CD44-Epidermal Growth Factor Receptor Interaction Mediates Hyaluronic Acid-promoted Cell Motility by Activating Protein Kinase C Signaling Involving Akt, Rac1, Phox, Reactive Oxygen Species, Focal Adhesion Kinase, and MMP-2*

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Hyaluronic acid (HA)2 is known to play an important role in motility of tumor cells. However, the molecular mechanisms associated with HA-promoted melanoma cell motility are not fully understood. Treatment of cells with HA was shown to increase the production of reactive oxygen species (ROS) in a CD44-dependent manner. Antioxidants, such as N-acetyl-L-cysteine and seleno-L-methionine, prevented HA from enhancing cell motility. Protein kinase C (PKC)-α and PKCδ were responsible for increased Rac1 activity, production of ROS, and mediated HA-promoted cell motility. HA increased Rac1 activity via CD44, PKCα, and PKCδ. Transfection with dominant negative and constitutive active Rac1 mutants demonstrated that Rac1 was responsible for the increased production of ROS and cell motility by HA. Inhibition of NADPH oxidase by diphenylene iodonium and down-regulation of p47^Phox^ and p67^Phox^ decreased the ROS level, suggesting that NADPH oxidase is the main source of ROS production. Rac1 increased phosphorylation of FAK. FAK functions downstream of and is necessary for HA-promoted cell motility. Secretion and expression of MMP-2 were increased by treatment with HA via the action of PKCα, PKCδ, and Rac1 and the production of ROS and FAK. Alamastat, an inhibitor of MMP-2, exerted a negative effect on HA-promoted cell motility. HA increased interaction between CD44 and epidermal growth factor receptor (EGFR). AG1478, an inhibitor of EGFR, decreased phosphorylation of PKCα, PKCδ, and Rac1 activity and suppressed induction of p47^Phox^ and p67^Phox^. These results suggest that CD44-EGFR interaction is necessary for HA-promoted cell motility by regulating PKC signaling. EGFR-Akt interaction promoted by HA was responsible for the increased production of ROS and HA-promoted cell motility. In summary, HA promotes CD44-EGFR interaction, which in turn activates PKC signaling, involving Akt, Rac1, Phox, and the production of ROS, FAK, and MMP-2, to enhance melanoma cell motility.

Hyaluronic acid (HA)2 is a glycosaminoglycan secreted by various tumor cells. HA has been shown to promote proliferation of some tumor cell lines (1, 2) and epithelial cells (3) and inhibits osteoclast cell differentiation (4). Tumor-specific accumulation of HA has been widely observed in human tumors, including colon cancer (5) and breast cancer (6). Inhibition of CD44 by anti-CD44 mAb or CD44-Fc fusion protein has been shown to block tumor growth, metastasis, and invasion (2, 7). HA interacts with receptors such as CD44 and RHAMM to regulate cellular proliferation and motility (8–10). HA enhances motility of glioma cells by inducing MMP-9 secretion through a pathway involving FAK (11). HA induces osteopontin via PI 3-kinase/Akt pathways (11). PTEN inhibits secretion of MMP-9 (12). PTEN interacts with FAK and suppresses PI 3-kinase/Akt survival pathways (13). The interaction of HA with CD44 promotes intracellular signaling pathways, including Ca2⁺ mobilization (14) and Rho signaling (15). HA-CD44 interactions with IQGAP-1 promote Cdc42 and ERK2 signaling (16). HA promotes Rac1 signaling by inducing an interaction between CD44 and Rac1-specific guanine nucleotide exchange factors, including Tiam1 and Vav2 (17, 18). The CD44-EGFR interaction is necessary for CD44-mediated cell motility (19). Epidermal growth factor enhances CD44 cleavage through activation of Rac GTPase to promote cell motility. Epidermal growth factor requires EGFR and CD44 to exert its effect on cell proliferation and motility (20).

Although it has been demonstrated that HA promotes cell motility, the mechanisms of its action have not been fully understood. In this study, we sought to further identify signal-

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2 The abbreviations used are: HA, hyaluronic acid; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; PKC, protein kinase C; MMP, matrix metalloproteinase; NAC, N-acetyl-L-cysteine; SL-MT, Seleno methionine; Phox, NADPH oxidase; ROS, reactive oxygen species; DPL, diphenylisothiourea; EMT, epithelial-mesenchymal transition; DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate; PI, phosphatidylinositol; siRNA, short interfering RNA; SOD, superoxide dismutase; ERK, extracellular signal-regulated kinase.
ing molecules involved in HA-promoted cell motility. We found that HA increased the production of ROS via the action of PKC and Rac1. NADPH oxidase activity was mainly responsible for the increased level of ROS by HA. We demonstrate that the CD44-EGFR interaction promoted by HA activates PKC signaling, involving Rac1, Phox, Akt, ROS, FAK, and MMP-2, to mediate HA-promoted cell motility. The functional role of EGFR in relation to HA-promoted cell motility has not been reported previously.

