17-Allylamino-17-Demethoxygeldanamycin Down-Regulates Hyaluronic Acid–Induced Glioma Invasion by Blocking Matrix Metalloproteinase-9 Secretion

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Abstract
Hyaluronic acid (HA) has been implicated in cell adhesion, motility, and tumor progression in gliomas. We previously reported that HA stimulates secretion of matrix metalloproteinase-9 (MMP-9) and induces glioma invasion. However, the molecular mechanism of HA action and therapeutic strategies for blocking HA-induced MMP-9 secretion remain unknown. Here, we report that the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) blocks MMP-9 secretion and that HA-induced nuclear factor-κB (NF-κB) activation is mediated by IκB kinase, which phosphorylates the NF-κB inhibitor IκBα and promotes its degradation. In addition, using an RNA interference approach, we show that the focal adhesion kinase plays a critical role in mediating HA-induced NF-κB activation, which resulted in increased MMP-9 expression and secretion, cell migration, and invasion. Importantly, we show that 17-AAG acts by blocking focal adhesion kinase activation, thereby inhibiting IκB kinase–dependent IκBα phosphorylation/degradation, NF-κB activation, and MMP-9 expression. This leads to suppression of HA-induced cell migration and invasion. Based on our data, we propose that 17-AAG is a candidate drug for treatment of highly invasive gliomas resulting from HA-induced, NF-κB–mediated MMP-9 secretion. (Mol Cancer Res 2008;6(11):1657–65)

Introduction
Gliomas are common malignant brain tumors characterized by invasive growth and extensive angiogenic vascularization (1). Synthesis and secretion of several proteases are up-regulated in gliomas, including the matrix metalloproteinases (MMP). One MMP, MMP-9, has received considerable attention because its expression can be correlated with glioma progression (2).

Hyaluronic acid (HA) is the principal glycosaminoglycan of the human brain extracellular matrix (ECM) and it facilitates cell adhesion, cell migration, cell proliferation, and tumor progression by interacting with receptors such as CD44 and RHAMM (3). Consequently, HA plays an important role in glioma cell motility and invasion (4, 5). Previously, we reported that PTEN suppresses HA-induced MMP-9 secretion and invasion in U87MG glioma cells, by dephosphorylation of focal adhesion kinase (FAK; ref. 6), and that HA-stimulated MMP-9 secretion is mediated by nuclear factor-κB (NF-κB). Alainz et al. (7) reported that MMP-9 activity can be modulated by HA and is dependent on NF-κB in lymphoma cell lines. However, the underlying molecular mechanisms were not identified.

The NF-κB transcription factor is a heterodimer consisting of p50/p105/NF-κB1, p52/p100/NF-κB2 or p65/RelA, and c-Rel or RelB. It is present in the cytoplasm of unstimulated cells, where it associates with IκB family inhibitors. On stimulation with growth factors, cytokines, hormones, or other agents, IκB is phosphorylated and degraded, resulting in dissociation from and nuclear translocation of NF-κB. NF-κB promotes expression of antiapoptotic genes, thereby protecting cancer cells from apoptosis and contributing to tumor growth (8, 9). NF-κB also regulates the expression and activation of MMPs, which play a significant role in ECM degradation and in facilitating cell motility, tumor growth, and metastasis (9). Thus, we predict that compounds that block NF-κB activity will be useful for inhibiting MMP-dependent tumor growth and invasion.

17-Allylamino-17-demethoxygeldanamycin (17-AAG) is an inhibitor of Hsp90 and it is well tolerated in most cancer patients as shown in several phase I clinical trials (10). 17-AAG–mediated inhibition of Hsp90 results in degradation of RIP and IκB kinase (IKK) β, which in turn blocks tumor necrosis factor–induced NF-κB activation in the human cervical cancer cell line HeLa and Hodgkin’s lymphoma cells (11, 12). Moreover, Zaggag et al. (13) showed that nontoxic concentrations of geldanamycin decreased migration of U87MG, LN229, and U251MG glioma cells and reduced phosphorylation of FAK in vitro. In the lung cancer cell line...
A549, IKKβ degradation and NF-κB inhibition could be induced by 17-AAG and 17-AAG synergistically potentiated tumor necrosis factor–induced lung cancer cell death by blocking the NF-κB pathway (14, 15).

