In Vivo and in Vitro Degradation of Heparan Sulfate (HS) Proteoglycans by HPR1 in Pancreatic Adenocarcinomas

LOSS OF CELL SURFACE HS SUPPRESSES FIBROBLAST GROWTH FACTOR 2-MEDIATED CELL SIGNALING AND PROLIFERATION

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Heparan sulfate proteoglycans (HSPGs) function as a co-receptor for heparin-binding growth factors, such as fibroblast growth factors (FGFs) and heparin-bound epidermal growth factor (HB-EGF). The HS side chain of HSPGs can be cleaved by HPR1 (heparanase-1), an endoglycosidase that is overexpressed in many types of malignancies. In the present study, we demonstrated that HPR1 expression in pancreatic adenocarcinomas inversely correlated with the presence of heparan sulfate (HS) in the basement membrane. In vitro cell culture study revealed that cell surface HS levels inversely correlated with HPR1 activity in five pancreatic cancer cell lysates and their conditioned media. Heparin and PI-88, two HPR1 inhibitors, were able to increase cell surface HS levels in PANC-1 cells in a dose-dependent manner. The ability of HPR1 to degrade cell surface HS was confirmed by showing that cell surface HS levels were increased in HT1080 cells stably transfected with the HPR1 antisense gene but was decreased in the cells overexpressing HPR1. Further studies showed that PI-88 and heparin were able to stimulate PANC-1 cell proliferation in the absence or presence of exogenous FGF2, whereas exogenous HPR1 was able to inhibit PANC-1 cell proliferation in a dose-dependent manner. Modulation of PANC-1 cell proliferation by HPR1 or HPR1 inhibitors corresponded with the inhibition or activation of the mitogen-activated protein kinase. Our results suggest that HPR1 expressed in pancreatic adenocarcinomas can suppress the proliferation of pancreatic tumor cells in response to the growth factors that require HSPGs as their co-receptors.

HSPGs2 are a group of glycoproteins encoded by 13 HSPG genes from five distinct classes, including three pericellular

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2The abbreviations used are: HSPG, heparan sulfate proteoglycan; BM, basement membrane; FGF, fibroblast growth factor; HS, heparan sulfate(s); IF, immunofluorescence; IHC, immunohistochemistry; HB-EGF, heparin-bound epidermal growth factor; FITC, fluorescein 5-isothiocyanate; mAb, monoclonal antibody; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; FACS, fluorescence-activated cell sorting.

Emerging evidence indicates that HS structure on tumor cells is altered. Jayson et al. (8) analyzed the structural difference of HS between colonic adenomas and carcinomas and found that in colon carcinomas 6-O-sulfation in the HS chain is increased, whereas 2-O-sulfation is reduced. Mundhenke et al. (9) conducted a stepwise reconstitution of the FGF-HSPG-FGFR-1 complex in situ and found that the complex formation is increased in breast carcinoma cells, compared with normal mammary epithelial cells, and the complex formation is independent of the levels of syndecan-1 expression. Nackaerts et al. (10) showed that HS content is reduced in squamous cell lung carcinomas. The molecular mechanisms of the structural changes of HSPGs in these malignancies remain poorly understood.

HPR1 is an endoglycosidase that specifically degrades HSPGs. HPR1 is overexpressed in a variety of malignancies (11–13). Using in situ hybridization, we have previously detected HPR1 mRNA in 78% of primary pancreatic adenocarcinomas (14). Koliopanos et al. (15) reported that HPR1 mRNA
levels detected by real time reverse transcription-PCR are elevated in 25 of 33 (83%) pancreatic cancers by an average of 30-fold, compared with that in normal pancreatic tissues. Increased HPR1 expression in pancreatic cancer cell lines by transient transfection of the HPR1 gene leads to an increase of their invasive potential. Rohloff et al. (16), using immunohistochemical staining, showed that HPR1 is expressed in 76% of pancreatic adenocarcinomas at moderate or high levels. Our present study aims to determine if HPR1 expression in pancreatic adenocarcinomas and tumor cell lines leads to the degradation of HSPGs, subsequently affecting the mitogenic activity of the growth factors that require HSPGs as their co-receptors.