EXPERIMENTAL PROCEDURES

Materials—Anti-Rac1 antibody was purchased from R&D Systems (Minneapolis, MN). Anti-p40<sup>Phox</sup>, anti-p47<sup>Phox</sup>, anti-p67<sup>Phox</sup>, anti-rhoGDI, anti-phospho-rhoGDI, anti-Akt, anti-phospho-Akt (Ser-473), anti-ERK, anti-phospho-ERK, anti-FAK, anti-phospho-FAK<sup>Y397</sup>, anti-phospho-FAK<sup>Y925</sup>, anti-CD44, anti-EGFR, anti-PKC<sub>a</sub>, anti-phospho-PKC<sub>a</sub><sup>T505</sup>, anti-snail, anti-vimentin, anti-E-cadherin, and anti-N-cadherin antibodies were purchased from Cell Signaling Technology Co. (Beverly, MA). Anti-actin antibody was purchased from Sigma. Hyaluronic acid (1 MDa), fibronectin, Matrigel, type IV collagen, N-acetyl-l-cysteine, seleno-l-methionine (sL-MT), LY294002, DCFH-DA were purchased from Sigma. Various sizes of HA (3 MDa, 100 kDa, and 6 kDa) were obtained from Dr. K. Han (Hanson Biotech, Korea). Streptomyces hyaluronidase was purchased from Sigma. PKC inhibitors, including Go6976 (α) and rottlerin (β), were purchased from Calbiochem. Ionomastat was purchased from Chemicon International (Temecula, CA). Anti-mouse and anti-rabbit IgG-horseradish peroxidase-conjugated antibodies were purchased from Pierce. Wild type and mutant MMP-2 promoter-luciferase constructs were kindly provided by Prof. H. Lee (Kangwon National University). An ECL kit was purchased from Amersham Biosciences. Lipofectamine and Plus<sup>TM</sup> reagent were purchased from Invitrogen. The transwell chamber system was purchased from Costar (Acton, MA). Bioneer (Daejeon, Korea) synthesized all primers used in this study. The LumiMax superoxide anion detection kit for the measurement of peroxynitrite (ONOO<sup>−</sup>), 5 μM DHR123 was used instead of DCFH-DA.

To examine the effect of CD44 on ROS generation by following HA exposure, melanoma cells were preincubated with a monoclonal anti-CD44 antibody (3 μg/ml) for 2 h. Cells were then treated with HA (200 μg/ml) for 20 min. Cells were further incubated with DCFH-DA for 10 min. The effect of PKC on ROS production was determined by transiently transfecting cells with a control vector (2 μg), or a dominant negative PKC<sub>a</sub> (2 μg), or dominant negative PKC<sub>b</sub> (2 μg). After transfection (48 h), cells were treated without or with HA (200 μg/ml). ROS measurements were performed as described. To ensure that the hyaluronic acid used in this study was free of contaminants, cells were treated with hyaluronic acid in the presence or absence of hyaluronidase (30 units/ml). Hyaluronidase was activated by preincubation at 37 °C for 30 min and was inactivated by preincubation at 65 °C for 30 min.

NADPH Oxidase Activity Assay—NADPH oxidase activity was measured according to the manufacturer’s recommendations (Stratagene). The assay medium, comprised of 100 μM luminol and enhancer, was added to melanoma cells. HA (200 μg/ml), in the presence or absence of DPI (10 μM), was then added. Incubation was continued for 30 min. Chemiluminescence was measured continuously in a luminometer. To examine the effect of down-regulation of p47<sup>Phox</sup> and p67<sup>Phox</sup> on NADPH oxidase, cells were transiently transfected with 10 nM of each of a control siRNA, p47<sup>Phox</sup> siRNA, or p67<sup>Phox</sup> siRNA. Superoxide dismutase (SOD) activity was measured by using xanthine oxidase/xanthine luminal solution according to the manufacturer’s instructions (Stratagene).

Western Blot Analysis—For PAGE and Western blot, cell lysates were prepared using lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, 0.01% (w/v) bromophenol blue, 10 mM NaF, 1% (v/v) protease inhibitor mixture, 1 mM sodium orthovanadate). The samples were boiled for 5 min, and equal amounts of protein (20 μg/well) were analyzed on a 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and subjected to immunoblotting. The antibodies and dilutions included anti-Rac1 (1:1,000), anti-rhoGDI (1:1,000), anti-phospho-rhoGDI (1:1,000), anti-FAK (1:1,000), anti-phospho-FAK<sup>Y397</sup> (1:1,000), anti-phospho-FAK<sup>Y925</sup> (1:1,000), anti-p40<sup>Phox</sup> (1:1,000), anti-p47<sup>Phox</sup> (1:1,000), anti-p67<sup>Phox</sup> (1:1,000), anti-CD44 (1:2,000), anti-EGFR (1:3,000), anti-Akt (1:2,000), anti-phospho-Akt<sup>S473</sup> (1:2,000), anti-snail (1:3000), anti-E-cadherin (1:2,000), and anti-vimentin (1:3,000), and anti-actin (1:2,000). After extensive washing, blots were further incubated with an anti-mouse or anti-rabbit IgG-horseradish peroxidase-conjugated antibody at 1:3,000 dilution for 1 h at room temperature and were developed using an enhanced chemiluminescence kit (Amersham Biosciences). For immunoprecipitation, cell lysates were immunoprecipi-
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To determine the effect of antioxidants on HA-promoted chemoinvasion, melanoma cells were pretreated with or without NAC (100 μM) or sL-MT (30 μM) for 1 h. The effect of PKC, Rac1, FAK, MMP-2, and EGFR on chemoinvasion was also determined. Results were analyzed for statistical significance using the Student’s t test. Differences were considered significant at \( p < 0.05 \).

Wound Migration Assays—Melanoma cells were cultured on 6-well plates coated with fibronectin, type IV collagen, or Matrigel (10 μg/cm² each) until confluent. Confluent cultures of melanoma cells were treated with hyaluronic acid. Wounds were made by scraping with micropipette tips. Photographs were taken at 48 h after a wound was made. The migration distance and the number of cells migrated was measured for the determination of cell motility. To determine the effect of antioxidants on HA-promoted wound migration, melanoma cells were pretreated with NAC (100 μM), DPI (10 μM), or sL-MT (30 μM) for 1 h, followed by treatment with HA (200 μg/ml). The direct effect of ROS on wound migration was determined by addition of H₂O₂ (10 μM) or ONOO⁻ (10 μM) for various time intervals. Wound migration assays were performed as described above. Results were analyzed for statistical significance using the Student’s t test. Differences were considered statistically significant at \( p < 0.05 \).

