Hepatocyte growth factor induces anoikis resistance by up-regulation of cyclooxygenase-2 expression in uterine endometrial cancer cells

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Abstract. Cyclooxygenase-2 (COX-2) has been implicated in the promotion of carcinogenesis. Although the role of COX-2 in endometrial cancer remains unclear, recent experiments suggest that COX-2 antagonizes cell apoptosis, increases the invasiveness of malignant cells, and promotes angiogenesis. Hepatocyte growth factor (HGF) is a mesenchymal-derived cytokine and the interaction between HGF and its tyrosine kinase receptor, c-Met proto-oncogene, is associated with tumor progression and metastasis. To investigate the molecular mechanism of HGF-induced anoikis resistance, we analyzed the signal transduction and COX-2 expression in endometrial cancer cells. Here, we show i) the expression of COX-2 protein significantly increased in a dose-dependent manner after HGF stimulation in endometrial cancer cell lines (HEC-IB and RL95-2), reaching 200-270% stimulation at the highest doses of HGF tested (40 ng/ml); ii) flow cytometry and TUNEL analyses revealed that HGF significantly inhibited anoikis of RL95-2 cells; iii) phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), but not mitogen-activated protein kinase/ERK kinase (MEK) inhibitor (PD98059), specifically blocked HGF-mediated anoikis resistance in RL95-2 cells; and iv) COX-2 inhibitor, Meloxicam, abrogated HGF-mediated anoikis resistance. Our data suggest that HGF induces anoikis resistance in endometrial cancer cells possibly through PI3K/Akt pathway-dependent up-regulation of COX-2 expression.

Introduction

Much attention has been focused on cyclooxygenase (COX), the rate-limiting enzyme that converts arachidonic acid to prostaglandins. Two COX isoforms have been characterized. COX-1 is expressed constitutively in most tissues, which facilitates a homeostatic function. COX-2 is not usually expressed in normal tissue, but is rapidly inducible by a variety of agents such as growth factors, oncogenes, prostaglandins, and cytokines, and plays a key role in the inflammatory response (1,2). COX-2 has also been implicated in the promotion of carcinogenesis. Up-regulation of COX-2 expression has been found in several epithelial cancers such as colon (3), head and neck (4), lung (5), breast (6), gastric (7), and prostate cancers (8). These findings suggest that COX-2 up-regulation may be a common mechanism in epithelial carcinogenesis. COX-2 is up-regulated in a variety of malignancies and favors the growth of malignant cells by stimulating proliferation and angiogenesis (9,10). Over-expression of COX-2 also reduces apoptosis (11,12) in several epithelial type cancers.

Uterine endometrial carcinoma is the fourth most frequent malignancy in females (13). Despite the high prevalence, the exact molecular mechanism of endometrial carcinogenesis is been poorly understood. COX-2 is overexpressed in endometrial cancer and is associated closely with parameters of tumor aggressiveness (14). Recent reports indicate that COX-2 inhibitors may be effective in the treatment of endometrial cancer via the suppression of angiogenesis (15).

Hepatocyte growth factor (HGF), known as a scatter factor, is a mesenchymal-derived cytokine (16) and the interaction between HGF and its tyrosine kinase receptor, c-Met proto-oncogene is associated with tumor progression and metastasis (17). The overexpression of HGF/c-Met has been observed in many malignancies, such as breast (18), gastrointestinal (19), renal (20), head and neck (21) and endometrial cancer (22). Recent studies suggest that the interaction between HGF and c-Met stimulates tumor growth (23) and angiogenesis (24,25), or suppresses tumor apoptosis (26) in various malignant tumors. In endometrial cancer, the interaction between HGF and c-Met is related to tumor invasion (27-29), angiogenesis (22) and anti-apoptosis (30). However, few studies have been reported in which the interaction between HGF and COX-2 in tumor progression and metastasis has been investigated. HGF triggers the activation of the COX-2 gene in gastric epithelial cells (31), demonstrating that angiogenesis is susceptible to the inhibition of COX-2 (32). The transcriptional and post-transcriptional regulation of COX-2 by HGF is well characterized in gastric epithelial (31) and head and neck

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squamous cancer cells (33). In a previous study, COX-2 selective nonsteroidal anti-inflammatory drugs inhibited HGF-induced angiogenesis with human umbilical vein endothelial cells (HUVECs) (34).

