

Signaling pathway involved in cyclooxygenase-2 up-regulation by hepatocyte growth factor in endometrial cancer cells

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Abstract. Hepatocyte growth factor (HGF) is up-regulated in tissue repair and has been implicated in playing a role in this process through its anti-apoptotic and proliferative activities. Cyclooxygenase-2 (COX-2) is an inducible enzyme in the biosynthetic pathway of prostaglandins, and its activation has been shown to play an important role in cell growth. We previously reported that HGF significantly inhibited anoikis, possibly through the up-regulation of COX-2 expression in the endometrial RL95-2 cancer cell line. Here, we report that i) treatment of RL95-2 cells with HGF resulted in phosphorylation of the HGF receptor c-Met, activation of Akt and I κ B, translocation of NF- κ B into the nucleus, and up-regulation of COX-2 mRNA; ii) the I κ B- α phosphorylation inhibitor BAY11-7082 and the selective COX-2 inhibitor CAY10452 blocked HGF-mediated anoikis resistance in RL95-2 cells; and iii) HGF induced migration and invasion in RL95-2 cells, while the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and CAY10452 blocked these effects of HGF stimulation. Our data suggest that HGF possesses chemotactic ability, has anti-apoptosis action, and induces cellular infiltration via the PI3K/Akt pathway; it also triggers NF- κ B activation and up-regulates COX-2 gene expression in endometrial cancer cells.

Introduction

Hepatocyte growth factor (HGF), also known as the scatter factor, was identified and cloned as a mitogen of hepatocytes (1,2). During organ development and tissue repair, HGF acts as a pleiotropic cytokine with regenerative, protective, and angiogenic properties, and it displays these functions by binding to its receptor, c-Met (3). HGF and c-Met are often overexpressed in human cancers, including gastrointestinal

(4), renal (5), head and neck (6), lung (7), pancreatic (8) and endometrial cancers (9). Recent studies suggest that the interaction between HGF and c-Met stimulates tumor growth (10) and angiogenesis (11,12) and suppresses tumor apoptosis in various malignant tumors (13). In endometrial cancer, it has been observed that HGF is secreted by endometrial stromal cells and that endometrial cancer cells express its receptor c-Met (14). The interaction between HGF and c-Met is involved in the effects of HGF on tumor invasion (14-16), angiogenesis (9) and anti-apoptosis (17).

Cyclooxygenase-2 (COX-2) is the rate-limiting step in the production of prostaglandins and is expressed at high levels only upon induction by growth factors, cytokines, and extracellular stimuli associated with cell activation (18). COX-2 is reportedly involved in chronic inflammation and cancer growth (19,20). COX-2 mediates a number of effects, including stimulation of invasion and motility, angiogenesis, and protection from apoptosis (21). All of these effects are also linked to HGF, suggesting that COX-2 may be an important downstream mediator of HGF effects (22). In endometrial cancer, the overexpression of COX-2 is closely associated with parameters of tumor aggressiveness (23). Moreover, recent reports indicate that COX-2 inhibitors may be effective in the treatment of endometrial cancer via the suppression of angiogenesis (24).

Anoikis is a form of apoptosis induced in epithelial cells through loss of their matrix attachment and is involved in a wide diversity of tissue-homeostatic, developmental, and oncogenic processes. In contrast to the fate of normal cells, tumor cells metastasize upon the loss of their matrix attachment. Hence, the inhibition of anoikis is a key element in tumor progression and metastasis (25-27).

We previously reported that HGF significantly inhibited anoikis, possibly through the up-regulation of COX-2 expression in endometrial cancer (28). Our previous report was the first to report the relationship between anoikis and gynecological cancer. However, the signaling pathway involved in the up-regulation of COX-2 expression by HGF stimulation in endometrial cancer cells remains unknown. In this study, we demonstrated that HGF up-regulates COX-2 through the NF- κ B and the PI3K/Akt pathways in endometrial cancer cells and that HGF stimulation enhances chemotaxis ability, cellular infiltration and anoikis resistance in uterine cancer cells.

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Materials and methods

Reagents. Recombinant human hepatocyte growth factor (HGF) was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). PD98059, an inhibitor of mitogen-activated protein kinase kinase activation, was obtained from Biomol Research Labs, Inc. (Plymouth Meeting, PA, USA). LY294002, a PI3K/Akt inhibitor, was purchased from Calbiochem. BAY11-7082, an inhibitor of I κ B- α phosphorylation, was from Sigma-Aldrich (St. Louis, MO, USA). CAY10452, a selective COX-2 inhibitor, was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fetal bovine serum (FBS) was obtained from Invitrogen, Carlsbad, CA, USA. Furthermore, 100 U penicillin and 100 U streptomycin were purchased from ICN Biomedicals Inc. (Aurora, OH, USA).

Cell culture. The human endometrial cancer cell line RL95-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). RL95-2 cells were grown in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 Ag/ml). DMEM/F-12 was purchased from Sigma-Aldrich. Cells were cultured in 75-cm² bottles at 37°C in 95% air and 5% CO₂. At the log growth phase, the cells were seeded into 6-well plates and then serum-starved for 12 h prior to the experiments.