Here, we investigated the possible signaling pathways involved in HA-induced, NF-κB–mediated MMP-9 expression in glioma cells and tested if 17-AAG blocks these pathways. Our results suggest that HA engages the NF-κB pathway by activating IKK via FAK phosphorylation, leading to phosphorylation and degradation of IkBα and NF-κB activation, which ultimately triggers MMP-9 secretion. Additionally, we show that 17-AAG potently suppresses HA-induced cell migration, invasion, and NF-κB–mediated MMP-9 secretion by blocking the FAK/IKK/IkBα/NF-κB signaling pathways in glioma cells. These results identify FAK-induced NF-κB signaling as a novel therapeutic target in glioma.

Results

HA Induces Nuclear Translocation of the NF-κB Subunit p65

To determine the time dependence of HA-induced nuclear translocation of p65, U251MG cells were treated with 200 μg/mL HA in basal medium for 0 to 6 hours at 37°C. Next, nuclear and cytoplasmic fractionation were prepared from the cells. In the untreated control cells, p65 primarily localized to the cytoplasm at all time points. Following HA treatment, p65 translocated to the nucleus in a time-dependent manner, and by the 3-hour time point, it was predominantly localized to the nucleus (Fig. 1A). Tubulin and Ref-1 were used as markers for the cytoplasmic and nuclear fractions, respectively. In a separate series of experiments, cells were fixed at various time points after stimulation, incubated sequentially with anti-p65 (primary) and FITC-conjugated anti-rabbit IgG (secondary) antibodies, and analyzed by immunofluorescence confocal microscopy. In untreated cells, the majority of p65 staining was cytoplasmic. One hour after HA stimulation, initial nuclear accumulation of p65 was observed, and at the 3-hour time point, the localization of p65 was almost entirely nuclear (Fig. 1B), consistent with the results of the Western blotting. Taken together, these results show that HA exposure results in nuclear translocation of the NF-κB subunit p65 in U251MG cells.

HA Induces NF-κB Transcriptional Activity

Next, we did a luciferase reporter gene assay to determine whether the HA-induced cell invasion is related to modulation of NF-κB transcriptional activity. U251MG cells were transiently transfected with a NF-κB–responsive luciferase reporter construct and subsequently stimulated with increasing
concentrations of HA (50-200 μg/mL). Luciferase activity was measured in cell lysates and showed a dose-dependent increase, suggesting that HA stimulates NF-κB transcriptional activity (Fig. 1C).

**HA Exposure Results in Increased IKK Activity and IκBα Phosphorylation**

Next, it was determined if HA-induced NF-κB activation was due to phosphorylation and subsequent degradation of IκBα. Cells were treated with HA (200 μg/mL) for 0 to 120 minutes and lysed, and the lysates were analyzed by Western blotting with an anti-phospho-IκBα antibody. The results show that HA exposure increased the amount of phospho-IκBα after 30 minutes and the increase continued until 120 minutes (Fig. 2A). Reprobing the blots with an anti-IκBα antibody revealed that HA-induced IκBα degradation reached a maximum at 120 minutes (Fig. 2A, middle). In additional experiments, HA-treated cell lysates were used for immunoprecipitation with an anti-IκBα antibody followed by Western blotting with a phosphoserine antibody. There was no phospho-IκBα–specific band in the HA-treated sample, suggesting that HA exposure leads to phosphorylation of IκBα but not IκBα in these cells (data not shown). Previous reports have shown that IKK plays a major role in cytokine-induced phosphorylation of the IκBα serine residues 32 and 36 (16, 17). Therefore, we tested if HA changes the phosphorylation status of IκBα by modulating the activity of IKK. Cells were treated with HA for 30 minutes, lysed, and immunoprecipitated with an anti-IKKα/β antibody. Half of each immunoprecipitate was used to assay for kinase activity using recombinant IκBα as substrate. The other half was analyzed by Western blotting using an anti-IKKα/β antibody. An isotope-labeled, phospho-IκBα–specific band was detected in the HA-treated cell lysates, showing that HA stimulates IKK kinase activity (Fig. 2B, top). However, HA exposure had no effect on the IKKα/β protein level (Fig. 2B, bottom). These results show that HA exposure increased IKK activity.