Rac1 Activity Assay—Cells were grown in 100-mm dishes and treated with HA (200 μg/ml) for the indicated times. Cells were quickly rinsed in phosphate-buffered saline and lysed using 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂) plus protease inhibitors. Cells were scraped, and cell lysates were centrifuged for 5 min at 14,000 rpm. To determine the levels of Rac1-GTP, 400 μl of each cell lysate was incubated with PAK (CRIB)-GST beads for 1 h with rocking at 4 °C. Samples were centrifuged for 2 min at 2000 rpm, and supernatant was discarded. The beads were then washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂). Finally, 50 μl of 2× Laemmli buffer was added to the beads, and the mixture was boiled for 10 min. The levels of active Rac1, Rac1-GTP, were detected by Western blotting using specific monoclonal anti-Rac1 antibody (BD Transduction Laboratories). Approximately 10 μg of total lysate was analyzed to examine the levels of total Rac1, rhoGDI, phospho-rhoGDI, or actin (Sigma).

tated with polyclonal anti-Rac1 antibody (5 μg), anti-EGFR antibody (5 μg), or anti-Akt antibody (5 μg) on ice for 1 h. Protein G-Sepharose was then added, and the reaction was performed at 4 °C for 2 h on a rotary shaker. Immune complexes were washed three times with lysis buffer, and 2× sample buffer was added to the beads. Boiled samples were then loaded on gels. Western immunoblot analyses were followed according to standard procedures.

Chemoinvasion Assay—The invasive potential of melanoma cells was determined by using a Transwell chamber system with 8-μm pore polycarbonate filter inserts (Costar, Acton, MA). The lower and upper sides of the filter were coated with gelatin and Matrigel, respectively. Melanoma cells treated with HA for 16 h were harvested by trypsinization and counted. Trypsinized cells (2×10⁴) in the serum-free RPMI 1640 medium containing 1% bovine serum albumin were then added to each upper chamber of the Transwell. RPMI 1640 medium supplemented with 10% fetal bovine serum was placed in the lower chamber, and cells were incubated at 37 °C for 16 h. The cells were fixed with methanol, and invaded cells were stained and counted. To determine effect of ROS on chemoinvasion, melanoma cells were treated with H₂O₂ (10 μM) or ONOO⁻ (10 μM) for 2 h. Trypsinized cells were then subjected to chemoinvasion assays.
Analysis of Interaction between Rac1 and Phox in the Membrane—Cells were resuspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and were lysed by sonication. The lysates were then centrifuged at 100,000 g for 1 h at 4 °C. The supernatants constitute the cytosolic fraction. The pellet was resuspended in the above buffer, which also contained 0.1% Triton X-100, and the mixture was lysed by sonication and centrifuged again at 100,000 × g for 1 h at 4 °C to obtain the membrane fraction (supernatant). The Rac1 levels in these membrane fractions were analyzed by Western blotting to determine the extent of Rac1 translocation to the membrane.

To determine the binding of the translocated Rac1 to rhoGDI and phox, the membrane proteins were immunoprecipitated with anti-Rac1 antibody (5 μg), and the levels of rhoGDI, p47phox, p67phox, and anti-p40phox in the precipitates were analyzed by Western blotting. For immunoprecipitation, cells treated with HA were washed with phosphate-buffered saline buffer and lysed in buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 0.5% Nonidet P-40) on ice. The lysates were clarified by centrifugation at 16,000 × g for 30 min at 4 °C. Anti-Rac1 antibody (5 μg/ml) was added to the cell lysates, and the mixture was incubated for 1 h on ice. After incubation, 50 μl of protein G-Sepharose beads was added and incubation continued for 1 h at 4 °C with constant rotation. Immunoprecipitates were collected by centrifugation at 16,000 × g for 5 min at 4 °C and washed with lysis buffer.
buffer. Immunoprecipitates were eluted with 2× sample buffer, and the proteins were loaded onto 10% SDS-PAGE for Western blot analysis.

Preparation of siRNA Duplexes—The siRNA duplexes were constructed with the following target sequences: p47Phox, sense (5'-AAATGTTCCCTATTGAGGCAGCCTGTCTC-3'), and antisense (5'-AACTGCCTCAATAGGGAACATCCTGTCTC-3'); p67Phox, sense (5'-AAGTACACGGTAGTCATGAAGCCTGTCTC-3'), and antisense (5'-AACTTCATGACCTACCGTGACCTGTCTC-3'); and control, sense (5'-AATTCTCCGAACGTGTCACGTCCTGTCTC-3'), and antisense (5'-AAACGTGACACGTTCGGAGAACCTGTCTC-3'). The construction of siRNAs was carried out according to the manufacturer's instructions (Ambion, Austin, TX). Transfection of the siRNA construct was performed by Lipofectamine.

Gelatin Zymography—Conditioned medium from cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer (40% (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% (w/v) bromphenol blue) and loaded onto a 7.5% SDS-PAGE containing type I gelatin (2 mg/ml). After electrophoresis, the gel was soaked in Triton X-100 and shaken for 30 min with one change of solution. The gel was rinsed and incubated for 24 h at 37 °C in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, and 0.02% NaN₃). After incubation, the gel was stained with 0.1% Coomassie Brilliant Blue G-250 and destained in 50% methanol, 10% acetic acid, and 40% water. To determine the effect of PKCθ and PKCδ on the secretion and induction of MMP-2, cells were pretreated with Go6976 (1 μM) or rottlerin (10 μM) for 1 h. Cells were then treated with or without HA (200 μg/ml) for 4 h. Gelatin zymography was performed as described. To determine the effect of Rac1 on the secretion and induction of MMP-2, cells were transiently transfected with a control vector (2 μg) or dominant negative Rac1 construct (2 μg) plasmid. After transfection (48 h), cells were then treated with or without HA (200 μg/ml) for 4 h. Gelatin zymography was performed as described.