Induction of anoikis. To prevent cell adhesion, culture dishes were coated with diluted poly-HEMA solution and ethanol solvent, and then evaporated overnight as described by Folkman and Moscona (29). Cultured cells were plated at a concentration of 7x10⁵/ml on culture dishes. Next, the cells were cultured in DMEM/F-12 supplemented with 1% FBS for 48 h in suspension.

Protein extraction. Total protein was extracted from whole RL95-2 cells plated in 6-well dishes using M-PER mammalian protein extraction reagent (Pierce, USA) with 1% HaltTM protease inhibitor cocktail (Pierce) according to the manufacturer's instructions. We added phosphatase inhibitor Cocktails B and C (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) upon detection of phosphorylation. The protein concentration was measured using a DC protein assay (Bio-Rad) according to the manufacturer's protocol.

Nuclear extractions. Nuclear extractions were prepared from whole RL95-2 cells plated in 6-well dishes using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) with 1% Halt protease inhibitor cocktail (Pierce), according to the protocol suggested by the manufacturer.

Western blot analysis. The lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins on the gel were transferred onto a polyvinylidene difluoride membrane. The membrane was then washed with Tris-buffered saline (TBS; Santa Cruz Biotechnology) containing 0.1% Tween-20 (TBST) and blocked with Blocking One-P (Nakarai Tesque Inc., Kyoto, Japan) at 4°C overnight. Next, the membrane was probed with primary antibodies for 12 h

at 4°C. The following primary antibodies were purchased from the following commercial sources. The anti-human I κ B- α , p-I κ B- α , c-Met, p-Met and COX-2 rabbit polyclonal antibodies (dilution, 1:1000) were purchased from Santa Cruz Biotechnology, and the anti-human NF- κ B p65 rabbit polyclonal antibody (ab31481; dilution, 1:10000) and anti-human Lamin A/C mouse monoclonal antibody (ab8984; dilution 1:2000) were from Abcam Cambridge, UK. The anti-human Akt and phospho-Akt rabbit monoclonal antibodies (dilution, 1:10,000) were from Cell Signaling Technology, Inc., USA, and the anti-human β -actin rabbit monoclonal antibody (dilution, 1:10000) was from Epitomics Inc. (Burlingame, CA, USA). After the membrane was washed with TBST, the antibodies bound to the membrane were detected with anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (HRP) (GE Healthcare UK Ltd., Buckinghamshire, UK) and anti-mouse immunoglobulins/HRP (dilution, 1:10,000; DakoCytomation, Glostrup, Denmark) and visualized with the ECL Plus Western blotting detection system (GE Healthcare UK Ltd.) according to the manufacturer's protocol. After the membrane was washed with TBST, the membrane was reprobed with RestoreTM Plus Western Blot stripping buffer (Pierce).

Densitometry of Western blot data. Images of blots were entered into a computer using a scanner (LAS-1000; FujiFilm Corp., Tokyo, Japan) and analyzed using the software program Image Gauge (FujiFilm Corp.).

Real-time polymerase chain reaction (real-time PCR). Total RNA was reverse transcribed in a 50- μ l volume with the TaqMan Gene Expression Cells-to-CtTM kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. After RL95-2 cells grown in 96-well plates were washed in PBS, they were incubated for 5 min at room temperature in the supplied lysis solution with DNase in order to lyse the cells and remove the gDNA. Next, the samples were incubated in stop solution for 2 min at room temperature. Samples were then processed directly in the culture well. Reverse transcription was carried out by using the Thermal Cycler Personal (Takara Bio Inc., Japan). Samples were incubated at 37°C for 60 min and then at 95°C for 5 min to inactivate the reverse transcriptase enzyme. Next, 4 μ l of the reverse transcription reaction mixture was used in each of the real-time PCR assays. Quantitative real-time PCR was performed with the TaqMan Gene Expression Cells-to-Ct kit according to the manufacturer's recommendations. The primers for COX-2 and the housekeeping gene GAPDH were used for the TaqMan Gene Expression Assay (Applied Biosystems). The assay ID of the primer for COX-2 was Hs01573471_m1 and that for GAPDH was Hs99999905_g1. Samples were denatured at 50°C for 2 min and at 95°C for 10 min, followed by 55 cycles each of 95°C for 15 sec and 60°C for 1 min, and then held at 25°C on the StepOneTM real-time PCR system (Applied Biosystems).

Data normalization. Relative quantification of gene expression was calculated using the 2^{- $\Delta\Delta$ Ct} method. The delta-delta method was used to determine the relative levels of mRNA expression between experimental samples and controls. The relative quantification is based on a reference mRNA, which

has a consistent level of mRNA expression under existing experimental sample and control sample conditions. In the present study, GAPDH was selected as the reference gene for the normalization of inter-PCR variation. Because of the exponential amplification of target genes by the real-time PCR reaction, a $\Delta\Delta CT=1$ was considered to represent a 2-fold change in target gene expression.