Anoikis is one form of apoptosis in which the apoptosis of epithelial cells is induced by a loss of matrix attachment. This mechanism is critical in maintaining the tissue architecture. Tumor cells lose matrix attachment during metastasis, thus the inhibition of anoikis is an important step for the tumor progression and metastasis (35-37). HGF inhibits anoikis by the induction of COX-2 in head and neck squamous cell carcinoma (34). However, there have been no reports on the relationship between anoikis and gynecological cancer. The aim of this study was to investigate the interaction between HGF and COX-2 in endometrial cancer, especially focusing on the resistance of anoikis.

Materials and methods

Reagent. Meloxicam, a selective COX-2 inhibitor, was provided by Boehringer Ingelheim, Laboratories, Ingelheim, Germany. PD98059, an inhibitor of MEK activation, was purchased from Biomol Research Labs, Inc. LY294002, a PI3K/Akt inhibitor, was from Calibiochem. Minimum essential medium (MEM), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12), and polyhydroxyethylmethacrylate (poly-HEMA) were from Sigma-Aldrich, St. Louis, MO, USA. Fetal bovine serum (FBS) was from Invitrogen, Carlsbad, CA, USA.

Cell culture. Human endometrial cancer cells (HEC-1B and RL95-2) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in 75 cm^2 bottles at 37˚C in 95% air and 5% CO_2. HEC-1B cells were maintained in MEM supplemented with 10% FBS. RL95-2 cells were grown in DMEM/F-12 and supplemented with 10% FBS. Cells were plated in log growth phase and onto 6-well plates, then serum starved for 12 h prior to the experiments.

Anoikis induction. To prevent cell adhesion, culture dishes were coated with poly-HEMA, as described by Folkman (38). Culture dishes were coated with the diluted poly-HEMA solution and ethanol solvent was left to evaporate overnight. Then, cultured cells were plated at a concentration of $1x10^6$/ml on culture dishes, cultured in DMEM/F-12 and supplemented with 1% FBS for 48 h in suspension.

MTS proliferation assay. Cell viability was determined by MTS proliferation assay using Cell Titer 96 Aqueous One solution cell proliferation assay (Promega, Corporation, Madison, WI, USA) according to the protocol. Briefly, MTS solution (20 μl/well) was added and after 2 h of culture at 37˚C in 95% air and 5% CO_2, the conversion of MTS to formazan was measured in a plate reader at 490 nm. Survival rate was represented by the percentage of the absorbance of formazan at 0 h from detachment.

Flow cytometry. Anoikis was detected using flow cytometry by staining cells with FITC-labeled Annexin V and propidium iodide (PI) labeling (MBL, Nagoya, Japan). RL95-2 cells were cultured in suspension for 48 h at 37˚C in the absence or presence of HGF, washed and re-suspended in 85 μl of binding buffer. The cells were then stained with 10 μl of FITC-conjugated Annexin V and 5 μl of PI and immediately analyzed by FACScan flow cytometry (Becton Dickinson, USA). Cells that were stained with both Annexin V-FITC and PI were recognized as late apoptotic or secondary necrotic, while those that were stained only with Annexin V-FITC were recognized as early apoptotic.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL). Suspended cells were placed on the silane coated slides and fixed in 4% paraformaldehyde/PBS solution. The slides were incubated with TdT labeling reaction (*in situ* Apoptosis detection kit, Takara Biomedical, Japan) for 60 min at 37˚C in a humidified environment, rinsed with PBS, incubated with anti-FITC-HRP and conjugated for 15 min at 37˚C. Color development was achieved by incubation in DAB substrate and the cells were counterstained with 3% methylgreen. Apoptotic cell percentage was calculated by counting TUNEL staining positive cells in 200 cells from each of the three independent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from each HEC-1B and RL95-2 cell line using the Micro-to-Midi total RNA purification system (Invitrogen) according to the protocol recommended by the manufacturer. Reverse transcription was carried out by using the thermoscript RT-PCR System (Invitrogen). The primers for single stranded cDNA were generated from 5 μg total cellular RNA using an avian reverse transcriptase and oligo-(dT) - primers. The primers for sequence were as follows: for COX-2, 5'-TCC TTG CTG TTC CCA CCC ATG-3' (forward) and 5'-CAT CAT CAG ACC AGG CAC CAG-3' (reverse) and for c-MET, 5'-CAG TGA TGA TCT CAA TGG GCA AT-3' (forward) and 5'-AAT GCC CTC TTC CTA TGA CTT C-3' (reverse). For the positive control, ß-actin primer pair was purchased from R&D Systems (Abingdon, UK). Samples were denatured at 94˚C for 2 min followed by 30 cycles each: 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 1 min, and ended with a 5 min extension at 72˚C and stored at 23˚C. PCR products were run by electrophoresis on a 3% agarose gel and DNA was visualized using ethidium bromide staining.