Transient Transfection and Luciferase Assay—pGL3 vector (2 μg; Promega, Madison, WI) containing wild type or mutant MMP-2 promoter was transiently transfected into melanoma cells, along with 0.6 μg of the pSV-β-galactosidase control vector. For transfection, Lipofectamine (Invitrogen) was used. Twenty four hours after the transfection, cells were treated with or without HA (200 μg/ml) for 4 h, and a luciferase activity assay was performed according to the manufacturer’s instructions (Tropix, Applied Biosystems). Luciferase activities were measured with the use of a luminometer (PerkinElmer Life Sciences). A pGL3-basic control vector, without an insert, was used as a negative control for the transfection experiments. Luciferase activities were normalized using β-galactosidase.

FIGURE 3. HA activates Rac1 and induces p47phox and p67phox via the action of CD44. A, melanoma cells were treated with or without HA 200 (μg/ml) for various time intervals. At each time point, cell lysates were prepared and subjected to GST-pulldown assays. Total cell lysates (10 μg) were subjected to Western blot analysis. B, same cells were treated with various concentrations of HA for 1 h and subjected to GST-pulldown assay. C, melanoma cells were treated with various molecular weight forms of HA at the same concentration (200 μm) for 1 h. Rac1 activity assay was performed. D, melanoma cells were pretreated with a monoclonal anti-CD44 antibody (10 μg/ml) or normal IgG (10 μg/ml) for 2 h. Cells were then treated with or without HA (200 μg/ml) for 1 h. Cell lysates were prepared, and GST-pulldown assays were performed. Total cell lysates (10 μg) were subjected to Western blot analysis. E, melanoma cells were treated with or without HA (200 μg/ml) for various time intervals. At each time point, cells were subjected to cellular fractionation. Cell lysates prepared from each fraction at each time point were immunoprecipitated (IP) with an anti-Rac1 antibody (Ab, 5 μg). The immunoprecipitates were then subjected to Western blot analysis.
Transfections of other constructs for luciferase activity assays were carried out as described.

Electrophoretic Mobility Shift Assay—AP-1-binding assays in nuclear extracts were performed with biotin-labeled AP-1 oligonucleotides (Panomics, Fremont, CA), and electrophoretic mobility shift assay was performed by using the electrophoretic mobility shift assay gel shift kit (Panomics). A reaction mixture containing 10 μg of nuclear protein was incubated for 30 min at room temperature in a final volume of 10 μl with a binding mixture containing 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 2 μg of poly(dI-dC), and 2 pmol of oligonucleotide probe, with or without a nonlabeled 10-fold excess of competitor oligonucleotides. Protein-DNA complexes were separated by electrophoresis on a 6% nondenaturing acrylamide gel containing 0.5 X TBE buffer (1 X TBE: 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA), transferred to Biodyne nylon membranes (Pierce), and then cross-linked on a Stratagene cross-linker. Protein gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection. For antibody supershift assay, 5 μg of anti-c-Jun antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reactions and incubated for 45 min at room temperature before gel loading. Nucleotide sequence of biotin-labeled AP-1 was 5'-CGCTTGATGACTCAGCCGGA-3'. The AP-1 recognition sequence is underlined.

RESULTS

HA Increases the ROS Level in Melanoma Cells and Cell Motility via the Action of CD44—We first examined whether HA promoted cell motility via the action of CD44. Inhibition of
CD44 exerted negative effects on HA-promoted chemoinvasion (Fig. 1A) and wound migration (Fig. 1B). HA-CD44 interaction plays a significant role in HA-promoted cell motility (21). Synthetic pep-1 that binds to and inhibits the function of HA significantly suppressed HA-promoted cell motility (Fig. 1, C and D). ROS mediated fibroblast migration by increasing phosphorylation of Cortactin (22). In addition, ROS mediated growth factor-induced migration of vascular smooth muscle cells (23).

HA Enhances Cell Motility by Increasing the Level of ROS—Because treatment of cells with HA enhances cell motility, we proposed that HA would affect intracellular ROS level. To determine the effect on ROS, melanoma cells treated with various concentrations of HA were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Hyaluronidase (30 units/ml) was added along with HA to determine specificity of HA. Hyaluronidase reversed the effect of HA on ROS production (Fig. 2A, upper panel) and chemoinvasion (Fig. 2B). The high molecular weight form of HA (1 MDa), but not the low molecular weight form, increased the level of ROS (Fig. 2A, lower panel). HA increased the level of ROS in melanoma cells in a CD44-dependent manner (Fig. 2C). To determine whether HA increased the level of peroxynitrite, cells treated with HA were incubated with DHR123. HA increased the level of peroxynitrite via the action of CD44. SL-MT, a peroxynitrite scavenger, decreased the level of ROS in melanoma cells treated with HA (data not shown). These results suggest that HA-promoted cell motility is accompanied by an increased level of ROS. ROS have been shown previously to play an important role in cell motility (24). Therefore, the effect of ROS on HA-promoted cell motility was examined. The antioxidant NAC (100 μM) prevented HA from enhancing invasion (Fig. 2D) and wound migration (Fig. 2E). SL-MT, a scavenger of peroxynitrite, also prevented HA in cells from enhancing chemoinvasion and wound migration. These results indicate that ROS are necessary for HA-promoted cell motility.