Methionyl-tRNA synthetase (MTS) proliferation assay. Cell viability was measured using the MTS proliferation assay using the Cell Titer 96 Aqueous One solution cell proliferation assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Briefly, MTS solution (20 μ l/well) was added, and after 2 h of culture at 37°C in 95% air and 5% CO₂, the conversion of MTS to formazan was measured on a plate reader at 492 nm. The percentage of cell viability/cell death was determined by a comparison with untreated controls.

Cell culture wound healing assay. RL95-2 cells were seeded onto 24-well dishes and grown to overconfluence for 24 h. Upon reaching overconfluence, the center of the culture dishes was scraped once with a 200- μ l yellow pipette tip. After washing away the debris, the cells were re-fed fresh medium with or without pharmacological inhibitor and HGF. Images of each well were examined under an Olympus microscope (Tokyo, Japan), and the width of each wound was photographed and measured after 0 and 24 h using an Olympus sc35 camera. The area of the denuded surface was quantified immediately after wounding and again after 24 h. The extent of wound closure was determined by calculating the ratio between the surface area of the wound for each time point and the surface of the initial wound. These data were then expressed as the percentage of wound closure relative to the control conditions for each experiment.

Invasion assay. The invasion assay was performed by using 24-well BD BioCoat Matrigel invasion chambers with 8- μ m polycarbonate filters (Becton-Dickinson, Bedford, MA, USA). Fifty thousand RL95-2 cells were seeded onto the upper chamber of the Matrigel invasion chamber plates and cultured in 500 μ l serum-free medium in the absence or presence of a pharmacological inhibitor. In the lower chamber, DMEM/F-12 with 5% FBS in the absence or presence of 10 ng/ml of HGF was loaded. Cells were incubated for 34 h at 37°C in a humidified incubator with 5% CO₂. Migrating cells on the upper surface of the filter were removed by wiping with a cotton swab. Invasive cells that penetrated through the pores and that migrated to the underside of the membrane were stained with Diff-Quik (Sysmex Corp., Kobe, Japan). The transwell membranes were cut, mounted on glass slides upside down, immersed in a diluted glycerol solution (1:10 in PBS), and observed under a microscope. After selecting 5 regions of each membrane randomly, the number of cancer cells that had migrated to be trapped in the pores was counted. Images of the chamber membranes were viewed under the Olympus microscope, BX41, and photographed using the Olympus DP20 camera and Olympus DP2-BSW software.

Statistical analysis. Each experiment was performed in triplicate. Values are expressed as the mean [standard deviation

(SD)]. Differences between groups were analyzed using the Student's t-test and analysis of variance (ANOVA). A value of $p<0.05$ was considered statistically significant.

Results

HGF phosphorylation of c-met, Akt and I κ B in RL95-2 cells. We re-examined whether the PI3/Akt and NF- κ B/I κ B signaling pathways are activated by HGF stimulation by using Western blot analysis. RL95-2 cells in 6-well dishes were incubated with varying concentrations of HGF for 5 min. Next, 10 μ g of the protein extracts was analyzed by Western blot analysis. A very low amount of phosphorylated c-Met (p-Met) was detected in the control cells, while the concentration of p-Met was increased in the extracts of cells stimulated with 5 and 10 ng/ml of HGF. Similarly, very low amounts of phosphorylated Akt (p-Akt) and phosphorylated I κ B (p-I κ B) were detected in the control cells. Meanwhile, the concentrations of p-Akt and p-I κ B were increased in the extracts of cells stimulated with 10 ng/ml of HGF. However, pretreatment with LY294002 before the addition of HGF did not increase the concentrations of p-I κ B and p-Akt, and PD98059 failed to inhibit the effect of HGF (Fig. 1).

RL95-2 cells in 6-well dishes were incubated with or without HGF for 5 min. Next, 5 μ g of nuclear extracts were analyzed by Western blot analysis. The concentrations of NF- κ B in the nucleus were significantly increased in the extracts of cells stimulated with 20 ng/ml of HGF ($p<0.05$). Pretreatment with LY294002 (10 μ M) and BAY11-7082 (10 μ M), an I κ B- α phosphorylation inhibitor, prior to the addition of HGF significantly decreased the concentration of NF- κ B in the nucleus ($p<0.05$). However, PD98059 (20 μ M) failed to block the effect of HGF (Fig. 2). These results suggest that HGF activates the PI3K/Akt and NF- κ B/I κ B signaling pathways in RL95-2 cells.

Effect of HGF on the expression of COX-2 mRNA in RL95-2 cells. We previously reported that HGF stimulates COX-2 protein expression in RL95-2 cells (28), but we have yet to examine COX-2 mRNA expression. The transcription factor NF- κ B was found to function upstream of COX-2 in colon cancer (30-32). Therefore, we tested whether HGF has an effect on the expression of COX-2 mRNA in RL95-2 cells. For this purpose, RL95-2 cells in 96-well plates were incubated with varying concentrations of HGF for 12 h, with some of the plates pretreated with BAY11-7082 (1 μ M) for 1 h prior to HGF stimulation. As shown by real-time PCR, the COX-2 gene expression levels significantly increased in the extracts of cells stimulated with 1, 5 and 10 ng/ml of HGF ($p<0.05$). Pretreatment with BAY11-7082 prior to the addition of HGF significantly decreased the gene expression levels of COX-2, relative to treatment with HGF alone ($p<0.05$) (Fig. 3A). These results suggest that HGF stimulates COX-2 mRNA expression through the activation of NF- κ B in RL95-2 cells.