**FAK is a critical intermediate in HA-induced, NF-κB–dependent MMP-9 secretion in U251MG cells**

FAK is a critical intermediate in HA-induced, NF-κB–dependent MMP-9 secretion in U251MG cells (6). The results shown in Figs. 1 and 2 show that HA is a potent activator of NF-κB, one of the transcription factors necessary for activation of MMP-9 gene expression. Therefore, we determined the role of FAK activation in HA-induced activation of NF-κB. As shown in Fig. 3A, FAK was activated within 5 minutes of HA treatment. To further confirm that FAK is an intermediate in the pathway that links HA exposure to NF-κB activation, endogenous FAK expression was silenced in U251MG cells using small interfering RNAs (siRNA). Robust FAK-specific knockdown was confirmed by Western blotting (Fig. 3B) and resulted in reduced HA-mediated NF-κB nuclear translocation compared with cells transfected with a control (scrambled) siRNA (Fig. 3C). To determine whether the FAK knockdown was associated with a decrease in NF-κB–dependent transcription, we used the NF-κB–responsive reporter gene assay. As shown in Fig. 3D, FAK knockdown diminished HA-induced NF-κB activity. To show FAK involvement in HA-induced MMP-9 secretion, we did zymography analysis and found that silencing FAK diminished HA-induced MMP-9 secretion (Fig. 3E). Furthermore, treatment with the NF-κB inhibitor BAY 11-7082 diminished MMP-9 secretion, indicating that NF-κB activation is essential for HA-induced MMP-9 secretion. Taken together, these results indicate that FAK is a critical mediator of HA-induced, NF-κB–dependent MMP-9 secretion in U251MG cells.

**17-AAG Treatment/Exposure Decreases FAK Phosphorylation in U251MG Cells**

FAK potentially plays an important role in glioma invasion, and we hypothesized that an agent that inhibits HA-induced FAK phosphorylation would inhibit MMP-9 secretion and invasion of glioma cells. To test this hypothesis, we used the geldanamycin derivative 17-AAG, which has been shown to block the NF-κB pathway. U251MG glioma cells were incubated with or without different concentrations of 17-AAG.
for 45 minutes and then treated with HA for 10 minutes. 17-AAG treatment resulted in a dose-dependent decrease in phospho-FAK (Fig. 4), indicating that 17-AAG inhibits FAK phosphorylation in HA-treated U251MG cells.

17-AAG Suppresses HA-Induced Nuclear Translocation of p65 and NF-κB Transactivation

To test if 17-AAG could inhibit HA-induced p65 translocation, U251MG cells were treated with 17-AAG for 45 minutes followed by treatment with HA for 3 hours. Pretreatment with 17-AAG prevented HA-induced nuclear translocation of p65 (Fig. 5A). In parallel experiments, cells were analyzed by immunofluorescence confocal microscopy. As above, HA exposure resulted in translocation of p65 from the cytoplasm to the nucleus. However, pretreatment with 0.1 μmol/L 17-AAG inhibited nuclear translocation of p65 following HA exposure (Fig. 5B). Together, these data indicate that 17-AAG suppresses HA-induced nuclear translocation of the NF-κB subunit p65 in U251MG cells. Next, the effect of 17-AAG on NF-κB transcriptional activity was monitored using the luciferase reporter gene assay. Cells were pretreated with different doses of 17-AAG (0-0.1 μmol/L), exposed to HA, and assayed for luciferase activity. The results show that 17-AAG inhibited HA-induced NF-κB transcriptional activity in a dose-dependent manner (Fig. 5C).

17-AAG Suppresses HA-Stimulated IKK Activity and IκBα Phosphorylation

Next, it was determined if 17-AAG suppresses HA-induced NF-κB activation through phosphorylation and degradation of IκBα. U251MG cells were treated with different concentrations of 17-AAG (0-0.1 μmol/L) for 45 minutes and then