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FIGURE 5. Rac1 is responsible for the increase of ROS and enhanced cell motility by HA. A, serum-starved melanoma cells on coverslip were transiently transfection with a control vector (2 μg) or a dominant negative Rac1 construct (2 μg). At 48 h after transfection, cells were treated with or without HA (200 μg/ml), and the ROS level was measured by using DCFH-DA. B, same as A except that cells were transiently transfected with a constitutive active mutant construct (Rac1V12). C, melanoma cells were transiently transfected with a control vector (V) (2 μg) or a dominant negative Rac1 construct (2 μg). At 48 h after transfection, cells were treated without or with HA (200 μg/ml) and subjected to chemoinvasion assays (left panel). The same cells were transiently transfected with a control vector (2 μg) or a constitutive active Rac1 construct (2 μg). At 48 h after transfection, cells were subjected to chemoinvasion assays (right panel). The columns represent mean of triplicate experiments; bars indicate S.D.; * indicates p < 0.05, statistically different from control vector transfection. ** indicates p < 0.005 versus control vector transfection. D, confluent cultures of melanoma cells on fibronectin-coated plates (10 μg/cm²) were transiently transfected with a control vector (2 μg) or a constitutive active Rac1 construct (2 μg). At 48 h after transfection, cells were subjected to wound migration assays. * indicates p < 0.05, statistically different from control transfection. E, melanoma cells were transiently transfected with a control vector (2 μg) or a constitutive active mutant Rac1 vector (2 μg). At 48 h after transfection, cell lysates were prepared and subjected to Western blot analysis.

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HA Activates Rac1 via the Action of CD44—The role of Rac1 in the generation of ROS has been previously reported (25). GST pulldown assay was performed to examine whether HA affected Rac1 activity. HA was found to activate Rac1, in a time-dependent (Fig. 3A) and dose-dependent manner (Fig. 3B). However, HA did not affect RhoA activity (data not shown).

Effects of various molecular weight forms of HA on Rac1 activity were examined. The high molecular weight forms of HA (100 kDa and 1 MDa), but not the low molecular weight form of HA (6 kDa), increased Rac1 activity (Fig. 3C). Monoclonal anti-CD44 antibody prevented HA from increasing Rac1 activity and the induction of p47^{phox} and p67^{phox} (Fig. 3D). NADPH oxidase complex is composed of three cytosolic proteins (p40^{phox}, p47^{phox}, and p67^{phox}), the catalytic subunit gp91^{phox}, and p22^{phox}. Rac1 increases the production of ROS through the activation of NADPH oxidase (26), and the above finding suggests that NADPH oxidase may be responsible for the ROS production. A cellular fractionation study revealed that HA induced translocation of Rac1, p47^{phox}, and p67^{phox} (Fig. 3E). The interaction between Rac1 and rhoGDI was inhibited by HA (Fig. 3E). This suggests that HA activates Rac1 by phosphorylation of rhoGDI. Hyaluronidase treatment reversed the effect of HA on Rac1 activity, phosphorylation of rhoGDI, PKC\textsubscript{H9251}, and PKC\textsubscript{H9254}, and induction of p47^{phox} and p67^{phox}, suggesting that HA increases ROS production by activating PKC and Rac1 and increasing NADPH oxidase activity through the action of CD44.

PKC\textsubscript{α} and PKC\textsubscript{δ} Mediate HA-promoted Cell Motility by Activating Rac1 and Increasing ROS Production—PKC is known to be responsible for ROS production (27). We examined whether HA affected PKC activity. HA increased phosphorylation of PKC\textsubscript{α} and PKC\textsubscript{δ} (Fig. 4A). However, HA did not
affect phosphorylation of other PKCs, such as PKCζ, PKCθ, PKCμ, and PKCβ (data not shown). HA is present as a high molecular weight form with an average molecular mass of several thousand kDa under physiological conditions (28). Treatment of cells with a low molecular weight from of HA did not affect PKCα or PKCδ (data not shown). Expression of a dominant negative PKCα and PKCδ exerted negative effects on Rac1 activity (Fig. 4A).

Dominant negative PKCα and PKCδ exerted negative effects on ROS generation (Fig. 4B) and cell motility (Fig. 4, C and D) in melanoma cells treated with HA. These results suggest that PKCα and PKCδ mediate HA-promoted cell motility by an increase of Rac1 activity and ROS production.

Rac1 Is Required for the Increase of ROS and Enhanced Cell Motility by HA—We determined the role of Rac1 in the production of ROS and enhanced cell motility by HA. Expression of a dominant negative Rac1 (Rac1N17) inhibited the increase of ROS in cells treated with HA (Fig. 5A). Expression of a constitutive active mutant Rac1 (Rac1V12) itself increased ROS production (Fig. 5B), suggesting that Rac1 is responsible for the induction of ROS by HA. The role of Rac1 in HA-promoted cell motility was also investigated. Expression of a dominant negative Rac1 (Rac1N17) exerted a negative effect on HA-promoted invasion (Fig. 5C) and motility (Fig. 5D). These results suggest that Rac1 mediates HA-promoted cell motility by increasing the level of ROS. Epithelial-mesenchymal transition (EMT) occurs during tumorigenesis. EMT effectors promote tumor formation and metastasis (29, 30). Overexpression of Rac1 affected expression of proteins associated with EMT. For example, Rac1 decreased expression of E-cadherin and increased expression of N-cadherin, Snail, and vimentin (Fig. 5E). The fact that Rac1 affects expression of EMT-related proteins implies that HA-promoted cell motility is closely related with EMT. Rac1 did not affect phosphorylation of PKCα or PKCδ (Fig. 5E), suggesting that PKC functions upstream of Rac1.

