Abstract. Protease-activated receptor-2 (PAR-2) has shown strong pro-angiogenesis activity physiologically and pathologically. This study aimed to explore PAR-2 regulation of pro-angiogenesis gene expression and the underlying molecular pathways in gastric cancer cells. MKN28 human gastric cancer cells were treated with trypsin, a PAR-2 activator, and subjected to real-time reverse transcription polymerase chain reaction (qRT-PCR), western blotting and ELISA for gene expression analyses. ERK1/2 phosphorylation and p38 MAP kinase inhibitors (PD98059 and SB203580, respectively) were used to block their gene activities. PAR-2 mRNA and protein were expressed in MKN-28 cells and activated by trypsin treatment. Trypsin-activated PAR-2 protein significantly enhanced expression of vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) mRNA and protein in gastric cancer cells in a dose- and time-dependent manner. PAR-2 activation also induced the phosphorylation of ERK1/2 and p38 MAP kinase, but the ERK1/2 and p38 inhibitors blocked the activated PAR-2-induced VEGF and COX-2 expression in gastric cancer cells. PAR-2-induced expression of VEGF and COX-2 mRNA and protein in gastric cancer MKN28 cells was mediated by activation of an ERK1/2- and p38 MAP kinase-dependent pathway. Thus, PAR-2 may serve as a promising target for anti-angiogenesis therapy to treat gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world. In China alone, ~463,000 new patients will be diagnosed with gastric cancer and 352,000 patients will die annually, accounting for nearly half of the new cases and deaths of gastric cancer in the world (1). Clinically, gastric cancer rarely presents with noticeable early signs and symptoms in the early stage; thus, most patients are diagnosed at advanced stages (2). To date, various treatment options, such as surgery, radiotherapy, and chemotherapy, are routinely used to manage gastric cancer patients, but the overall 5-year patient survival rate still remains at only ~26% (3,4). The significant failure of the treatment and the poor survival rate are due to diagnosis at the later stages, which make surgical resection impossible; therefore, innovative treatment strategies and approaches to early gastric cancer diagnosis could significantly improve survival and treatment outcome of this disease.

It has been well documented that angiogenesis plays an important role in growth and metastasis of gastric cancer (5,6). Thus, anti-angiogenesis has been used as a potential and promising strategy in gastric cancer treatment by developing anti-angiogenesis drugs in clinical trials against gastric cancer (6-8). A comprehensive understanding of angiogenesis mechanisms responsible for gastric cancer progression could lead to novel treatment approaches. To this end, our research has focused on the crucial molecules involved in gastric cancer angiogenesis.

Protease-activated receptors (PARs) are a recently described subfamily of G-protein-coupled receptors with seven transmembrane-spanning domains and are comprised of four proteins, i.e., PAR-1, PAR-2, PAR-3, and PAR-4 (9). Among the four receptors, PAR-2 is mainly activated by trypsin and other trypsin-like serine proteases through cleavage of the extracellular N-terminal domain, which in turn enables the N-terminus of the protein to bind to the receptor itself as a tethered ligand to activate G-protein-coupled signal transduction pathways (10). PAR-2 is widely expressed in a
The PCR was set at 94˚C for 3 min, followed by 30 cycles of using a Takara PCR amplification kit (Takara) in a total volume manufacturer's instructions. Next, PCR amplification was performed then reverse transcribed into cDNA using a PrimeScript™ RT Reagent kit (Takara, Dalian, China) according to the manu-

Beckman Coulter DU 530 spectrophotometer. The RNA was measured by the optical densities at 260 and 280 nm using a 

CA, USA) according to the manufacturer's protocol. The 

cultured cells using the TRIzol reagent (Invitrogen, Carlsbad, 

USA). 

Materials and methods

Cell lines and culture. A well-differentiated human tubular gastric adenocarcinoma MKN-28 cell line was kindly provided by Professor Fang-Dong Men of The Institute of Oncology, China Medical University, Shenyang, China. The human lung adenocarcinoma A549 cell line was purchased from the Chinese Academy of Sciences, Shanghai, China. These cell lines were cultured in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37˚C in a humidified atmosphere containing 95% air and 5% CO₂. Cells were seeded at 1x10⁶ cells per well in 6-well plates for reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis, or at 5x10⁵ cells per well in 12-well plates for ELISA. In each experiment, MKN-28 cells were first deprived of FBS for 2 h and then treated with trypsin, SB20358 (a p38 MAP kinase inhibitor), or PD98059 (a specific inhibitor to block ERK1/2 phosphorylation). Trypsin was purchased from Amresco (Solon, OH, USA), and SB20358 and PD98059 were from Sigma Chemical Co. (St. Louis, MO, USA).