HGF stimulation of COX-2 expression in RL95-2 cells through the PI3/Akt and NF- κ B/I κ B pathways. Next, we used pharmacological inhibitors to elucidate the signaling pathway for COX-2 induction. RL95-2 cells in 6-well dishes were incubated with or without 5 ng/ml of HGF for 24 h, with some

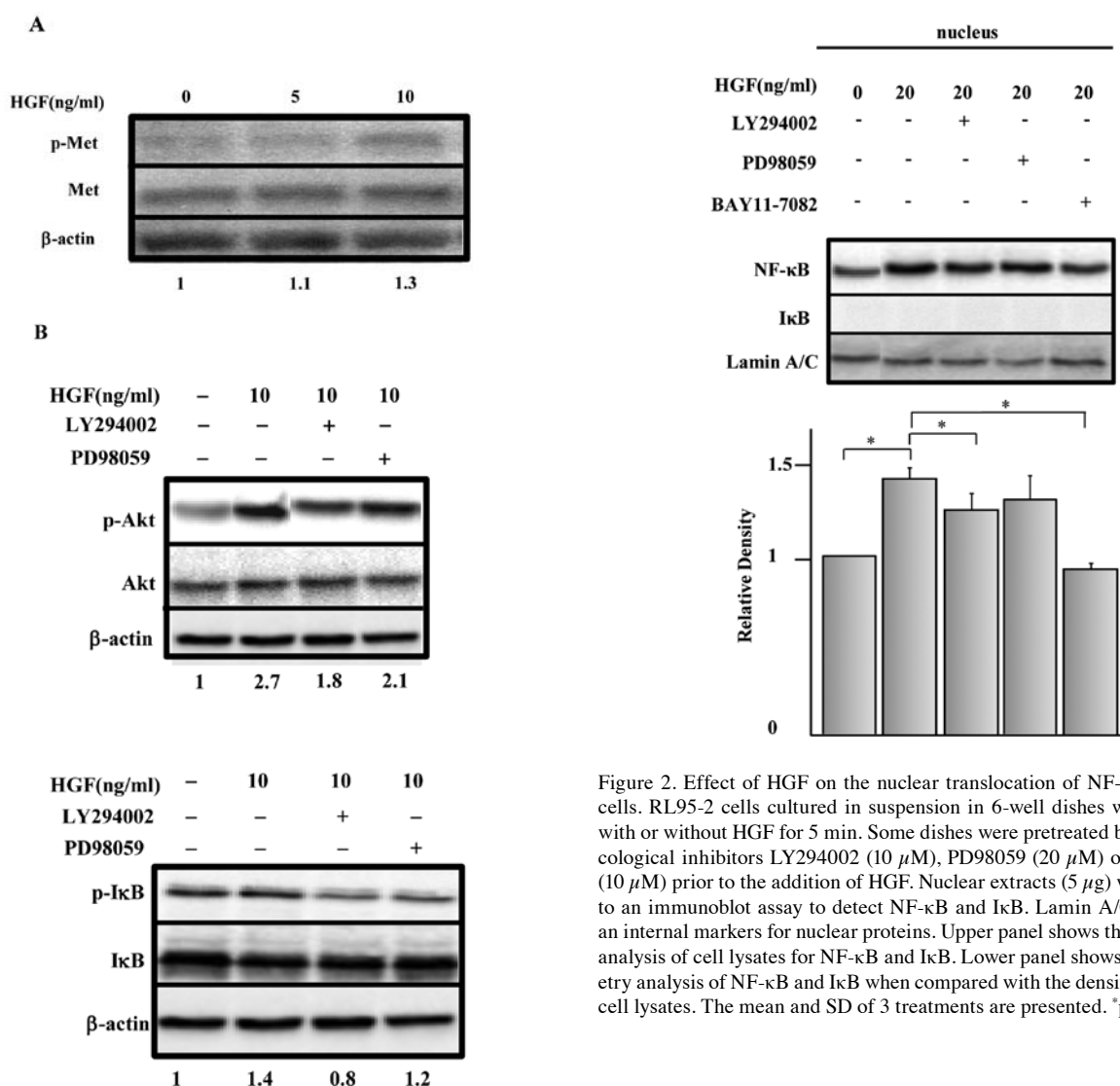


Figure 1. Effect of HGF on the phosphorylation of c-Met, Akt, and IκB in RL95-2 cells. RL95-2 cells cultured in suspension in 6-well dishes were incubated with varying concentrations of HGF for 5 min. Protein extracts (10 μg) were subjected to an immunoblot assay to detect the phosphorylation of (A) c-Met and (B) Akt and IκB. β-actin served as an equal loading control. The numbers below each gel band represent the densitometric band density ratios of the phosphorylated forms of c-Met, Akt and IκB. P-Met, phosphorylated c-Met; p-Akt, phosphorylated Akt; and p-IκB, phosphorylated IκB.

dishes pretreated with the pharmacological inhibitors before the addition of HGF. Protein extracts were then analyzed by Western blot analysis. Relative to the control, which showed a very low amount of COX-2 protein, the levels of COX-2 protein were higher in the extracts of cells stimulated with 5 ng/ml of HGF. Pretreatment with LY294002 (10 μM) and BAY11-7082 (1 μM) before the addition of HGF did not increase COX-2 protein concentrations. PD98059 (20 μM) failed to inhibit the effect of HGF (Fig. 3B). These results suggest that HGF stimulates COX-2 expression in RL95-2 cells through the PI3K/Akt and NF-κB/IκB pathways.