NADPH Oxidase Is Responsible for ROS Production—We determined the main source of ROS in melanoma cells treated with HA. HA increased the level of superoxide in melanoma cells (Fig. 6A). DPI, an inhibitor of NADPH oxidase, exerted negative effect on the generation of superoxide in cells treated with HA (Fig. 6A). Phorbol 12-myristate 13-acetate, an activator of NADPH oxidase, stimulated NADPH oxidase activity...
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HA induces secretion and expression of MMP-2 via PKCα and PKCδ, Rac1, and FAK. A, melanoma cells were transiently transfected with a wild type (W.T.) MMP-2 promoter-luciferase construct (2 μg), a mutant MMP-2 promoter-luciferase construct (2 μg), or a pGL3-Basic control vector (v) (2 μg) together with a pSV-β-galactosidase vector (0.6 μg). Twenty-four hours after transfection, cells were treated with or without HA (200 μg/ml) for 8 h, and luciferase activity was measured and normalized to β-galactosidase activity. The promoter activity was expressed relative to that of the empty pGL3-Basic control vector after normalization to the co-transfected pSV-β-galactosidase vector. B, serum-starved melanoma cells were pretreated with various PKC inhibitors, such as Go6976 (1 μM) and rottlerin (10 μM) for 1 h. Cells were then treated with HA (200 μg/ml) for 1 h. Supernatants and cell lysates were subjected to gelatin zymography and Western blot analysis, respectively. D, dimethyl sulfoxide. C, melanoma cells were transiently transfected with a control vector (V) (2 μg) or a dominant negative Rac1 mutant construct (2 μg). At 24 h after transfection, cells were treated with or without HA (200 μg/ml) for 8 h. Gelatin zymography and Western blot analysis were performed. D, same as C except that melanoma cells were transfected with a FRNK construct (2 μg). E, melanoma cells were transiently transfected with 2 μg each of constructs expressing Rac1N17 or FRNK in the presence or absence of Go6976 (1 μM) or rottlerin (10 μM) for 24 h. At 24 h after transfection, cells were treated with or without HA (200 μg/ml) for 8 h. Luciferase activity assays were performed. Statistical analyses were carried out by Student's t test. F, specific DNA binding activity of AP-1 factor was determined by an electrophoretic mobility shift assay in the presence of absence of a 10-fold excess of cold competitors. c-Jun antibody (Ab) (5 μg) was employed for supershift analysis. Open circle denotes DNA-protein complex. Filled circle denotes c-Jun-DNA complex.

(data not shown). Down-regulation of p47^phox^ and p67^phox^ by the use of siRNAs diminished chemiluminescence in melanoma cells treated with HA (Fig. 6B). Down-regulation of p47^phox^ and p67^phox^ with the siRNAs decreased ROS level in melanoma cells treated with HA (Fig. 6C). It is still possible that superoxide can be converted into H_2O_2. We examined whether HA affected SOD activity to convert superoxide into H_2O_2. Addition of purified SOD clearly inhibited endogenous SOD activity, as expected (Fig. 6D). HA did not affect SOD activity (Fig. 6D). These results suggest that NADPH oxidase may be the main source for generating ROS, such as H_2O_2 and peroxynitrite (ONOO^-), in melanoma cells treated with HA.

FAK Functions Downstream of Rac1 and Mediates HA-promoted Cell Motility—FRNK, an endogenous inhibitor of FAK, did not suppress the induction of ROS by HA (Fig. 7A) suggesting that FAK functions downstream of Rac1. Expression of a dominant negative Rac1 mutant (Rac1N17) suppressed phosphorylation of FAK by HA (Fig. 7B). Expression of a constitutive active mutant Rac1 (Rac1V12) activated FAK (Fig. 7C). FRNK did not affect Rac1 activity in melanoma cells treated with HA (Fig. 7D). Subsequently, we determined whether FAK was required for HA-promoted cell motility. Overexpression of FRNK inhibited HA-promoted invasion (Fig. 7E) and wound migration (Fig. 7F). These results indicate that FAK functions downstream of Rac1 to mediate HA-promoted cell motility.

HA Induces Secretion and Expression of MMP-2 via PKC, Rac1, and FAK—MMPs play significant roles in cellular invasion and metastasis (31–34). HA induced secretion and expression of MMP-2 in a time- and dose-dependent manner (data not shown). An MMP-2 promoter-luciferase activity assay showed that with HA treatment wild type MMP-2 promoter activity increased, but the activity of a mutant MMP-2 promoter did not increase (Fig. 8A). Inhibition of PKCα, PKCδ (Fig. 8B), Rac1 (Fig. 8C), and FAK (Fig. 8D) exerted a negative effect on secretion and induction of MMP-2 in cells treated
with HA. Overexpression of a constitutive active Rac1 mutant increased secretion and induction of MMP-2 (data not shown). Blocking of CD44 by an anti-CD44 antibody suppressed induction and secretion of MMP-2 by HA (data not shown). MMP-2 promoter-luciferase activity assay confirmed that HA induced expression of MMP-2 via PKC, Rac1, and FAK (Fig. 8E). The MMP-2 promoter contains AP-1-binding consensus sequences. We found an increased phosphorylation of JNK with HA treatment (data not shown). Specific DNA binding activity of AP-1 was determined. HA increased DNA binding activity of c-Jun to MMP-2 promoter (Fig. 8F, upper panel). Supershift analysis showed specific binding of c-Jun to MMP-2 promoter (Fig. 8F, upper panel).

ROS Are Responsible for Induction of MMP-2—As Rac1 was necessary for the increased secretion and induction of MMP-2, it was necessary to examine whether ROS affected secretion and expression of MMP-2. Antioxidants, such as NAC and sL-MT, exerted a negative effect on the secretion and induction of MMP-2 in melanoma cells treated with HA (Fig. 9A). MMP-2 promoter-luciferase activity assay confirmed that NAC and sL-MT suppressed the induction of MMP-2 promoter activity in melanoma cells treated with HA (Fig. 9B). Ilomastat, an inhibitor of MMP-2, suppressed HA-promoted invasion (Fig. 9C) and wound migration (Fig. 9D). These results suggest that ROS promote cell motility by regulating secretion and expression of MMP-2.

CD44 Interacts with EGFR to Regulate PKC Signaling and Cell Motility—HA activates multiple receptor tyrosine kinases, including EGFR (35). The HA-CD44 interaction with EGFR promotes Rac1 activity, phosphorylation of rhoGDI, PKCα, p47^phox, and p67^phox (Fig. 10A). AG1478, an inhibitor of EGFR, decreased Rac1 activity, phosphorylation of rhoGDI, PKCα, PKCβ, and the expression level of p47^phox and p67^phox (Fig. 10B). AG1478 inhibited the interaction between CD44 and EGFR.