RNA isolation and RT-PCR. Total RNA was isolated from the cultured cells using the TRIzol reagent (Invitrogen, Carlshad, CA, USA) according to the manufacturer's protocol. The concentration and purity of the freshly isolated RNA were measured by the optical densities at 260 and 280 nm using a Beckman Coulter DU 530 spectrophotometer. The RNA was then reverse transcribed into cDNA using a PrimeScript™ RT Reagent kit (Takara, Dalian, China) according to the manu-
factory's instructions. Next, PCR amplification was performed using a Takara PCR amplification kit (Takara) in a total volume of 25 µl containing 2 µl of cDNA (0.5 µg), 200 µM each dNTP, 1.25 units of Taq polymerase, and 0.2 µM each primer (Takara). The PCR was set at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 30 sec, and a final extension at 72˚C for 10 min. The amplified PCR products were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The primers used for detection of PAR-2 mRNA were 5'-GGC CAA TCT GGC CTT GGC TGA C-3' (sense) and 5'-GGG CAG GAA TGA AGA TGG TCT GC-3' (antisense), and for β-actin they were 5'-GCC AAC CTG GAA AAG ATG-3' (sense) and 5'-CCA GGA TAG AGC CAC CAA T-3' (antisense).

Real-time RT-PCR. To quantify PAR-2 mRNA levels, real-time RT-PCR (qRT-PCR) was performed with the LightCycler thermal cycling system (Roche Diagnostics Corp., Indianapolis, IN, USA) using a SYBR® Premix Ex Taq™ II (Perfect real-
time) kit (Takara) as described by the manufacturer. Briefly, the total qPCR volume was 20 µl, which contained 2 µl of cDNA template (100 ng), 10 µl of 2X SYBR Premix Ex Taq II, 0.8 µl of primers (10 µM each), and 6.4 µl of ddH₂O. After an initial denaturation at 95˚C for 3 min, PCR was performed for 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The primer sequences for human VEGF were 5'TGA CGG ACA GAC AGA CAG ACA CC-3' (sense) and 5'AGA ACA GCC CAG AAG TTT TTC TAC CAG A-3' (antisense), for COX-2 they were 5'TCA CAG GCT TCC ATT GAC CAG-3' (sense) and 5'CCG AGT TTC TTG GCC GTA AG-3' (antisense), and for β-actin they were 5'TCA CCA CTA TTG GCA ATG AG-3' (sense) and 5'AGA GCC GAC TGT GGT TTG CTT AAC-3' (antisense). All primers were synthesized by Takara. The qPCR data were analyzed by using the Roche Molecular Biochemicals LightCycler software (version 3.5). Specificity of the amplification reactions was confirmed by analyzing their corresponding melting curves. The relative expression of each gene was normalized with the β-actin control and then estimated as values of 2^(-ΔΔCt).

Protein extraction and western blotting. Total cellular protein was extracted from the cultured tumor cells with ice-cold lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na3 vanadate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin (pH 7.3), 10 µg/ml leupeptin, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, 0.5% deoxycholate, and 1% NP-40 for 15 min, followed by centrifugation at 21,000 x g for 15 min at 4˚C. The concentrations of these protein samples were determined using a BCA Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) standardized to bovine serum albumin (BSA) according to the manufacturer's protocol.

The protein samples (50 µg for each lane) were resolved by electrophoresis in 7.5 or 12.5% SDS-PAGE precast gels (Bio-Rad). Protein samples were then electro-transferred onto nitrocellulose membranes. The membranes were incubated in 5% (w/v) non-fat dry milk in 20 mM Tris-buffered saline with 0.1% Tween (TBS-T) for 2 h at room temperature and then with the primary antibodies overnight at 4˚C. These primary antibodies were human PAR-2 (sc-8205, 1:1000), VEGF (sc-7269, 1:1000), COX-2 (sc-7951, 1:1000), ERK1/2 (sc-93, 1:2000), p38 (sc-7973, 1:2000), phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38), the phospho-specific antibodies targeting p-ERK1/2 (Tyr 204) (sc-7383, 1:1000), p-p38 (Tyr182) (sc-7973, 1:2000), or β-actin (sc-1615, 1:1000), all of which were