Protein extraction. Total protein were extracted from whole HEC-1B and RL95-2 cells in 100-mm dishes using M-PER mammalian protein reagent (Pierce, USA) with 1% Halt™ protease inhibitor cocktail (Pierce), following the protocol suggested by the manufacturer. Protein concentration was measured using a DC protein assay (Bio-Rad) according to the manufacturer's protocol.

Western blot analysis. The lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins on the gel were transferred onto a nitrocellulose membrane. The membrane was then washed with PBS containing 0.1% Tween-20 (PBST) and blocked in PBST containing 5% skim

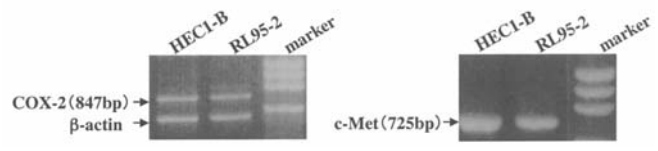

Figure 1. COX-2 mRNA and c-Met mRNA were detected in human endometrial cancer cells by RT-PCR. ß-actin was used as an indicator for equal lane loading. The 847 and 725 bp bands each represent COX-2 mRNA and c-Met mRNA, respectively.

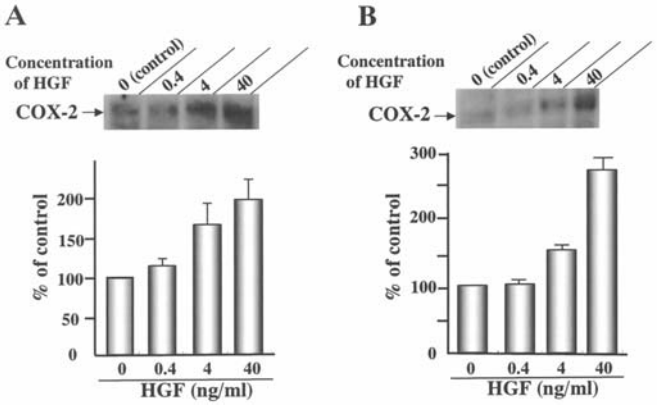

Figure 2. Effects of HGF on expression of COX-2 protein in HEC1-B (A) and RL95-2 (B) cells. COX-2 protein expression levels were measured by Western blot analysis. Incubation of HGF at concentrations of 4 to 40 ng/ml significantly increased COX-2 protein levels after 6 h of incubation in both cell lines compared with untreated cultures (control). The experiment was repeated three times. Values are the mean ± SD (n=3). *P<0.01.

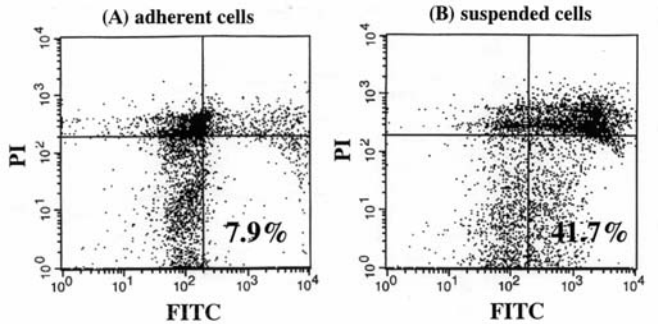

Figure 3. Detection of apoptosis by flow cytometry using FITC-conjugated Annexin V and PI staining. To induce anoikis, RL95-2 cells were plated at a concentration of $1x10^6$/ml on the tissue culture dishes coated with 10 mg/ml poly-HEMA. After cell suspension for 48 h, the suspended cells were collected and analyzed by flow cytometry.

milk. After blocking the membrane, the membrane was probed with an anti-human COX-2 mouse monoclonal antibody diluted 1:5000 (IBL, Gunma, Japan) for 12 h at 4˚C. After the membrane was washed with PBST, bound antibodies on the membrane were detected with the use of anti-mouse immunoglobulin G conjugated to horseradish peroxidase and visualized with the use of enhanced chemiluminescence (Amersham, Aylesburg, UK).