FIGURE 3. FAK is the critical mediator of HA-induced, NF-κB-dependent MMP-9 secretion in U251MG cells. A, U251MG cells were starved by culturing in serum-free medium for 24 h and then treated with 200 μg/mL HA. Western blotting was done on cell lysates collected at the indicated times using an anti-phospho-FAK antibody. B, Western blotting of U251MG cell lysates after transfection with FAK-specific (50 nmol/L) or control siRNAs. C, Top, cytoplasmic (C) and nuclear (N) extracts from untreated cells and cells treated with 200 μg/mL HA for 3 h were immunoblotted with a rabbit polyclonal anti-p65 antibody; bottom, cells grown on glass slides were treated with 200 μg/mL HA for 0 to 3 h, fixed, incubated sequentially with anti-p65 (primary) and FITC-conjugated anti-rabbit IgG (secondary) antibodies, and analyzed by immunofluorescence confocal microscopy. D, A NF-κB-responsive luciferase reporter assay of siRNA-transfected cells cultured in the presence or absence of HA for 12 h. Each experiment was repeated thrice with similar results. Columns, mean; bars, SD. *, P < 0.005 versus control; **, P < 0.005 versus HA alone by unpaired t test. E, FAK siRNA-transfected U251MG cells were treated with 200 μg/mL HA (top) with or without 45-min pretreatment with different doses of BAY 11-7082 (bottom). The conditioned media were collected, and MMP-2 and MMP-9 activities were analyzed by gelatin zymography.
stimulated with HA for 120 minutes. The results show that 17-AAG inhibited HA-induced IκBα phosphorylation and degradation in these cells (Fig. 6A). Similarly, in kinase assays, IKK activity was undetectable in both untreated and 0.5 μmol/L 17-AAG–treated cells, indicating that HA-induced IKK activity was blocked by 17-AAG (Fig. 6B). Taken together, these data show that 17-AAG effectively suppresses HA-induced IκK/IκBα signaling.

17-AAG Suppresses HA-Induced In vitro Cell Migration and ECM Invasion

We previously showed that HA enhances cell migration and ECM invasion in a dose-dependent manner. In this study, we assessed the effect of 17-AAG on HA-induced cell migration and ECM invasion using the modified Boyden chamber method. After pretreatment with different concentrations of 17-AAG (0-0.1 μmol/L), cells were placed in the upper chamber followed by addition of HA (200 μg/mL) to the lower chamber. Figure 7A shows how 17-AAG suppressed HA-induced cell migration in a dose-dependent manner. The number of cells migrating to the lower chamber in the absence of HA was used to define maximum (i.e., 100%) migration. There was a dramatic reduction in HA-induced ECM invasion in cells pretreated with different concentrations of 17-AAG (Fig. 7B).

17-AAG Blocks HA-Induced MMP-9 Expression

Next, we determined whether 17-AAG reduced HA-induced MMP-9 secretion. U251MG cells were exposed to different doses of 17-AAG (0-0.1 μmol/L) for 45 minutes followed by treatment with 200 μg/mL HA for 12 hours. The conditioned media were collected and the levels of MMP-9 were determined by gelatin zymography. Consistent with our previous results (see Fig. 3), HA treatment resulted in significantly increased MMP-9 secretion (Fig. 8A, lanes 1 and 2), and 17-AAG blocked the HA-induced MMP-9 secretion (lanes 3-5). Next, the effect of 17-AAG on MMP-2 and MMP-9 expression was determined by reverse transcription-PCR analysis (Fig. 8B).

FIGURE 4. 17-AAG decreases FAK phosphorylation in glioma cells. U251MG glioma cells were incubated for 45 min in increasing concentrations of 17-AAG and then treated with 200 μg/mL HA for 10 min. The lysates were subjected to Western blotting with a phospho-FAK antibody. The data represent three independent experiments.

