Hyaluronic Acid Induces Osteopontin via the Phosphatidylinositol 3-Kinase/Akt Pathway to Enhance the Motility of Human Glioma Cells

Mi-Suk Kim,1,3 Myung-Jin Park,2 Eui-Jung Moon,1 So-Jeong Kim,1 Chang-Hun Lee,1 Heon Yoo,1 Sang-Hoon Shin,1 Eun-Sook Song,3 and Seung-Hoon Lee1

1Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi, Korea; 2Laboratory of Cell Biology, Korea Institute of Radiological and Medical Sciences, and 3Department of Life Science, Sookmyung Women's University, Seoul, Korea

Abstract

Hyaluronic acid (HA) binds to cell-surface receptors such as CD44, and seems to be involved in cell adhesion, motility, and tumor progression in brain. To identify gene expression changes that are initiated by HA, we explored human cytokine arrays in U87MG glioma cells and identified osteopontin, a secreted matrix protein, as a transcriptional target of HA. Interestingly, expression of osteopontin was induced by HA in glioma cells lacking functional PTEN, a tumor suppressor gene (U87MG, U251MG, and U373MG), but not in wild-type (wt)-PTEN-harboring cells (LN18 and LN428). To confirm the role of PTEN, adenoviral (Ad)-wt-PTEN was used to induce ectopic expression of wt-PTEN in U87MG cells, leading to reduced HA-mediated osteopontin induction. Reciprocally, transfection with dominant-negative Akt repressed HA-induced osteopontin expression. Furthermore, HA promoted the motility of glioma cells, and down-regulation of induced osteopontin activity via a neutralizing anti-osteopontin antibody repressed HA-induced motility in vitro. Together, these results strongly suggest that induction of osteopontin expression by HA is dependent on activation of the phosphatidylinositol 3-kinase/Akt pathway. Furthermore, our data indicate that PTEN can effectively modulate the expression of osteopontin, and HA-induced osteopontin plays an important role in the motility response induced by HA in human glioma cells. (Cancer Res 2005; 65(3): 686-91)

Introduction

Glioblastoma, a severe type of primary brain tumor, is often lethal due to local invasion into the brain parenchyma (1). Hyaluronic acid (HA), a principal glycosaminoglycan found in the extracellular matrix of human brain, is distributed in the white matter fiber tracts that form the most frequent route of glioma dissemination (2). HA facilitates cell adhesion, cell motility, cellular proliferation and tumor progression through interactions with receptors such as CD44 and RHAMM (3). Several studies have suggested that HA plays an important role in glioma cell motility and invasion (4, 5), but despite recent advances in understanding the proximal signal transduction pathways activated by HA, the molecular mechanisms of HA-associated motility and invasion are not well understood. Thus, identification of HA-regulated genes is essential to clarifying the molecular basis of glioma invasion and motility. Accordingly, we explored transcriptional responses to HA in glioma cells using the human cytokine array method, and found that the transcription of osteopontin was highly induced by HA. Osteopontin, an arginine-glycine-aspartate cell binding sequence-containing phosphorylated glycoprotein (6), is also known as gene-secreted phosphoprotein 1 (7) and early T cell activation 1 (8). Interestingly, HA-induced osteopontin expression was dependent on PTEN status in various glioma cells. PTEN, a tumor-suppressor gene located on human chromosome 10q23.3, interacts with the extracellular matrix and inhibits cell migration, spreading, and focal adhesion (9). Recently, Sugatani et al. (10) reported that PTEN regulated osteopontin-stimulated signal transduction during osteoclast differentiation and cell motility. Taken together, these studies indicate that PTEN regulates both osteopontin induction and osteopontin-stimulated signal transduction. Osteopontin and its cell surface receptors, CD44 (11) and the α-containing integrins (12, 13), have been implicated in the progression of cancer toward an invasive-metastatic phenotype (14–16). Therefore, we hypothesized that osteopontin induction is a main event in the motility and invasive growth responses induced by HA in glioma cells, and further conjectured that PTEN regulates glioma motility by reducing osteopontin expression. Accordingly, we examined the effect of HA on the expression of osteopontin, role of PTEN in these events, and role of osteopontin in HA-induced cell motility. Our results indicate that HA specifically promotes osteopontin transcription via the PI3-K/Akt/mTOR pathway. In addition, PTEN can effectively modulate the expression of osteopontin by HA, and stimulated osteopontin seems to play an important role in HA-induced motility in human glioma cells. Taken together, these results suggest that osteopontin may be a potentially useful target for inhibition of motility in glioma cells.