Statistical analysis. Each experiment was performed in duplicate or triplicate. Values are expressed as mean ± standard deviation (SD). The difference between the groups was analyzed by using the Student's t-test and ANOVA. $P<0.05$ was considered as statistically significant.

Results

Expression of COX-2 and c-Met mRNA in endometrial cancer cell lines. To confirm the expression of COX-2 and c-Met in HEC1-B and RL95-2 cell lines at the mRNA level, we carried out RT-PCR analysis. We recognized an 847 bp band representing COX-2 mRNA and a 725 bp band representing c-Met mRNA in both cell lines (Fig. 1).

Effect of HGF on expression of COX-2 protein in HEC1-B and RL95-2 cells. HEC1-B and RL95-2 cells were incubated with varying concentrations of HGF for 6 h. A very low amount of COX-2 protein was detected by Western blot analysis in the controls. Concentrations of COX-2 were increased in extracts of cells stimulated with 4 and 40 ng/ml HGF. COX-2 production in HEC-IB cells was enhanced by HGF in a dose-dependent manner, reaching 198±25% up-regulation at the highest doses of HGF tested (40 ng/ml) (Fig. 2A). Similar results are shown in RL95-2 cells (Fig. 2B). All these results from the assays together suggest that COX-2 expression was stimulated by HGF.

Detection of anoikis. Anoikis was induced in RL95-2 cells plated on the tissue culture dishes coated with poly-HEMA. To confirm anoikis of the suspended cells, flow cytometric analysis using annexin V and PI double staining was conducted. A loss of cell adhesion strongly increased Annexin V-positive (early apoptotic) cells at a 48 h time point (41.7%, Fig. 3B) compared with the adherent cells (7.9%, Fig. 3A). In addition, TUNEL analysis was performed to confirm nuclear fragmentation. We found significantly more apoptotic cells in suspended cells at 48 h after the loss of matrix contact (33.6±3.4%, Fig. 4A2) than in adherent cells (10.6±3.5%, Fig. 4A1).

HGF inhibited anoikis in RL95-2 cells. To examine the effect of HGF on anoikis resistance, RL95-2 cells were plated on the tissue culture dishes coated with poly-HEMA in the absence or presence of HGF (4 ng/ml). After 48 h of incubation, floating cells were collected for TUNEL assay to count apoptotic cells. As shown in Fig. 5, treatment of HGF at the concentration of 4 ng/ml significantly decreased apoptotic cells of RL95-2 cells (23.0±2.7%) compared with the control cells (33.6±3.4%). To further confirm this finding, viability of a floating cell was determined by MTS proliferation assay. Incubation with HGF at the concentration of 4 ng/ml significantly increased cell survival of RL95-2 cells (76.7±3.4%) compared with the control samples (63.3±7.6%) (Fig. 6).

HGF inhibited anoikis through PI3K/Akt pathway. We then examined whether the ERK or PI3K/Akt signaling pathway is involved in the HGF-mediated inhibition of anoikis in RL95-2 cells. Cells were pretreated with MEK inhibitor PD98059 (20 μM) or PI3K inhibitor LY294002 (10 μM) for 30 min and then stimulated by HGF for 48 h. The suspended cells were collected and apoptotic cells were counted by the TUNEL

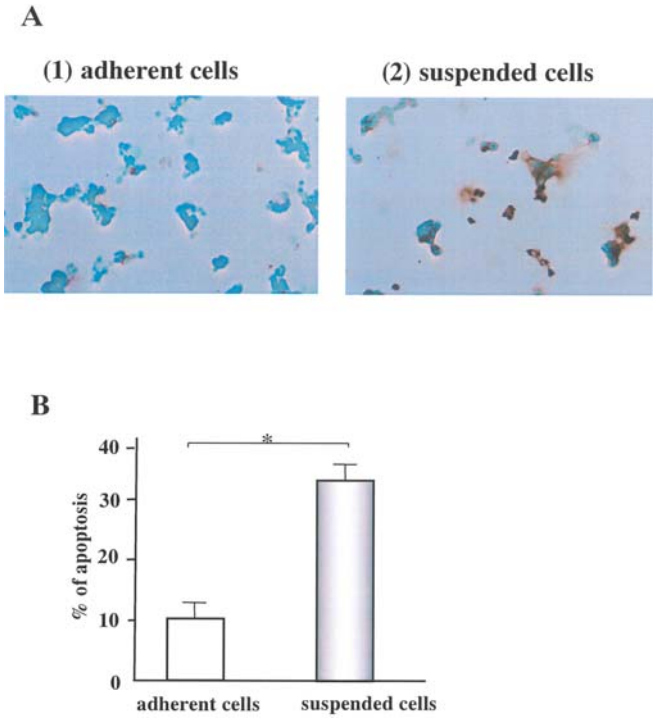

Figure 4. Nuclear fragmentation of RL95-2 cells was confirmed by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). (A) Representatives of DNA nick end-labelling of RL95-2 cells (1) adherent cells (2) suspended cells which were incubated for 48 h in poly-HEMA coated dishes (x100 magnification). (B) Data represent the mean ± SD (n=3). *P<0.05.