HGF inhibition of anoikis by activation of NF-κB and up-regulation of COX-2. We previously reported that HGF inhibited anoikis through the PI3K/Akt pathway in RL95-2

Figure 2. Effect of HGF on the nuclear translocation of NF-κB in RL95-2 cells. RL95-2 cells cultured in suspension in 6-well dishes were incubated with or without HGF for 5 min. Some dishes were pretreated by the pharmacological inhibitors LY294002 (10 μM), PD98059 (20 μM) or BAY11-7082 (10 μM) prior to the addition of HGF. Nuclear extracts (5 μg) were subjected to an immunoblot assay to detect NF-κB and IκB. Lamin A/C was used as an internal markers for nuclear proteins. Upper panel shows the Western blot analysis of cell lysates for NF-κB and IκB. Lower panel shows the densitometry analysis of NF-κB and IκB when compared with the density of untreated cell lysates. The mean and SD of 3 treatments are presented. *p<0.05.

cells (28). Here, we examined downstream of the PI3K/Akt signaling pathway and investigated whether activation of NF-κB is involved in the HGF effect on anoikis. RL95-2 cells were plated on tissue culture dishes coated with poly-HEMA in the absence or presence of HGF (10 and 20 ng/ml). Some dishes were pretreated with either BAY11-7082 (8 μM) or CAY10452 (10 μM), a selective COX-2 inhibitor, for 1 h before HGF stimulation. After 48 h of incubation, the viability of floating cells was measured by the MTS proliferation assay. Incubation with HGF significantly increased cell survival [10 ng/ml HGF: 80.8% (SD, 5.6%); 20 ng/ml HGF: 75.8% (6.8%)] compared with the control samples [68.1% (8.5%)] (Fig. 4). Pretreatment with BAY11-7082 significantly blocked HGF-mediated anoikis resistance [10 ng/ml HGF: 71.6% (6.9%); 20 ng/ml HGF: 68.6% (8.3%)]. Pretreatment with CAY10452 also succeeded in inhibiting HGF-mediated anoikis resistance [10 ng/ml HGF: 69.0% (5.8%); 20 ng/ml HGF: 68.3% (6.3%)].

Effects of HGF on cell migration. We examined the effect of HGF on the migratory capacity of RL95-2 cells. This cell line has been shown to migrate but not proliferate after treatment with HGF *in vitro* (14). We confirmed that RL95-2 cells did not proliferate after the addition of HGF (data not shown). Involvement of the c-Met-PI3K-Akt cascade in the migration

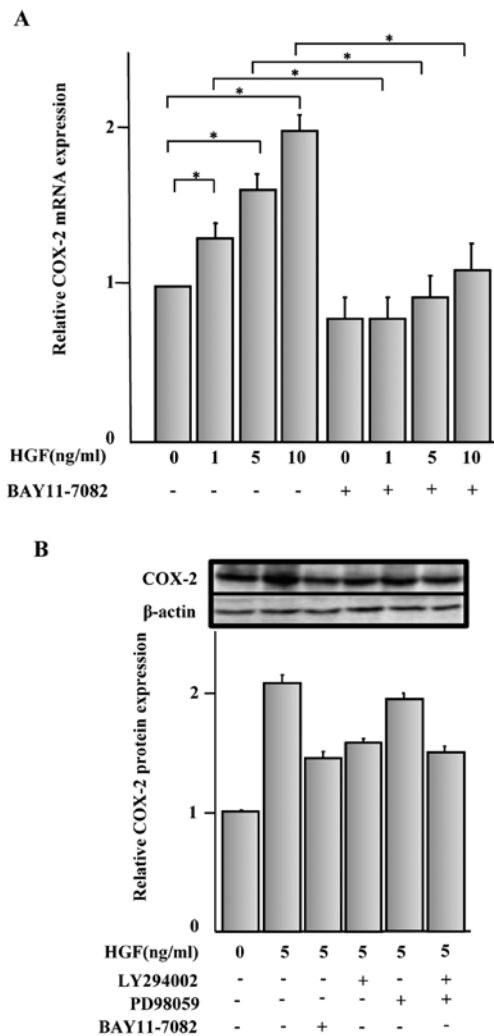


Figure 3. HGF stimulates COX-2 protein expression through the PI3/Akt and NF- κ B/I κ B pathways. (A) RL95-2 cells in 96-well plates were incubated with varying concentrations of HGF for 12 h. Some of the plates were pretreated with BAY11-7082 (1 μ M) for 1 h before HGF stimulation. Gene expression levels of COX-2 were analyzed by real-time PCR. Each value represents mean and SD from 3 independent experiments. * p <0.05. (B) RL95-2 cells in 6-well dishes were incubated with or without 5 ng/ml of HGF for 24 h. Some dishes were pretreated with the pharmacological inhibitors LY294002 (10 μ M), PD98059 (20 μ M), or BAY11-7082 (10 μ M) prior to the addition of HGF. Protein extracts were analyzed by Western blot analysis. Protein levels were determined by densitometry. β -actin served as an equal loading control. Each value represents the mean and SD from 3 independent experiments. * p <0.05.