Materials and Methods

Reagents and Cell Culture. HA was purchased from Sigma Chemical Co. (St. Louis, MO) and reconstituted in DMEM (Life Technologies, Inc., Grand Island, NY). rh-OPN and the neutralizing osteopontin antibody were obtained from R&D Systems, Inc. (McKinley Place, NE). The specific signaling inhibitors (PD98059, LY294002, and wortmannin) were purchased from Calbiochem (La Jolla, CA). Polyclonal antibody against osteopontin was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA) and polyclonal antibody to phosphorylated Akt and rapamycin were obtained from Cell Signaling Biotechnology (Beverly, MA). Human glioma cell lines U87MG, U373MG, and U251MG were obtained from American Type Culture Collection, LN428 and LN18 glioma cells were kindly provided by Dr. Frank Funari (Ludwig Institute for Cancer Research, La Jolla, CA). Glioma cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO₂-humidified atmosphere.

Requests for reprints: Seung-Hoon Lee, National Cancer Center, 809 Madu-dong, Ilsan-gu, Goyang, Gyeonggi 411-764, Korea. Phone: 82-31-920-1660; Fax: 82-31-920-1520; E-mail: mshlee@ncc.re.kr.

©2005 American Association for Cancer Research.
Hydration Acid Up-Regulates Osteopontin Expression

RNA Extraction and Cytokine cDNA Microarray Analysis. Total RNA was isolated from U87MG cells treated with or without 200 μg/mL HA for 24 hours using the RNeasy kit (Qiagen, Valencia, CA). Poly(A)+ RNA was isolated by two rounds of purification on Oligotex mRNA Purification System (Qiagen). The cytokine expression patterns of cells stimulated with HA or with control medium were compared by using human Cytokine Expression Array (R&D Systems) containing 847 different cDNAs, according to the manufacturer's protocol. The microarray image files were analyzed using Quantity One software from Bio-Rad Laboratories (Hercules, CA). Signal values on a spreadsheet file were entered into Microsoft Excel for subsequent normalization averaging and comparative analysis. Normalized values for individual from two microarrays (HA-stimulated and control) were compared to determine the relative mRNA expression of each gene.

Western Blot Analysis. Protein lysates (70 μg) from cells treated with or without 200 μg/mL HA for 24 hours were used to determine the effect of HA on osteopontin expression. Western blot analysis was done as described by Park et al. (17). Immunoblots were visualized using the ECL Plus Western blotting detection system (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. Bands were quantified by densitometry with the help of an EDAS290 device (Kodak, Japan).

Reverse transcription-PCR. Total RNA was isolated from U87MG cells treated with or without 200 μg/mL HA for 24 hours using the RNeasy kit (Qiagen), and first-strand cDNA was generated using the manufacturer's protocol. PCR was done using osteopontin-specific primers (OPN-S, 5'-ACC ATG AGA ATT GCA GTG ATT TG3'-3' and OPN-AS, 5'-ATC AGT GAC CAG TTC ATC AGA TTC-3') and the PCR Master kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. The PCR conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute. The PCR products were resolved in a 2% agarose gel containing ethidium bromide. Bands were quantified by densitometry with the help of an EDAS290 device (Kodak, Japan).