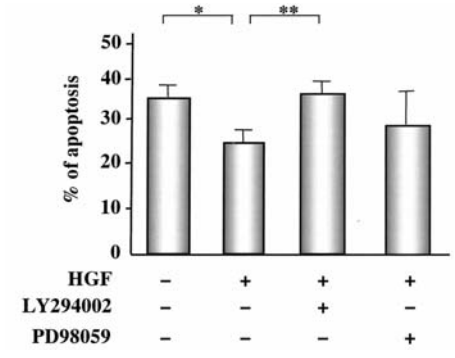

Figure 5. To determine the effect of HGF on anoikis, apoptotic cells were counted by TUNEL assay. Cells were pretreated with pharmacological inhibitors (PD98059, 20 μM or LY294002, 10 μM) for 30 min and then stimulated with HGF (4 ng/ml) for 48 h. The mean ± SD of the three treatments are presented. *P<0.05 and **P<0.01.

method. Pretreatment of LY294002 before the addition of HGF significantly increased apoptotic cells (34.9±3.4%) compared with the only cells incubated with HGF (23.0±2.7%) (Fig. 5). PD98059 failed to affect the HGF-induced inhibition of apoptosis (28.8±5.2%, P=0.102). A similar result was obtained from the MTS assay. Fig. 6 demonstrates that LY294002 significantly blocked HGF-mediated anoikis resistance in RL95-2 cells, whereas PD98059 does not.

Meloxicam inhibited HGF-mediated anoikis resistance in RL95-2 cells. We then examined the involvement of COX-2

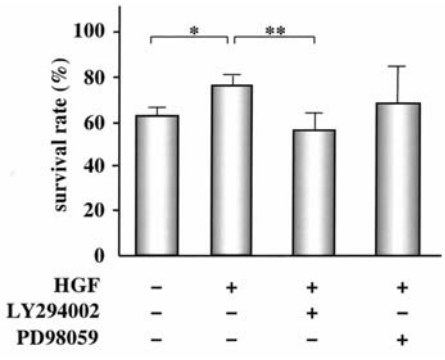

Figure 6. To determine the effect of HGF on anoikis, MTS proliferation assay was conducted. RL95-2 cells treated with HGF at the concentration of 4 ng/ml were plated at a concentration of $1x10^6$/ml on the tissue culture dishes coated with 10 mg/ml poly-HEMA. Then, 48 h after cell suspension, the cells were collected and cell viability was determined by MTS proliferation assay. The assays were performed in triplicate, and the result represents one of three independent experiments. Data represent the mean ± SD (n=3). *P<0.05 and **P<0.01.

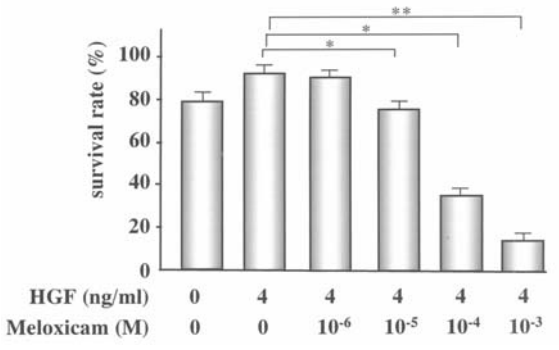

Figure 7. Effect of selective cyclooxygenase-2 inhibitor, Meloxicam, on HGF-mediated anoikis resistance in RL95-2 cells. Cells were pretreated with Meloxicam at concentrations from 10^{-5} M to10^{-3} M before the administration of HGF (4 ng/ml) and then treated with HGF 48 h in suspension. The cells were then collected and cell viability was determined by MTS proliferation assay. The assays were performed in triplicate, and the result represents one of three independent experiments. Data represent the mean ± SD (n=3). *P<0.05 and **P<0.01.

on HGF-mediated anoikis resistance in RL95-2 cells. RL95-2 cells pretreated with a selective COX-2 inhibitor, Meloxicam, for 30 min were stimulated with HGF for 48 h in suspension. The suspended cells were collected and cell viability was determined by MTS proliferation assay. Fig. 7 shows Meloxicam at concentrations from 10^{-5} M to10^{-3} M which significantly blocked HGF-mediated anoikis resistance in RL95-2 cells in a dose-dependent manner.