FIGURE 5. 17-AAG blocks HA-induced NF-κB p65 subunit nuclear localization (A and B) and NF-κB transactivation (C) in U251MG cells. A. Cytoplasmic (C) and nuclear (N) extracts from cells treated with 200 μg/mL HA for 3 h, with or without 45-min pretreatment with different doses of 17-AAG, were immunoblotted with a rabbit polyclonal anti-p65 antibody. B. Cells grown on glass slides were treated as in A, fixed, incubated sequentially with an anti-p65 (primary) and a FITC-conjugated anti-rabbit IgG (secondary) antibody, and analyzed by immunofluorescence confocal microscopy. DAPI, 4,6-diamidino-2-phenylindole. C. Luciferase reporter gene assay. Cells transiently transfected with a NF-κB–responsive luciferase reporter construct were pretreated with different doses of 17-AAG for 45 min and stimulated with HA for 24 h. The cells were lysed and luciferase activities were measured. Each experiment was repeated thrice. Columns, mean; bars, SD. *, P < 0.005 versus control; **, P < 0.005 versus HA alone by unpaired t test.
MMP-9 secretion by glioma cells. MMP expression is a hallmark of tumor invasion, as exemplified by the HA-induced secretion of ECM-modifying MMPs is an important determinant of tumor invasion, and the surrounding brain and angiogenic activity associated with the development of glioblastoma multiformes. In many cell types, overexpression of FAK leads to constitutive activation of NF-κB and up-regulation of survival genes. In human leukemic HL-60 cells, for example, FAK overexpression protects the cells from apoptosis caused by oxidative stress, etoposide phosphate, and ionizing radiation (26, 27). Our previous reports showed that FAK is important in HA-induced secretion of MMP-9 and suggested that FAK may be important in linking invasion and malignant progression.

**Discussion**

In this study, we characterized the mechanism of HA-induced, NF-κB-mediated MMP-9 secretion in U251MG glioma cells. Our results show that HA induces IκBα phosphorylation by increasing the activity of IKK. Ultimately, this leads to phosphorylation and degradation of IκBα and to NF-κB nuclear translocation and transcriptional activation. Moreover, inhibition of FAK phosphorylation by 17-AAG resulted in decreased HA-induced NF-κB activation and MMP-9 secretion. These results indicate that HA acts through FAK to activate NF-κB and stimulate MMP-9 secretion. Therefore, we propose that 17-AAG is a therapeutic candidate for the treatment of highly invasive gliomas.

Glioma invasion seems to depend on both cellular properties and ECM composition. The expression and secretion of ECM-modifying MMPs is an important determinant of tumor invasion, as exemplified by the HA-induced MMP-9 secretion by glioma cells. MMP expression is regulated at the transcriptional level and the MMP promoters contain putative binding sites for activator protein-1 and/or NF-κB (18, 19), which have been shown to regulate MMP-9 promoter activity. The NF-κB transcription factor family regulates numbers of genes involved in diverse cellular processes, including inflammation, immune response, cell proliferation, and apoptosis (20, 21). NF-κB activity is regulated by the endogenous inhibitor IκBα and interaction with IκBα blocks the nuclear localization of NF-κB, keeping it sequestered in the cytoplasm. Following an appropriate stimulus, IκBα is phosphorylated at Ser32 and Ser36, leading to ubiquitin-dependent degradation. Free NF-κB then translocates to the nucleus where it activates transcription of its target genes, including MMPs (22). Phosphorylation of IκBα is mediated by the multisubunit kinase complex IKKα/β, which itself is regulated by several upstream kinases, including the NF-κB–inducing kinase, a phosphatidylinositol 3-kinase 3-kinase, and mitogen-activated protein kinase kinase kinase IKK, which leads to degradation of IκBα and to NF-κB nuclear translocation and transcriptional activation in glioma cells.

FAK is up-regulated in glioblastoma multiformes, particularly in invasive zones, and hyperplastic vessels show strong FAK immunoreactivity (13, 25). These observations are consistent with a dual role for FAK in the invasion of glioma cells into the surrounding brain and in the angiogenic activity associated with the development of glioblastoma multiformes. In many cell types, overexpression of FAK leads to constitutive activation of NF-κB and up-regulation of survival genes. In human leukemic HL-60 cells, for example, FAK overexpression protects the cells from apoptosis caused by oxidative stress, etoposide phosphate, and ionizing radiation (26, 27). Our previous reports showed that FAK is important in HA-induced secretion of MMP-9 and suggested that FAK may be important in linking invasion and malignant progression.

**Supplementary Figure 6.** 17-AAG blocks HA-induced IκBα phosphorylation (A) and IKK activity (B). A, U251MG cells were pretreated with different doses of 17-AAG for 45 min and then stimulated with 200 μg/mL HA for 120 min. Western blotting was done on cell lysates using an anti-phospho-IκBα or a phospho-IKK antibody. To verify expression of IκBα or equal loading, the blots were probed with antibodies against IκBα or actin. B, Cells were treated with 200 μg/mL HA with or without 45-min pretreatment with different doses of 17-AAG. Top, cell lysates were immunoprecipitated with an anti-IKKα/β antibody. To verify expression of IKKα/β as a substrate; bottom, IKKα/β was identified by Western blotting. Each experiment was repeated thrice and representative results are shown.
HA-induced activation of NF-κB to MMP-9 gene expression in glioma cells (6). In this report, we confirm this link using FAK knockdown, which leads to reduced NF-κB activation, MMP-9 secretion, and invasion. This suggests that FAK is involved in modulating HA-induced MMP-9 secretion and invasion.