**FIGURE 9.** **A**, melanoma cells were pretreated with NAC (100 μM) or sL-MT (30 μM) for 1 h. Cells were then treated with HA (200 μg/ml) for 8 h. Gelatin zymography and Western blot analysis were performed. **B**, melanoma cells were pretreated with NAC (100 μM) or sL-MT (30 μM) for 2 h. Cells were then transiently transfected with an MMP-2 promoter-luciferase construct (2 μg), a mutant MMP-2 promoter-luciferase construct (2 μg), or a pGL3-Basic control vector (2 μg), together with a pSV-β-galactosidase vector (0.6 μg). Statistical analyses were carried out by Student’s t test. Melanoma cells were pretreated with or without Ilomastat (20 μM) for 2 h. Cells were then treated without or with HA (200 μg/ml) for 4 h and subjected to chemoinvasion assays (C) and wound migration assays (D). The columns represent mean of triplicate experiments; bars indicate S.D.; * indicates p < 0.05, statistically different from controls. DMSO, dimethyl sulfoxide.
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EGFR, as expected (data not shown). Furthermore, treatment with AG1478 decreased ROS production in melanoma cells treated with HA (Fig. 10C). Treatment with AG1478 exerted a negative effect on HA-promoted cell motility (Fig. 10, D and E). These results indicate that CD44-EGFR interaction is responsible for HA-promoted cell motility by regulating PKC signaling.

**HA Induces EGFR-Akt Interaction to Enhance Cell Motility**—Treatment of cells with HA promotes the CD44-EGFR interaction to enhance cell motility (Fig. 10A). HA has been shown to activate PI 3-kinase/Akt signaling (13). Therefore, it was of interest to examine whether the EGFR-Akt interaction is necessary for HA-promoted cell motility. HA induced an EGFR interaction with Akt, whereas treatment with HA did not induce an EGFR-ERK interaction (Fig. 11A). This result implies that EGFR interaction with Akt is necessary for HA-promoted cell motility. The high molecular weight form of HA, but not the low molecular weight form, induced the EGFR-Akt interaction (Fig. 11B). Inhibition of Akt activation by LY294002 exerted a negative effect on HA-promoted cell motility (Fig. 11, C and D).

**HA Induces the Interaction between Akt and Rac1 to Increase the Level of ROS**—The inhibition of Akt by LY294002 did not affect phosphorylation of PKCα or PKCδ, suggesting that Akt functions downstream of PKC (Fig. 12A). Inhibition of PKCα and PKCδ suppressed activation of Akt in cells treated with HA (data not shown). The inhibition of Akt activation by LY294002 decreased phosphorylation of rhoGDI and Rac1 activity in melanoma cells treated with HA (Fig. 12A), suggesting that Akt functions upstream of Rac1. HA induces an interaction between Akt and Rac1 (Fig. 12B), suggesting that Akt-Rac1 interaction is necessary for the increased ROS by HA. The inhibition of Akt activation by LY294002 decreased the level of ROS (Fig. 12C). These results indicate that Akt is responsible for the increased ROS levels and is responsible for enhanced cell motility by HA.

**DISCUSSION**

In this study, we investigated in detail the mechanisms associated with HA-promoted cell motility. Surprisingly, HA increased the intracellular level of ROS (Fig. 2, A and C). Estrogen enhances cell motility by increasing the level of ROS (36).
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HA-binding CD44 was responsible for the enhanced cell motility (Fig. 1B) and increased production of ROS (Fig. 2C). Antioxidants, such as NAC, inhibited HA-promoted cell motility (Fig. 2, D and E). Hydrogen peroxide and peroxynitrite enhanced cell motility. These results suggest that ROS mediate HA-promoted cell motility. Rho GTPases, such as Rac, Rho, and Cdc42, coordinate the actin cytoskeleton, modulate the formation of signaling reactive oxygen species, and control gene transcription. Rac1 in resting cells is bound to GDP and is associated with the inhibitor protein rhoGDI in the cytosol (37). Activation of rho GTPase signaling pathways requires the regulated release of rho GTPases from rhoGDI complexes. Phosphorylation of rhoGDI serves as mechanism for regulating the formation and/or dissociation of rho GTPase-GDI complexes (37). We proposed that HA would increase the level of ROS by regulating phosphorylation of rhoGDI. HA increased phosphorylation of rhoGDI and activated Rac1 (Fig. 3A, A and B). HA-binding CD44 was responsible for increased Rac1 activity and phosphorylation of rhoGDI (Fig. 3D). Cytosolic Rac1 is translocated into the membrane upon its activation (38). HA inhibited the Rac1-rhoGDI interaction and induced cytosolic translocation of Rac1 (Fig. 3E). PKCa and PKCδ are important regulators in mediating activation of Rac1 in the cyclic stretch-induced hypertrophic process (39). An association of HA with CD44 has been shown to promote interaction between PKCγ with Rac1 and increased Rac1 activity in keratinocytes (14). PKCa and PKCδ regulate Rac1 activity through phosphorylation of rhoGDI (Fig. 4A). As reported previously, PKCδ induces up-regulation of NADPH oxidase (40).