Discussion

A growing body of evidence has accumulated that suggests that the interaction between HGF and c-Met stimulates tumor growth, invasion and angiogenesis or suppresses tumor apoptosis in endometrial cancer (27-30). However, few studies have been reported on the interaction between HGF and COX-2 on the tumor progression and metastasis. Jones *et al* reported that HGF triggers the activation of COX-2 gene in gastric epithelial cells (31). Therefore, in the present study,

we investigated the effect of COX-2 on the HGF-mediated anoikis resistance in endometrial cancer. Previous reports reported that endometrial cancer cells, HEC-1B and RL95-2, express COX-2 mRNA (41). As expected, we were able to confirm COX-2 and c-Met mRNA expression in both HEC-1B and RL95-2 endometrial cancer cell lines using RT-PCR, and that COX-2 protein significantly increased 6 h after HGF stimulation in these cells. To the best of our knowledge, this is the first report about the interaction between HGF and COX-2 in endometrial cancer. It has been observed that HGF is secreted by endometrial stromal cells, and endometrial carcinoma cells express its receptor, c-Met (28), suggesting that the HGF/c-Met pathway through COX-2 expression may play a role in endometrial cancer progression. HGF inhibits anoikis by the induction of COX-2 in head and neck squamous cell carcinoma cells (HNSCC) (33). In this study, we confirmed that HGF inhibits anoikis possibly through the PI3K/Akt pathway. Hasegawa *et al* reported that the selective COX-2 inhibitor, etodolac, produced inhibition of cell proliferation through G1-phase cell cycle arrest and telomerase activity in an endometrial cancer cell line (39). In addition, COX-2 inhibitor, NS 398, inhibited the anti-apoptotic effect of COX-2 in human bladder cancer cells, focusing on anoikis, apoptosis which is induced by a loss of matrix attachment (40). Furthermore, we reported that a selective COX-2 inhibitor, Meloxicam, inhibited HGF-mediated anoikis resistance in endometrial cancer cells. Taken together, the present data allow us to hypothesize that HGF significantly inhibited anoikis, possibly through the PI3K/Akt-mediated up-regulation of COX-2 expression in endometrial cancer.

COX-2 inhibits apoptosis through the up-regulation of Mcl-1 expression, which is one of the Bcl-2-family proteins (11). In addition, increased PGE2 levels modulate apoptosis and Bcl-2 expression in colon cancer cells (45). Thus, it is possible that HGF-mediated anoikis resistance may be related to Bcl-2-family protein expression mediated by COX-2 up-regulation in endometrial cancer.

We did not investigate the upstream target (s) of COX-2 gene and protein expression. Previous studies have reported that HGF activates mainly two kinase cascades, the extracellular signal-related kinase (ERK) and PI3K/Akt signaling pathways (42-44). HGF inhibits anoikis in HNSCC cells by the activation of both ERK and Akt signaling pathways (43). In RL95-2 cells, however, the PI3K/Akt signaling pathway may play a crucial role in HGF-mediated anoikis resistance. Akt signals induce COX-2 expression through the NF-κB/IκB pathway in mutated PTEN endometrial cancer cells (41). The transcription factor NF-κB can function upstream of COX-2 in colon cancer (46). Thus, it is possible that HGF stimulation activates Akt and the NF-κB/IκB pathway upstream of COX-2 for the inhibition of anoikis in endometrial cancer. Further examination is needed to elucidate the molecular mechanism.

In conclusion, we demonstrated, for the first time, that HGF inhibits anoikis by the activation of the PI3K/Akt signaling pathway-dependent COX-2 overexpression and the COX-2 selective inhibitor inhibits HGF-mediated anoikis resistance in endometrial cancer cells. Therefore, COX-2 expression through the PI3K/Akt pathway may play an important role of HGF-mediated anoikis resistance in endometrial cancer cells. The present data provide a new therapeutic option whereby COX-2 inhibitors may be candidates for the treatment of endometrial cancer.

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