Fluorescence Microscopy. A standard immunostaining procedure was carried out to observe endogenous osteopontin expression. Briefly, cells were grown to 50% to 70% confluency onto 12 wells containing 18-mm² slide glasses. After incubation in the presence or absence of 200 μg/mL HA for 24 hours, 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 hour in blocking solution containing 2% bovine serum albumin, washed, incubated in 1:300 anti-osteopontin antibody for 1 hour and then washed again. Cells were then incubated in 1:500 Alexa Fluor 488-labeled Donkey anti-Goat IgG antibody (Molecular Probes, Inc., Eugene, OR), and washed. Preparations were examined and photographed using a camera-equipped fluorescence microscope (Olympus, Tokyo, Japan).

Preparation and Infection of Recombinant Adenovirus. To construct Ad-PTEN vectors, 1.2-kb fragments of wild-type or C124S mutant PTEN cDNAs were cloned into the KpnI and XhoI sites of pShuttle-cytoplasmalovirus vector (AdEasy Adenoviral Vector System; Stratagene, La Jolla, CA). PTEN cDNAs were kindly provided by Dr. Young E. Whang (University of North Carolina School of Medicine, Chapel Hill, NC). Recombinant adenovirus expressing PTEN was prepared as previously described (17). The recombinant adenoviruses were infected into U87MG cells with 50 plaque-forming units per cell in serum-free medium for 90 minutes at 37°C. The following day, transfected cells were incubated for 24 hours with 200 μg/mL HA. Cells were then washed with cold PBS and lysed, and PTEN expression was determined by Western blot analysis as above.

Plasmid Constructs and Cell Transfection. Hemagglutinin-tagged wild-type (HA-Akt) or dominant-negative Akt (HA-DN-Akt) expression plasmids were provided by Dr. Richard A. Roth (Stanford University School of Medicine, Stanford, CA). U87MG cells were transfected with HA-Akt and HA-DN-Akt in triplicate dishes and in three independent experiments by using the Effectene transfection reagent (Qiagen) following the supplier's instructions.

Motility Assay. Cells (2 × 10⁵) suspended in serum-free DMEM containing 0.1% bovine serum albumin were seeded in triplicate into the upper part of a transwell filter (diameter 6.5 mm, pore size 8 μm; Costar,

Figure 1. U87MG cells respond to HA by increasing osteopontin mRNA and protein. A, time course reverse transcription-PCR analysis of osteopontin mRNA expression. Total RNA was isolated from U87MG cells treated with or without HA (200 μg/mL) for different times (as indicated) and used for reverse transcription-PCR. B, Western blot analysis of osteopontin protein expression after different durations of HA (200 μg/mL) stimulation (as indicated). Total proteins were extracted from U87MG cells treated with or without HA, and subjected to Western blotting. Columns, relative band intensities measured by densitometric analysis. Each experiment was reproduced thrice with similar results and typical data are shown.
Cambridge, MA) and the lower compartment was filled with serum-free DMEM containing 0.1% bovine serum albumin with or without HA (200 μg/mL) or rh-OPN (1 μg/mL). After incubation for 24 hours at 37°C, nonmigrated cells of the upper surface of the filter were wiped with a cotton swab, and migrated cells in the lower surface of the filter were fixed and stained with Diff-Quick Kit. Motility rate was determined by counting cells in at least 20 random fields per well, and the extent of motility was expressed as the average number of cells per microscopic field.

Results
Hyaluronic Acid Up-regulates Osteopontin Expression in Glioma Cells. We used human cytokine cDNA array technology to investigate transcriptional changes initiated by HA in U87MG glioma cells. Several genes including transforming growth factor-β, BMP-2, IGFBP-8 and osteopontin were up-regulated by more than 2-fold. In contrast, MIF, PIN, and PTN exhibited reduced expression (data not shown). Among these genes, we focused on the highly increased gene, OPN, as a major HA transcriptional target. To validate and further characterize regulation of osteopontin expression, we did time course reverse transcription-PCR and Western blot analysis on U87MG cells stimulated with HA (Fig. 1A and B). Osteopontin induction was detectable after 8 hours of treatment with 200 μg/mL HA and was increased 6.5-fold at 24 hours. To confirm that HA-mediated induction of osteopontin expression is a universal phenomenon in glioma cells, we examined this response in several glioma cell lines. Interestingly, HA markedly induced expression of osteopontin in glioma cell lines harboring mutant PTEN (U87MG, U251MG, and U373MG) although induction range is variable, but not in those harboring wild-type PTEN (LN18 and LN428; Fig. 2A-C). We confirmed the status of PTEN competence of each cell line by detection of HA-induced Akt phosphorylation. Akt activation was observed only in PTEN mutant cell lines (data not shown). These results prompted us to examine the involvement of PTEN in HA-induced osteopontin expression.