Next, we used 17-AAG to further document the central role of FAK and the NF-κB pathway in HA-induced MMP-9 secretion and invasion. This pharmacologically safe, relatively nontoxic compound has anti-inflammatory and anticarcinogenic properties and was previously shown to potentiate tumor necrosis factor–induced lung cancer cell death by blocking the NF-κB pathway (15). We found that pretreatment of cells with 17-AAG inhibited HA-induced NF-κB activation, blocking p65 nuclear translocation. This suggests that 17-AAG inhibits HA-induced NF-κB activity by suppressing HA-induced FAK phosphorylation and IKK-dependent IκBα phosphorylation, consistent with the molecular mechanism revealed by the FAK RNA interference experiments.

Glioblastoma infiltrates the normal central nervous system by the interaction with ECM (28). It has been shown that HA-rich environment in brain ECM significantly correlates with cancer aggressiveness (29-33). Glioma cells express high levels of the HA receptors CD44 and RHAMM. Both of these HA receptors were known to play an important role for glioma migration and invasion (4, 5, 34). Therefore, blocking of HA-induced signaling pathways could reduce tumor aggressiveness and would provide a new anticancer strategy. Among anticancer agents, we showed 17-AAG as a strong inhibitor for HA-induced glioma invasion. Although several groups have reported 17-AAG as anticancer drug in glioma, we showed for the first time that 17-AAG inhibits HA-induced signaling pathway by blocking HA-induced FAK activation. This result, in fact, agrees with the finding that 17-AAG decreased the growth of glioma cells (13, 35). Our results expanded the understanding of 17-AAG on HA-mediated malignant mechanisms in glioma cells.

In summary, we have shown that HA induces NF-κB activation by stimulating IKK activity and promoting IKK-dependent phosphorylation and degradation of IκBα. Additionally, we propose a critical role for FAK in mediating HA-induced NF-κB activation and increased MMP-9 expression, cell migration, and ECM invasion. Importantly, 17-AAG inhibits FAK activation, IκBα phosphorylation, and degradation, and it is an effective inhibitor of HA-induced cellular changes. This suggests that 17-AAG represents a potential therapeutic candidate for cancers such as glioma.

Materials and Methods

Reagents and Cell Culture

HA and 17-AAG were purchased from Sigma Chemical Co. and reconstituted in DMEM (Life Technologies, Inc.). Rabbit polyclonal anti-NF-κB p65, anti-Ref-1, mouse anti-α-tubulin and goat polyclonal anti-actin antibodies, and IκBα recombinant protein were purchased from Santa Cruz Biotechnology. The rabbit polyclonal anti-IκBα, anti-IKKα/β, and monoclonal phospho-specific anti-IκBα were purchased from Cell Signaling Biotechnology. The FITC-conjugated goat anti-rabbit IgG was obtained from Molecular Probes, Inc. Human glioma cell lines U251MG, U373MG, and U87MG were obtained from the American Type Culture Collection. Glioma cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Nuclear and Cytoplasmic Extracts and Western Blot Analysis

The cells were treated either with HA (200 μg/mL) for a period of 0 to 6 h or with 17-AAG for 45 min followed by HA (200 μg/mL) for 3 h at 37°C. The cytoplasmic and nuclear extracts were prepared using CelLytic NuCLEAR Extraction kit...
from Sigma according to the manufacturer’s protocol. The nuclear
and cytoplasmic extracts (30 μg) were resolved by SDS-PAGE and
then electrotransferred to the nitrocellulose membrane (Schleicher
& Schuell). Western blot analysis was done as described by Park
et al. (6). Immunoblots were visualized using the enhanced
chemiluminescence Plus Western blotting detection system
(Amersham) according to the manufacturer’s protocol.