**EXPERIMENTAL PROCEDURES**

**Tissues**—The use of specimens from human subjects was approved by the Institutional Review Board of Rush University Medical Center. Paraffin-embedded tissue blocks derived from patients with either primary pancreatic adenocarcinomas and/or metastases were obtained from the Department of Pathology. The stage of the pancreatic adenocarcinomas was classified according to the TNM (T, tumor size; N, spread to lymph nodes; M, metastasis) scheme used by the American Joint Committee on Cancer (AJCC). Forty-two pancreatic cancer specimens from patients (male/female, 21:21) with a mean age of 64 ± 11 (median age 65, range 37–80 years) were analyzed for HPR1 expression by immunofluorescence (IF) staining. These 42 samples included 36 primary adenocarcinomas (35 from pancreatectomy and one from pancreatic biopsy) and six metastatic implants (three on the small bowel, one on the peritoneum, one in the liver, and one on the gallbladder). Fifty-two primary adenocarcinoma specimens from patients (male/female, 28:24) with a mean age of 64 ± 11 (median age 65, range 34–81 years) were analyzed for HPR1 expression by immunohistochemical staining (IHC).

**IF Staining**—The sections of tissue specimens were dewaxed and then fixed with 1% paraformaldehyde. The slides were incubated with an anti-HPR1 mAb (3–12 clone, working concentration 50 μg/ml) at room temperature for 30 min. The same concentration of normal mouse IgM was used as a negative control. The slides were washed three times with PBS, followed by incubation for 45 min with goat anti-mouse IgM conjugated with FITC (1:50) (ICN Biomedicals, Aurora, OH), and then washed and incubated for 45 min with rabbit anti-goat IgG conjugated with FITC (1:50) (ICN Biomedical, Aurora, OH). The slides were sealed with 50% glycerin in PBS containing 2364 JOURNAL OF BIOLOGICAL CHEMISTRY 25 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin. The supernatants were collected; cell lysates were prepared and analyzed by using a Bio-Rad protein assay kit (Bio-Rad). Protein concentrations in the cell lysates were determined based on the standard curve of serially diluted purified platelet HPR1 buffer (0.1 mg/ml bovine serum albumin, 0.01% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin). The supernatants were preincubated at 9:1 with 10× HPR1 buffer. Protein concentrations in the cell lysates were analyzed by using a Bio-Rad protein assay kit (Bio-Rad). Equal amounts of cell lysates (100 μg/well) and supernatants were then added to the Matrigel precoated ELISA plate and analyzed as previously reported (17). HPR1 activity was calculated based on the standard curve of serially diluted purified platelet HPR1 buffer (starting at 1:200) at a concentration of 1 μl of HPR1 with the activity of degrading 0.133 μg of heparan sulfate/h at 37 °C in HPR1 buffer. HPR1 purification and characterization from human platelets were conducted as previously reported (20). HPR1 activity was designated as per 100 units capable of degrading 1 ng of heparan sulfate at 37 °C/h in HPR1 buffer.

**Flow Cytometric Analysis**—Single cell suspensions of pancreatic cancer cell lines were prepared by using cell dissociation solution. Cells (5 × 10^6/sample) were stained by incubation for 30 min at 4 °C with an anti-HS mAb (0.5 μg/sample) (clone HPR1 signal in more than 10% of tumor cells. HS-negative tumors were defined as no HS signal (−) or weak signal (+) in less than ~20% of tumor ducts. HS-positive tumors were defined as moderate (++) or strong (+++) HPR1 signal in more than 20% of tumor ducts.

**Immunohistochemistry**—Tissue sections of paraffin-embedded blocks were dewaxed with xylene and rehydrated. Slides were processed for IHC staining with an anti-HPR1 anti-rabbit serum (1:500 dilution) as previously reported (17–19). Slides were counterstained with Mayer’s hematoxylin for 2 min, rinsed, dehydrated, and mounted. Two investigators (X. X. and R. M. Q.) and a pathologist (P. G.) graded HPR1 expression in a blinded fashion. HPR1-negative tumors were defined as no HPR1 signal (−) or weak signal (+) in less than ~10% of tumor ducts. HPR1-positive tumors were defined as moderate (++) or strong (+++) HPR1 signal in more than 10% of tumor cells.