PTEN Blocks Hyaluronic Acid–Induced Osteopontin Expression in U87MG Cells. To investigate the role of PTEN, we infected U87MG cells with Ad-wt-PTEN and Ad-C124S (both protein and lipid phosphatase–deficient)-PTEN. The expression

Figure 2. The effect of HA on expression of osteopontin in various glioma cell lines. A, the various glioma cell lines were incubated with or without HA (200 μg/mL) for 24 hours. Total RNA were extracted and subjected to reverse transcription-PCR analysis. B, Western blot analysis of osteopontin expression under the same conditions. C, fluorescence microscopic analysis of osteopontin expression. Cells were grown to 50% to 70% confluency on slides, and treated with or without HA (200 μg/mL) for 24 hours. Standard immunostaining procedures were used to visualize endogenous osteopontin expression. Each experiment was reproduced thrice with similar results and typical data are shown.
and functional activity of the expressed PTENs were confirmed by Western blot analysis using PTEN and p-Akt antibody, respectively. The results of further Western blot analysis and reverse transcription-PCR showed that osteopontin expression was increased in Ad-LacZ and Ad-C124S-PTEN-infected U87MG cells following HA treatment, whereas Ad-wt-PTEN-infected cells showed suppressed HA-induced osteopontin expression (Fig. 3A and B). These data indicate that transient expression of wt-PTEN efficiently inhibits the induction of osteopontin by HA in functional PTEN-deficient U87MG cells.

**Hyaluronic Acid Induces Osteopontin Expression via the PI3-K/Akt/mTOR Pathway.** Because the above data strongly implicated the involvement of PTEN signaling in HA-induced osteopontin induction, we investigated the role of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway in HA-stimulated osteopontin expression through the use of specific pathway inhibitors in U87MG cells. As expected, treatment of the cells with PI3-K inhibitors (LY294002 and wortmannin), but not an ERK1/2 inhibitor (PD98059), significantly suppressed HA-induced osteopontin expression (Fig. 3C). These results show that the PI3-K pathway is involved in the HA-induced modulation of osteopontin expression in U87MG cells. Next, we investigated the role of Akt. Hemaglutinin tagged-Akt (Akt) or dominant-negative-Akt (DN-Akt) vectors were transfected into U87MG cells and expression was confirmed by Western blot analysis using an anti-hemagglutinin antibody. HA-dependent osteopontin expression was enhanced 7-fold in nontransfected cells and 11-fold in wild-type Akt overexpression cells compared with nontreated cells. But overexpression of dominant-negative Akt significantly inhibited the HA-dependent osteopontin induction (Fig. 3D). We also examined the impact of mTOR, which is downstream of Akt signaling (18), on HA-induced osteopontin enhancement. The activity of mTOR is controlled by amino acids, as well as by the hormones and growth factors that activate the Akt protein kinase. As shown in Fig. 3E, the mTOR inhibitor, rapamycin, effectively inhibited HA-induced expression of osteopontin in a dose-dependent manner. Taken together, these results show that the
PI3-K/Akt/mTOR pathway is involved in the stimulation of osteopontin production by HA, and that PTEN efficiently modulates these events in U87MG human glioma cells.