variety of tissues, with a high expression level in the gastro-

intestinal tract, where physiological trypsin is highly expressed (11). Functionally, PAR-2 has been implicated in inflammation, pain, tissue injury, as well as in the regulation of gastrointestinal functions and diseases (12). Furthermore, PAR-2 protein is also highly expressed in various cancers, such as breast, colorectal, pancreatic, gastric, gallbladder, kidney, lung, uterine, cervical cancer and melanoma, and glioblastoma (13,14).

Recently, increasing attention has focused on the role of PAR-2 in angiogenesis (15). For example, PAR-2 activation in vascular endothelial cells was able to stimulate angiogenesis during tissue repair and promote neovascularization of the retina (15,16). PAR-2 activation also was shown to induce proliferation of endothelial cells, enhance production of proangiogenesis cytokines (such as vascular endothelial growth factor, namely VEGF), and upregulate expression of cyclooxygenase-2 (COX-2) protein in human endothelial cells and other types of cells (17-21). In gastric cancer (22), overexpression of PAR-2 could be the key inducer of angiogenesis. However, the underlying molecular mechanism responsible for PAR-2 action has yet to be determined. In the present study, we hypothesized that PAR-2 activation by trypsin contributes to gastric cancer angiogenesis, which may be through upregulation of VEGF and COX-2 expression.
from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The next day, the membranes were washed three times with TBS-T and then incubated with the HRP-conjugated anti-rabbit, anti-mouse, or anti-goat secondary antibodies (Santa Cruz Biotechnology Inc.) at a dilution of 1:2000 for 1 h at room temperature. The membranes were washed extensively with TBS-T three times and positive signals were detected by using the enhanced chemiluminescence detection system (Beyotime, China) and exposed to X-ray film.

**ELISA analysis.** The levels of VEGF in the conditioned cell culture media were determined by using ELISA. Briefly, gastric cancer MKN28 cells were seeded at 5x10^5 cells per well into 12-well plates and grown to reach a confluence of 60-70%. Next, the cells were washed three times with phosphate-buffered saline (PBS), and the culture medium was replaced with serum-free medium and treated with increased concentrations of trypsin (0-100 nM; Amresco) for 6 h, or treated with 10 nM trypsin for up to 24 h. At the end of the experiments, the conditioned cell culture medium was harvested and centrifuged for ELISA. A commercially available sandwich ELISA kit (Jinmei Biotechnology Co., Ltd., Shenzhen, China) was used to assess VEGF concentration in the supernatant of the conditioned growth medium according to the manufacturer’s instructions. The data were normalized to the kit controls and the number of producing cells and then expressed as pg of VEGF protein per 10^5 cells.

**Statistical analysis.** All experiments were performed in triplicate and repeated at least three times. Data are expressed as means ± standard deviation (SD). The Student's t-test was used to compare the means of two groups. P<0.05 was considered statistically significant. All analyses were performed using SPSS 10.0 statistical packages (SPSS Inc., Chicago, IL, USA).

**Results**

**Expression of PAR-2 in gastric cancer MKN28 cells.** Expression of PAR-2 mRNA and protein was determined in MKN28 gastric cancer cells by using RT-PCR and western blot, respectively. As shown in Fig. 1A, a 320-bp PAR-2 target band was amplified by RT-PCR, indicating that MKE28 expressed PAR-2 mRNA. Moreover, western blot data showed that the anti-PAR-2 antibody can specifically detect a 55-kDa expressed PAR-2 target band (Fig. 1B). Lung cancer A549 cells, highly expressing PAR-2, were used as a positive control.