of endometrial cancer cells was confirmed by the wound healing assay. Moreover, abrogation of the wound healing process was observed after pretreatment with LY294002 (5 μ M) and CAY10452 (10 μ M) (Fig. 5), further providing evidence that the HGF-induced migration of endometrial cancer cells may be regulated by COX-2 through the PI3K/Akt signal cascade.

Effect of HGF on cell invasion. We examined the effect of HGF on the invasive ability of RL95-2 cells. As seen in Fig. 6, HGF significantly increased the invasiveness of RL95-2 cells relative to the control (p <0.05). Pretreatment with LY294002 (5 μ M) or CAY10452 (10 μ M) significantly blocked the effect of HGF.

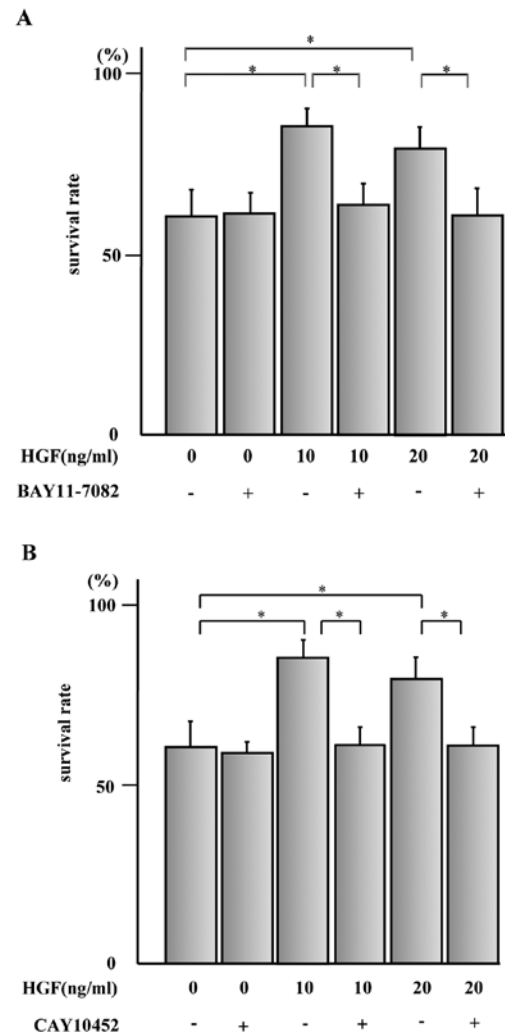


Figure 4. Effects of BAY11-7082 and CAY10452 on HGF-mediated anoikis resistance in RL95-2 cells. RL95-2 cells cultured in suspension in 6-well dishes were incubated with or without HGF. Some dishes were pretreated with BAY11-7082 (8 μ M) and CAY10452 (10 μ M), a selective COX-2 inhibitor, for 1 h before HGF stimulation. After a 48-h incubation, the viability of the floating cells was measured by the MTS proliferation assay. The survival rate was determined by setting the absorbance of the non-treated control cells to 100%. Each value represents the mean and SD of triplicates from 3 independent experiments. * p <0.05.

Discussion

We showed here that HGF up-regulates COX-2 through the NF- κ B and PI3K/Akt pathways in endometrial cancer cells and that HGF increases invasion, migration and resistance to anoikis by up-regulating COX-2 expression.

We previously reported that HGF significantly inhibited anoikis in endometrial cancer cells by up-regulating COX-2 expression (28). However, the intracellular signal transduction of COX-2 up-regulation by HGF stimulation has not yet been elucidated. Previous studies have reported that activation by HGF occurs primarily through two kinase cascades: the extracellular signal-related kinase (ERK) and PI3K/Akt signaling pathways (18-20). It has been reported that HGF inhibits anoikis in head and neck squamous cell carcinoma cells by activation of both the ERK and Akt signaling pathways (19). Sequencing of the human COX-2 promoter region