The inhibition of PKCδ and PKCa by their respective dominant negative construct suppressed induction of ROS (Fig. 4B) and decreased cell motility (Fig. 4, C and D). A dominant negative Rac1 mutant prevented HA from increasing the production of ROS (Fig. 5A), whereas a constitutive active Rac1 mutant further increased ROS production in the presence of HA (Fig. 5B). These results indicate that Rac1 mediates ROS generation by HA. Rac1 was found to be responsible for HA-promoted cell motility (Fig. 5, C and D). An epithelial-mesenchymal transition (EMT) is involved in various cellular processes, such as metastasis, embryonic development, and wound healing (41, 42). PKCa, PKCβ, and PKCγ are involved in the EMT (43). Rac1 and ROS are involved in the MMP-3-induced EMT (44). Snail has been shown previously to up-regulate MMP-2 (45). Snail is induced by NF-κB and has an important role in the EMT (46). The loss of E-cadherin is a hallmark of the EMT (47). Overexpression of Rac1 was found to affect expression of EMT-related proteins, such as E-cadherin, N-cadherin, and vimentin (Fig. 4E). It is therefore plausible that HA affects expression of proteins involved in EMT. For example, HA decreased expression of E-cadherin, increased that of snail, vimentin, and N-cadherin, and the effect of HA on expression levels of these proteins were mediated by CD44, PKCa, and PKCδ. HA induced morphological transformation. Therefore, it is probable that HA affects the EMT through PKC signaling, involving Rac1. As treatment of cells with HA increases Rac1 activity, it is likely that HA affects NADPH oxidase activity. NADPH oxidase has been shown to be responsible for the generation of ROS in response to transforming growth factor-β (48). The role of p47phox in ROS production has been demonstrated (49). DPI, an inhibitor of NADPH oxidase, decreased the level of superoxide in cells treated with HA.
Superoxide production is dependent on PI 3-kinase and PKC. Down-regulation of p47Phox and p67Phox decreased the superoxide level in cells treated with HA (Fig. 6B) and decreased the level of ROS, such as H2O2 and peroxynitrite (Fig. 6C).

FAK is a tyrosine kinase found in focal adhesions. Upon integrin engagement, FAK is autophosphorylated on Tyr397, creating a binding site for Src. Phosphorylation of Tyr397 promotes the binding of a number of SH2-containing proteins, including phospholipase C, Grb7, and PI 3-kinase. FAK-deficient fibroblasts have motility defects, and restoration or overexpression of FAK reportedly restores the motility defect. These findings indicate that FAK has a role in cell motility. Rac1 activates FAK signaling. FAK potentiates Rac1 activation and localization to matrix adhesion sites. These results indicate an interaction between Rac1 and FAK has a role in cell motility. In this study, FAK was found to function downstream of Rac1 and mediate HA-promoted cell motility.

We examined whether HA-promoted cell motility was accompanied by induction of MMPs. HA induced expression of MMP-2 (Fig. 8A). PKC has been shown to be required for secretion of MMP-2. The inhibition of PKCα and PKCδ suppressed induction and secretion of MMP-2 in melanoma cells treated with HA (Fig. 8B). Rac1 (Fig. 8C) and FAK (Fig. 8D) showed the same effects as PKCα and PKCδ on the induction and secretion of MMP-2 in cells treated with HA.

Addition of anti-CD44 antibody suppressed induction and secretion of MMP-2 in melanoma cells treated with HA (data not shown). Hydrogen peroxide and peroxynitrite increased expression and secretion of MMP-2 (data not shown). Treatment of cells with HA increased secretion and expression of MMP-2 via the production of ROS (Fig. 9, A and B). Oxidative stress enhances the invasion potential of hepatic stellate cells through the induction of MMP-2. Several reports have suggested an important role of ROS in cell motility. Peroxynitrite has been shown to induce secretion of MMP-2 in human hepatic stellate cells. NADPH oxidase is responsible for the increase in MMP-2. Peroxynitrite has been shown to promote cell motility. Ilomastat, an inhibitor of MMP-2, suppressed HA-promoted cell motility (Fig. 9, C and D). HA activated PKCδ (Fig. 3A), and EGFR has been shown to activate PKCδ. These results suggest that the interaction between CD44 and EGFR regulates PKC signaling. HA induced a CD44-EGFR interaction (Fig. 10A), and inhibition of EGFR exerted a negative effect on PKC signaling Rac1, p47Phox, and p67Phox (Fig. 10B), ROS (Fig. 10C), and cell motility (Fig. 10, D and E). EGFR is thought to have a role in EMT. The role of EGFR in EMT was confirmed by the fact that CD44 mediated effect of HA on expression of EMT-related proteins.

Increasing evidence suggests that HA serves as an extracellular signaling molecule. For example, HA binding to CD44 has been shown to activate NF-κB through Ras and protein kinase...
C (68). HA enhances cell motility by activating the PI 3-kinase/Akt pathway (69). PRL-3 acts through PI 3-kinase/Akt signaling to promote EMT (70). As overexpression of Rac1 affected expression of EMT-related proteins (Fig. 4E), it is possible that Akt is necessary for EMT. Akt has been shown to interact with Rac1 to inactivate JNK3 signaling, providing protection against brain ischemic injury (71). In our data, Akt was necessary for increased Rac1 activity (Fig. 12A). Overexpression of Rac1 did not affect phosphorylation of Akt (data not shown), confirming that Rac1 functions downstream of Akt. Inhibition of Akt suppresses phosphorylation of rhoGDI by HA (Fig. 12A), indicating that the increased Rac1 activity is accompanied by the phosphorylation of rhoGDI. Akt was necessary for the increased ROS level in melanoma cells treated with HA (Fig. 12C). Akt may have a role in the increased Rac1 activity by HA. HA promoted interaction between Akt and Rac1 (Fig. 12B). This finding implies that the Akt-Rac1 interaction is responsible for the activation of Rac1 by HA. As Rac1 affects proteins involved in EMT, it is necessary to investigate the further relationship between Akt and EMT. Based on the results obtained in this study, we propose the following HA signaling model: CD44-EGFR interaction (Fig. 10A), induced by HA, activates PKC signaling involving Akt, Rac1, ROS, FAK, and MMP-2 (Fig. 8E). The results presented in this study provide new insights toward a better understanding of HA-promoted cell motility.

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