Fluorescence Microscopy

The U251MG cells were grown to 50% to 70% confluency
onto 12-well dishes containing 18-mm slide glasses and then
treated with 200 μg/mL HA for a period of 0 to 3 h. In separate
experiments, the cells were pretreated with 17-AAG (50 μmol/L)
for 45 min and then treated with 200 μg/mL HA for 3 h at 37°C.
Cells were washed in ice-cold PBS and fixed in 3.7%
formaldehyde. After permeabilization with 0.2% Triton X-100,
cells were blocked for 1 h in blocking solution containing 2%
bovine serum albumin, washed, incubated in 1:300 rabbit
polyclonal anti-p65 antibody for 1 h, and then washed again.
Cells were then incubated in 1:500 Alexa Fluor 488–labeled
goat anti-rabbit IgG antibody and washed. Preparations were
examined and photographed using confocal microscopy (Zeiss).

Luciferase Assay

U251MG cells were plated onto 24-well plates and incubated
for 20 h at 37°C in 5% CO₂, pGL3 vector DNA (0.2 μg)
containing the MMP-2 or MMP-9 promoter regions, or similar
amounts of NF-κB reporter vectors (purchased from Clontech
Co.), was cotransfected into U251MG cells with the pRL-SV40
vector using the Effectene Transfection Reagent (Qiagen) for
20 h. Then, cells were treated with 200 μg/mL HA alone for 3 h or
with various concentrations of 17-AAG. Luciferase activity was
measured using the Dual-Luciferase Reporter Assay System
(Promega) according to the manufacturer’s protocol.

IKK Kinase Assay

The semiconfluent cells were treated either with 200 μg/mL
HA alone for 30 min or with various concentrations of 17-AAG
for 45 min and then treated with 200 μg/mL HA for 30 min at
37°C. Cell lysates were clarified by centrifugation, and equal
amounts of the lysate proteins (500 μg) were immunoprecipitated
with an antibody against anti-IKKα/β and conjugated to protein
A/G plus-agarose (Santa Cruz Biotechnology). The immune
complexes were washed twice with PBS (pH 7.4) containing 1%
NP40 and 100 μmol/L Na₃VO₄ and twice with kinase buffer
from Cell Signaling Biotechnology. Half of the immunoprecipi-
tated samples were incubated with recombinant IκBα (4 μg) in
kinase buffer containing 10 μmol/L ATP and 5 μCi [γ-³²P]ATP
at 30°C for 1 h. The reaction was terminated by addition of SDS
sample buffer, and the samples were subjected to 10% SDS-
PAGE. Phospho-IκBα was visualized by autoradiography. The
remaining half of the immunoprecipitated samples were
subjected to SDS-PAGE and analyzed by Western blot analysis
using anti-IKKα/β antibody.

Transfection

The siRNA against FAK was purchased from Santa Cruz
Biotechnology, and transfections of cells with siRNA molecules
(50 nmol/L) were done using HiperFect transfection reagent
(Qiagen) as described by the manufacturer.
Invasion and Migration Assays

U251MG invasion and migration assays were done using modified Boyden chambers with polycarbonate Nucleopore membrane (Corning) as described previously (6). Invasiveness was determined by counting cells in five microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field.

Gelatin Zymography

Glioma cells (~ 70-80% confluent) were washed and replenished with serum-free DMEM. Cells were then pretreated with various concentration of 17-AAG for 45 min followed by stimulation with 200 μg/mL HA for 24 h. The volume of conditioned medium was normalized by cell number. The enzymatic activity of electrophoretically separated gelatinolytic enzymes in the conditioned medium of glioma cells was determined by gelatin zymography as described previously (6). Zones of gelatinolytic activity were detected as clear bands against a blue background.

Reverse Transcription-PCR

Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer’s protocol. First-strand cDNA was generated using the Reverse Transcription System kit (Promega) according to the manufacturer’s protocol. PCR amplification was carried out with the following primers: MMP-2, 5′-ATCTGGTGTCTCCCTTACGG-3′ and 5′-GTGACGTATGTCGCCACAC-3′; MMP-9, 5′-AGTGGTGTTGCCTCGGAGGCAC-3′ and 5′-TACATGAGCCGTCGCCAAC-3′. PCR was done using the PCR Master kit (Roche) according to the manufacturer’s protocol.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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