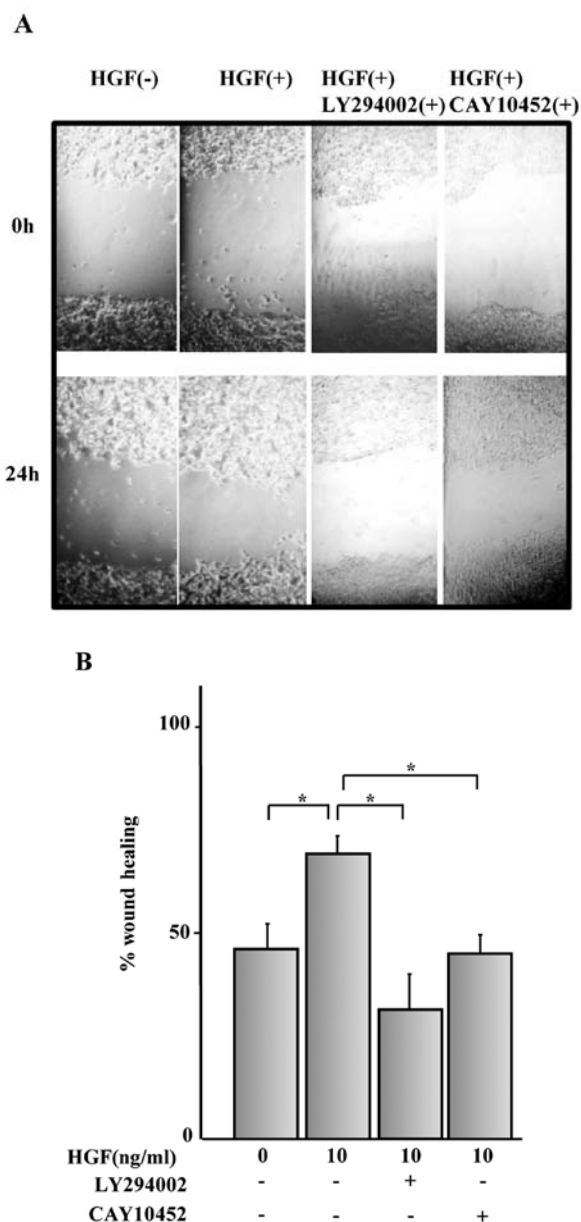


Figure 5. Effects of HGF on cell migration in RL95-2 cells. RL95-2 cells were grown to overconfluence. The center of the culture dishes was scraped once with a pipette tip. The migration of cells into the wound area was documented and evaluated after 24 h. Quantitative analysis of the percentage of wound healing was calculated using distances across the wound at 0 and 24 h. (A) Images of the cultures were captured immediately after the incision and after 24 h in culture. HGF(+): HGF at 10 ng/ml; HGF(+) LY294002(+): HGF at 10 ng/ml with 10 μ M LY294002; HGF(+) CAY10452(+): HGF at 10 ng/ml with 10 μ M CAY10452. (B) The extent of wound closure was determined after 24 h of incubation. Each value represents the mean and SD of triplicates from 3 independent experiments. * p <0.05.

has revealed the presence of NF- κ B consensus sites, which are important in the induction of COX-2 gene expression (33). NF- κ B is an essential transcription factor in the expression of vital genes as well as a number of genes involved in immune, inflammatory and growth responses (34,35). NF- κ B is present in the cytosol in an inactive form as a heterodimer of p50 and p65 subunits bound to one of the I κ B inhibitory proteins. Akt rapidly phosphorylates I κ B- α by an I κ B kinase (IKK), resulting in its ubiquitination and proteolytic degradation

