMA (lane A); cells grown as spheroids and treated with PMA (lane B); and cells grown as cell aggregates and treated with PMA (lane C). The right panel shows the morphology of cells grown as monolayers, spheroids, and aggregates. SNB19 cells grown in three-dimensional configuration failed to express MMP-9 in response to PMA.

**Fig. 8.** Induction of NF-κB binding is altered by cytochalasin-D. Cells were plated (1 × 10^5 cells/well) in six-well tissue culture plates and treated with PMA (50 ng/ml) during spreading. Cells were also treated with cytochalasin-D as described in Fig. 2 and colchicine-B as described in Fig. 3. Nuclear extracts were prepared as described under “Experimental Procedures,” and mobility shift assays were performed. The figure shows that PMA induced NF-κB binding, which was almost absent in untreated control cells. PMA-induced NF-κB binding was inhibited by cytochalasin-D but not by colchicine-B.

**Fig. 9.** MMP-9 induction is inhibited by the PKC inhibitor calphostin-C. Cells were plated (1 × 10^5 cells/well) in six-well tissue culture plates and treated with PMA (50 ng/ml) during spreading. Cells were also treated with calphostin-C, cAMP, genistein, and HA1004 for 1 h before the addition of PMA. In a separate set of experiments, cells were also treated with dexamethasone after PMA treatment, or cells were treated with PMA and dexamethasone followed by cytochalasin-D. Conditioned medium was collected, and samples containing an equal amount of protein (20 μg) from each treatment were run on SDS-polyacrylamide gels containing gelatin. PMA-induced expression of MMP-9 and to some extent MMP-2 was inhibited by calphostin-C.

**Fig. 10.** In vitro invasion of SNB19 cells is inhibited by cytochalasin-D and calphostin-C. 200 μl of a single-cell suspension (1 × 10^6 cells/ml) of SNB19 cells was placed in the upper wells of individual Transwell inserts containing 8-μm pore size polycarbonate membranes precoated with Matrigel (0.78 mg/ml). Before placing the cells in Transwell inserts, cells were also treated with PMA (50 ng/ml) alone or in combination with cytochalasin-D, colchicine-B, or calphostin-C. Cells were allowed to invade for 48 h at 37 °C followed by the fixation and staining of cells with Hema-3. Cells on the upper surface were removed with a cotton swab, and the cells that passed through the polycarbonate membrane were mounted onto microscope slides and photographed under a light microscope at × 20 magnification. The figure shows that invasion of SNB19 cells was inhibited significantly by cytochalasin-D and calphostin-C.
(28) that an alteration in cell shape influences MMP-9 and as well as MMP-2 in different cell types.

In the current study, we performed experiments to understand whether changes in cytoskeleton polymerization, a dynamic process that occurs during tumor cell invasion, modulate the expression of MMPs using agents that change the cell shape in vitro. Gelatin zymographic analysis of medium from the SNB19 glioma cell line revealed that the expression of MMP-9 is induced by PMA, which is normally absent in this cell line (Fig. 1). Treatment of the cells with cytochalasin-D, which causes disruption of actin stress fibers, resulted in decreased or loss of MMP-9 expression (Fig. 2). In contrast, treatment of cells with colchicine-B, an inhibitor of tubulin polymerization, had no effect on the expression of MMP-9 or MMP-2 (Fig. 3). To understand whether the constitutive expression of MMP-9 in other cell types is altered by cell shape changes, we used HT1080 fibrosarcoma cells, which constitutively express both MMP-2 and MMP-9, because glioma cells do not express constitutive MMP-9 in vitro culturing conditions. Cytochalasin-D inhibited constitutive expression of MMP-9 in HT1080 cells (Fig. 4), similar to the results observed in SNB19 glioma cells (Fig. 2), whereas colchicine-B treatment had no effect on the expression of either MMP-9 or MMP-2. These results confirm that induced production of MMP-9 in SNB19 cells and constitutive expression of MMP-9 in HT1080 cells were lost when the cytoskeletal organization was altered, suggesting that the loss of MMP-9 could be caused by the alteration in actin polymerization and subsequent shape modulation of SNB19 cells. Total actin content by fluorescent estimation of rhodamine phalloidin showed that the total quantity of the actin is not changed during the treatment conditions (results not shown), ruling out the idea that the observed effects are caused by changes in actin content. Cells grown as three-dimensional spheroids were treated with PMA to find out whether these cells (which do not spread and do not form polymerized actin) respond to PMA and express MMP-9. Interestingly, these spheroids failed to express MMP-9 (Fig. 7), unlike monolayer cultures.