**Effects of trypsin-activated PAR-2 on enhanced expression of VEGF and COX-2 in gastric cancer cells.** Next, we determined different gene expressions after gastric cancer cells were treated with trypsin at different doses or time points. We found that different treatment doses of trypsin for 6 h were able to activate PAR-2 expression (Fig. 2A) and in turn to upregulate VEGF and COX-2 expression in MKN28 cells. As shown in Fig. 2A, with increased concentrations of trypsin up to 100 nM, expression levels of VEGF and COX-2 mRNA were increased in a dose-dependent manner, e.g., 10 nM trypsin induced levels of VEGF and COX-2 mRNA by 7.7-fold and 8.3-fold, respectively, compared to their corresponding basal levels (P<0.05). Similar data were confirmed by using western blotting (Fig. 2B). Furthermore, VEGF protein expression in the conditioned media was also induced in a dose-dependent manner (Fig. 2C). Particularly, 10 nM trypsin treatment of MKN28 cells increased expression of VEGF protein to 81.7 pg/ml in the conditioned growth medium, while 100 nM trypsin treatment upregulated VEGF levels to 101.7 pg/ml (P<0.05). Taking all the findings into consideration, a concentration of 10 nM trypsin was used for further experiments.

Furthermore, induction of VEGF and COX-2 expression by activated PAR-2 at different time points was also analyzed in MKN28 cells. As shown in Fig. 3A, up to 10 h of treatment of MKN28 cells with 10 nM trypsin upregulated both mRNA and protein levels of VEGF and COX-2 expression in a time-dependent manner. A 24-h treatment of tumor cells with trypsin induced expression of VEGF and COX-2 to the peak mRNA levels, whereas the peak levels of their protein expression were reached after a 12-h treatment, although there was no statistical difference in expression of VEGF protein after 6, 12, or 24 h of treatment (Fig. 3B). The maximum COX-2 protein expression was found at 24 h of treatment, without statistical difference between 12 and 24 h (Fig. 3B). The VEGF concentration in the conditioned media was also time-dependent (Fig. 3C).

**Effects of trypsin-activated PAR-2 on induction of ERK1/2 and p38 MAP kinase phosphorylation.** To determine whether the MAP kinase signaling pathway participates in the upregulation of VEGF and COX-2 expression, we assessed the total and phosphorylated ERK1/2 and p38 MAP kinase proteins after trypsin-induced PAR-2 activation in MKN28 cells. As shown in Fig. 4, expression levels of the total ERK1/2 protein showed no significant changes from 0 to 60 min, whereas phosphorylated...
ERK1/2 protein was induced by trypsin-induced PAR-2 activation, which peaked at 15 min and returned back to the basal level at 60 min. A similar time-dependent manner for p38 MAP kinase phosphorylation was also noted, i.e., trypsin-induced PAR-2 activation upregulated significant and sustained phosphorylation of p38 MAP kinase that began at 5 min, reached the maximum at 30 min with nearly a 4-fold increase compared to the untreated controls, and then returned to the basal level after 60 min.

**ERK1/2 and p38 inhibitors antagonize the effects of trypsin-activated PAR-2 on induction of VEGF and COX-2 expression.** Next, we determined the role of the MAP kinase signaling pathway in mediating the effects of trypsin-activated PAR-2 on the induction of VEGF and COX-2 expression by pretreating...
MKN28 cells with specific ERK1/2 and p38 MAP kinase inhibitors (25 µM PD98059 or 20 µM SB203580, respectively) 1 h prior to PAR-2 activation with 10 nM trypsin for 6 h. We found that compared to the PBS control, treatment with 10 nM trypsin resulted in 7.0- and 7.5-fold induction of VEGF and COX-2 mRNA, respectively, whereas these increases in VEGF and COX-2 mRNA and protein levels were completely blocked by treatment with PD98059 or SB203580 in gastric cancer MKN28 cells (Fig. 5). However, there was no significant difference in VEGF and COX-2 expression between the control cells and PD98059 or SB203580-treated tumor cells alone (Fig. 5). The data suggested that the role of PAR-2 was mediated by the MAP kinase signaling pathway to upregulate expression of VEGF and COX-2 mRNA and protein.

Discussion

In this study, we chose gastric cancer cells to demonstrate that PAR-2 activation was able to upregulate expression of proangiogenesis genes and defined the underlying molecular pathway. Our data showed that trypsin-activated PAR-2 induced expression of VEGF and COX-2 in gastric cancer cells in a dose- and time-dependent manner. The ability of PAR-2 to induce VEGF and COX-2 expression was mediated by ERK1/2 and p38 gene signaling, i.e., pretreatment with ERK1/2 and p38 inhibitors blocked the effects of trypsin-activated PAR-2 on the induction of VEGF and COX-2 expression. Since angiogenesis plays an important role in gastric cancer development and progression, this study might provide a novel anti-angiogenesis target for future treatment of gastric cancer patients.