**Osteopontin Up-regulation Contributes to Hyaluronic Acid–Induced Motility.** Using the modified Boyden chamber method, we tested the contribution of osteopontin to the HA-induced *in vitro* motility of U87MG and LN428 cells. As shown in Fig. 4B and E, in the presence of 200 μg/mL of HA in the bottom chamber, U87MG cells migration increased 5-fold compared with that of untreated control cells (Fig. 4A), and treatment of U87MG cells with an osteopontin-neutralizing antibody decreased HA-dependent activation of cell motility in a dose-dependent manner (Fig. 4C and E). In contrast, exogenous addition of rh-OPN alone significantly increased the motility of U87MG cells through the membrane (Fig. 4D). In LN428 cells containing wild-type PTEN, cell motility did not change by treatment with HA or with an osteopontin-neutralizing antibody (Fig. 4F). These results suggest that activation of cell motility by HA at least partially requires expression of osteopontin in glioma cells.

**Discussion**

Even though the early downstream activation events of HA-CD44 signaling are relatively well understood, and are known to result in the stimulation of cell motility and proliferation *in vitro* (3, 4), the molecular mechanisms of this HA-induced cell motility stimulation remain poorly characterized. Accordingly, we sought to identify and functionally characterize downstream target proteins that may be involved in HA-induced cell motility and invasiveness. Here, we report for the first time that osteopontin is a major gene product induced by HA in glioma cells, and partially define the role of osteopontin in HA-induced cell motility. Our study revealed that HA up-regulated expression of osteopontin in glioma cells in a PTEN-modulated fashion.

PTEN is one of the most frequently mutated tumor suppressors in human cancer. Recently we reported that PTEN suppresses HA-induced secretion of MMP-9 and inhibits HA-induced invasion in U87MG cells, probably via dephosphorylation of FAK (17). PTEN also suppresses migration; genetic deletion of the *Pten* tumor suppressor gene promotes cell motility (19), PTEN reconstitution or overexpression inhibits cell motility in a variety of cell types (9). Mechanistically, PTEN reduces cell motility through a variety of pathways. Recent reports reveal that PTEN overexpression suppressed RANKL-mediated osteoclast differentiation and osteopontin-stimulated cell migration (10). Here, we showed that PTEN modulated cell motility by inhibiting HA-induced osteopontin expression in glioma cells.

Signals transduced by cell adhesion molecules play important roles in tumor cell attachment, motility, and invasion, whereas cell-matrix interactions are major factors in tissue remodeling, cell survival, and tumorigenesis. Osteopontin is a highly phosphorylated...
glycoprotein of the extracellular matrix (8), having an unusually wide spectrum of cell surface receptors and biological activities. By interacting with α3β1, α5β1, and αvβ5 integrins, osteopontin promotes cell attachment, spreading and motility, and more complex events like vascular remodeling, bone mineralization, and tumor metastasis (15, 20). Osteopontin also binds the cell surface hyalurionate receptor CD44 (11), a protein that has been implicated in cell-cell and cell-extracellular matrix interactions (21), and tumor cell metastasis (14, 16). Interestingly, many of the basic cellular functions affected by osteopontin are also controlled by HA. Indeed, osteopontin and HA are frequently overexpressed in a variety of malignant cells, and the expression levels of osteopontin and HA have been correlated with degrees of glioma malignancy (22, 23). Therefore, we hypothesized that osteopontin induction may be involved in HA-induced cell motility stimulation in glioma cells. Functional depletion of osteopontin by a neutralizing antibody showed that attenuation of osteopontin expression inhibited HA-induced motility in vitro, indicating that osteopontin activity is at least partially required for HA-induced cell motility. In summary, our results indicate that HA up-regulates osteopontin expression via the PI3-K/Akt/mTOR pathway. Furthermore, PTEN can effectively modulate the expression of osteopontin, and induced osteopontin contributes to HA-induced cell motility stimulation in glioma cells. Thus, inhibition of osteopontin activity may offer an alternative therapeutic strategy for reducing the motility of human glioma cells.

Acknowledgments

Received 7/7/2004; revised 11/21/2004; accepted 11/30/2004.

Grant support: National Cancer Center grant 0410052.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


Hyaluronic Acid Induces Osteopontin via the Phosphatidylinositol 3-Kinase/Akt Pathway to Enhance the Motility of Human Glioma Cells

Mi-Suk Kim, Myung-Jin Park, Eui-Jung Moon, et al.