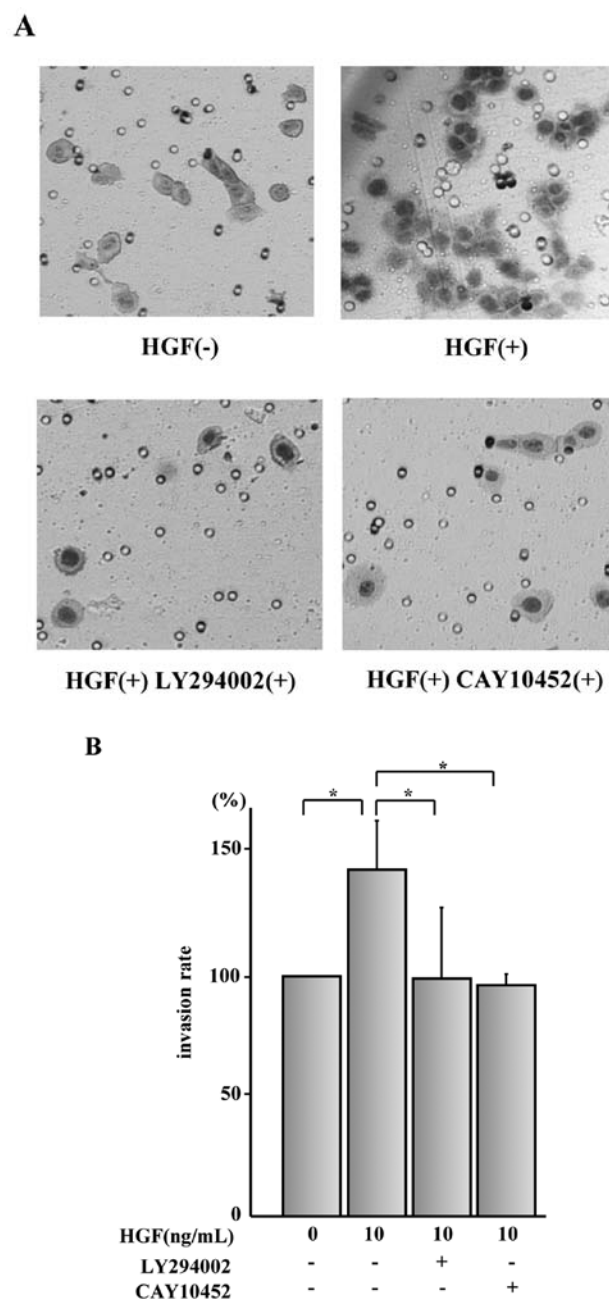


Figure 6. Effect of HGF on cell invasion in RL95-2 cells. The invasion assay was performed. RL95-2 cells were seeded on upper chamber plates and cultured in a serum-free medium in the absence or presence of a pharmacological inhibitor. In the lower chamber, DMEM/F-12 with 5% FBS in the absence or presence of HGF was loaded. Cells were incubated for 34 h. Invasive cells that penetrated through the pores and migrated to the underside of the membrane were stained with Diff-Quik. After selecting 5 regions randomly, the number of cancer cells that had migrated was counted. (A) The cell images are representatives of membranes of the chamber insert. HGF(+): HGF at 10 ng/ml; HGF(+) LY294002(+): HGF at 10 ng/ml with 10 μ M LY294002; HGF(+) CAY10452(+): HGF at 10 ng/ml with 10 μ M CAY10452. (B) Quantitative analysis of invaded cells. The percentage of control invasive cells was determined by setting the average invaded cell number of the control, non-treated cells as 100%. Each value represents mean and SD of triplicates from 3 independent experiments. * p <0.0.

(36,37). The NF- κ B/I κ B- α complex subsequently translocates NF- κ B into the nucleus, where it activates apoptosis-related gene transcription (38-40). The activation of PI3K/Akt may trigger the expression of several survival genes via NF- κ B.

A previous report has shown that NF- κ B functional activity is required for HGF-cMet-mediated cell protection in human prostate cancer (DU-145) and Madin-Darby canine kidney (MDCK) epithelial cells (41). Similarly, our results indicate that the PI3K/Akt and NF- κ B/I κ B signaling pathways in endometrial cancer RL95-2 cells may play a crucial role in the expression of COX-2 by HGF stimulation. In this study, we clarified that the stimulation by HGF enhances migration and invasion in endometrial cancer cells and showed that these effects were further attenuated by the addition of an inhibitor of I κ B- α phosphorylation (BAY11-7082) and a selective COX-2 inhibitor (CAY10452).

Knockdown of COX-2 expression by shRNA or COX-2 inhibitors (celecoxib) has been shown to have potent antitumor and chemosensitization activities, comparable to the effects of taxanes in laryngeal cancer (42) and to doxorubicin in breast cancer (43). COX-2 may also become an important target for regulating the progression of uterine cancer. However, the effects of the knockdown of COX-2 on invasion, migration, and anoikis in endometrial cancer cells will need to be further examined, and it will be necessary to consider what type of material changes occur downstream of COX-2. It has been reported that PGE₂ produced in the tumor microenvironment by the overexpression of COX-2 in tumoral and inflammatory cells may promote the growth of head and neck squamous carcinoma cells (44). Hence, in endometrial cancer, PGE₂ produced in the tumor microenvironment by the overexpression of COX-2 may be involved in tumor progression. Further studies are needed to clarify these issues.

HGF has been shown to induce a transient expression of Snail, which is required for the induction of cell scattering and epithelial-mesenchymal transition (45). E-cadherin-mediated cell-cell adhesion is frequently lost during malignant tumor progression by gene mutation, transcriptional repression, or protein degradation (46-48). Recently, it has been shown that down-regulation of E-cadherin expression is mediated by the zinc-finger transcription factor Snail (49,50). By binding to specific DNA sequences known as E-boxes in the E-cadherin promoter, Snail represses E-cadherin transcription, leading to the disruption of adherens junctions (51,52). Indeed, our data showed that HGF inhibits anoikis resistance and induces migration and invasion. We also analyzed the change in apoptosis-related genes by the addition of HGF to suspended RL95-2 cells by using cDNA microarray analysis. Snail mRNA levels were approximately 2-fold up-regulated, and E-cadherin mRNA levels were reduced in RL95-2 cells stimulated with HGF (data not shown). Collectively, the results showed that HGF induced cell scattering and epithelial-mesenchymal transition, inhibited anoikis, and stimulated migration and invasion in RL95-2 cells.

In summary, HGF exhibited an anti-apoptotic action that was exerted by the PI3K/Akt pathway, triggered NF- κ B activation and up-regulated COX-2 gene expression. Stimulation by HGF enhanced anoikis resistance and migration and invasion in endometrial cancer cells. This is the first report to clarify the intracellular signaling pathway through which HGF up-regulates COX-2 gene expression in endometrial cancer cells.

Our present study provides a novel therapeutic option for molecular-targeted treatment of endometrial cancer via

the suppression of anti-apoptosis mechanisms during cancer metastasis.

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