PAR-2 is a member of the protease-activated receptor family. Various proteases can cleave PAR-2 within the extracellular N-terminal domain to expose a tethered ligand that binds to and activates the cleaved receptor. Among these different proteases, trypsin is a potent PAR-2 activator that cleaves and triggers PAR-2 activation, although trypsin was traditionally regarded as an enzyme with the principal function of degrading dietary proteins (23). A wide expression of PAR-2 has been observed in the gastrointestinal tract in physiological conditions and malignant gastrointestinal diseases (24-26). Trypsin, the most prominent ligand of PAR-2, is not only maintained at a higher concentration in the gastrointestinal tract naturally, but also can be secreted by a few types of cancer cells derived from the gastrointestinal tract by an autocrine mechanism (27-29).

Accumulating evidence has demonstrated that PAR-2 is involved in the development and progression of human
cancers. For example, PAR-2 overexpression has been associated with the depth of tumor invasion, lymphatic involvement, early metastasis, and poor prognosis of gastric cancer and other types of cancer (22,26,30,31). Activated PAR-2 enhanced the growth of gastric cancer cells via trans-activation of epidermal growth factor receptor (EGF-R) (24). Activated PAR-2 also promoted the proliferation of colon cancer cells with a similar mechanism (25). In addition to the mutagenic function, a potential proangiogenesis effect of PAR-2 has been confirmed in breast cancer and uterine endometrial cancer (19,30). Particularly, in human breast cancer cells, activation of PAR-2 with an agonist peptide, trypsin, or FVIIa resulted in a robust increase in expression of VEGF mRNA and protein, the most prominent inducer of angiogenesis (19). Additionally, in human bile duct cancer cells, PAR-2 agonist peptide activated expression of COX-2 mRNA and protein, and the latter is responsible for prostaglandin biosynthesis, which also contributes to tumor angiogenesis (32). Moreover, COX-2 can increase VEGF production, and a link between COX-2, PGE2, and VEGF was proposed in AGS gastric cancer cells (33,34). Therefore, VEGF and COX-2 were presumed to mediate the proangiogenesis capacity of PAR-2 in gastric cancer.

In the present study, we first determined the effect of PAR-2 activation on the intracellular expression of VEGF and COX-2 in MKN28 gastric cancer cells and secreted VEGF in the conditioned medium. Our data revealed that trypsin-activated PAR-2 was able to upregulate the expression of COX-2 mRNA and protein in a dose- and time-dependent manner. Thereafter, we demonstrated that trypsin-activated PAR-2 could also increase the expression of VEGF mRNA and protein in gastric cancer cells. These data demonstrated and confirmed the effects of PAR-2 on increasing the expression of angiogenesis-related genes in gastric cancer cells.

Furthermore, previous studies have reported that PAR-2 activated by trypsin modulated the downstream signal transduction pathway through the activation of the MAP kinase cascade (19,32,35,36). In this study, we showed that trypsin-activated PAR-2 increased the phosphorylation of ERK1/2 and p38 MAP kinase. It is to be determined whether the MAP kinase cascade is the upstream gene pathway that regulates the expression of VEGF and COX-2. As shown in Fig. 5, at both the mRNA and protein level, PD98059 (ERK1/2 inhibitor) and SB203580 (p38 MAP kinase inhibitor) dramatically blocked the expression of VEGF and COX-2 induced by trypsin. These data indicate that PAR-2 induced VEGF and COX-2 expression is through the MAP kinase pathway. However, a striking difference has been noted in the mechanism responsible for activation of the MAP kinase signal transduction pathway by PAR-2 among a variety of cells and tissues. For example, trypsin-activated PAR-2 was able to strongly activate ERK1/2 protein but weakly activate p38 by trypsin-activated PAR-2 in rat aortic smooth-muscle cells with both endogenous and exogenous PAR-2 expression (35,37,38). In contrast, in human keratinocytes with PAR-2 overexpression, trypsin-activated PAR-2 strongly induced p38 MAP kinase activation, but weakly induced ERK1/2 activation (39).

Additionally, ERK1/2, p38, and JNK MAP kinases also could simultaneously be involved in the signal transduction following trypsin-induced PAR-2 activation, with indepen-

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**References**