Because cytochalasin-D is known to alter the polymerization of actin, experiments were performed to examine the changes in cell spreading and actin polymerization by TRITC-phalloidin staining. Cytochalasin-D-treated cells showed inhibition of actin polymerization when the cells were treated during spreading, whereas cells treated after spreading retained efficient cytoskeleton. In contrast, colchicine-B-treated cells, although showing some morphological changes, expressed organized cytoskeleton efficiently (Fig. 5). Actin cytoskeletons terminate at focal adhesion contacts in fully spread cells, and it has been shown that expression of FAK, phosphorylation of FAK, or both modulate the expression of a variety of events including migration and invasion (41). Results in Fig. 6 show that control cells as well as PMA-treated cells expressed FAK protein as observed on Western blots. Interestingly, FAK expression was lost completely in cytochalasin-D cells only when the cells were treated during spreading, whereas cells allowed to spread and then treated with cytochalasin-D retained FAK expression. On the other hand, colchicine-B-treated cells always expressed FAK irrespective of when colchicine-B was added. These results suggest that cytoskeletal organization and FAK expression are essential for the induction of MMP-9 but not of MMP-2 as MMP-2 is always expressed in these cells, including HT1080 cells.

To understand whether NF-κB (an upstream regulator that induces the expression of MMP-9) was altered by changes in actin polymerization, nuclear extracts prepared from various treatment conditions were examined for the NF-κB expression by gel mobility shift assays. Interestingly, PMA-induced NF-κB expression was reduced to some extent in cytochalasin-D-treated cells but not in colchicine-B-treated cells, showing that NF-κB expression also is regulated by actin polymerization. Although the exact biochemical process by which depolymerization of actin leads to activation of NF-κB remains to be elucidated, the present findings establish a role for NF-κB in sensing the changes in the state of the cytoskeleton and converting them to changes in gene activity. Because cytoskeletal changes are likely to occur when cell-substrate and cell-cell interaction, this process would provide signal transduction pathways by which these physical interactions can modulate gene expression and thereby affect glioma tumor cell invasion.

PKC, an enzyme essential to the cellular response of phorbol ester (19), plays an important role in MMP signal transduction. In previous studies by other investigators, abnormally high levels of PKC activity in glioblastomas were reported compared with those of nontransformed glia (42, 43). Moreover, PKC plays an important role in cell migration, invasion (44, 45) and metastatic spread of tumors (46), and glioma cell invasion (47). In our study, treating cells with calphostin-C (a highly specific inhibitor of PKC) led to a decrease in MMP-9 and to some extent in MMP-2 in SNB19 cells (Fig. 9) with a concomitant decrease in invasion through Matrigel (Fig. 10). Other highly selective inhibitors of tyrosine kinase and cAMP-dependent kinase inhibitors had no effect on MMP expression (Fig. 9).

These results are similar to the earlier reports wherein phorbol ester induced the expression of MMP with a concomitant increase in glioma cell invasion (47). Moreover, earlier studies showed that PMA-induced PKC also controlled actin polymerization by maintaining the cell shape and F-actin levels (48). Our results show that cell shape alteration, particularly inhibition of actin polymerization, results in inhibition of MMP-9 but not MMP-2 in glioblastomas; this indicates that these two enzymes are activated by distinct signaling mechanisms.

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