**Cells**—CAPAN-1, COLO-587, HPAF-II, Panc-1, and MPANC-96 cell lines were purchased from the American Type Culture Collection (Manassas, VA). CAPAN-1 and HPAF-II cells were grown in complete minimal essential medium containing 10% FBS, nonessential amino acids, sodium pyruvate, Hepes, penicillin, and streptomycin. COLO-587 cells were grown in complete RPMI 1640 medium containing 10% FBS. Panc-1 cells were grown in complete RPMI 1640 medium containing 10% FBS, nonessential amino acids, sodium pyruvate, Hepes, penicillin, and streptomycin. MPANC-96 cells were grown in complete Dulbecco’s modified Eagle’s medium containing 10% FBS. HT1080 cells were stably transfected with pcDNA3.1 empty vector. The vector encoding the human HPR1 gene in the sense or antisense orientation was produced earlier in our laboratory. Cells were maintained in the complete minimal essential medium containing 10% FBS, nonessential amino acids, sodium pyruvate, Hepes, penicillin, and streptomycin in the presence of 250 μg/ml G418.
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HepSS; Seikagaku Corp., Chuo-ku, Tokyo, Japan) or mouse IgM as a negative control. Cells were incubated with FITC-labeled goat anti-mouse IgM (5 μl/sample) for 30 min at 4 °C followed by rabbit FITC-labeled anti-goat IgG (5 μl/sample) for 30 min at 4 °C. Cell surface HS expression was analyzed in a BD Biosciences flow cytometer. Since the binding of anti-HS antibody to HS also depends on specific structures of HS, detection of HS in different cell lines and tumor tissues, in particular in those from different origins, may not only reflect the quantity of HS but also be affected by HS structure. A second anti-HS mAb (clone F58-10E4, mouse IgM; Seikagaku Corp., Chuo-ku, Tokyo, Japan) was used to analyze cell surface HS levels in HPAF-II and PANC-1 cells. The bindability of these two anti-HS monoclonal antibodies was compared. To detect cell surface HPR1 expression in transfected HT1080 cells, cells were similarly stained with an affinity-purified anti-HPR1 mAb and rabbit IgG (5 μl/sample each) followed by the use of FITC-labeled goat anti-mouse IgG or goat anti-rabbit IgG, respectively.

**Cell Proliferation**—Single cell suspension of PANC-1 cells was prepared by using cell dissociation solution and washed twice with PBS. Cells were seeded in 96-well plates at a density of 1 × 10^4/well in the complete RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. After incubation overnight, cells were washed with Hanks’ balanced salt solution and then incubated for 48 h with serum-free RPMI 1640 containing 0.1% bovine serum albumin, 5 μg/ml transferrin, 5 ng/ml sodium selenite in the absence or presence of the indicated concentrations of HPR1 inhibitors and/or FGF2. Cell proliferation was monitored by using a CellTiter 96 nonradioactive cell proliferation assay kit following the manufacturer’s instructions (Promega, Madison, WI).

**Western Blot**—A single cell suspension of PANC-1 cells was prepared by using the cell dissociation solution. Cells were seeded in a 6-well plate. Upon 80% confluence, cell monolayer was washed with Hanks’ balanced salt solution and then incubated in serum-free RPMI1640 medium in the absence of presence of various concentrations of PI-88 or heparin. For stimulation with FGF, cells were starved in serum-free medium for 24 h and then stimulated with FGF (10 ng/ml) for 15 min. Cell lysates were prepared in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 150 mM NaCl, 5 mM each EDTA and EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml each leupeptin and aproatin). Cell lysates were run on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. MAPK activation was analyzed by Western blot by probing with anti-phospho-ERK1/2. The membrane was then stripped and reprobed with an anti-ERK1/2 rabbit IgG (Cell Signaling, Beverly, MA).

**Statistical Analysis**—The relationship of HPR1 expression with the deposition of HS in the BM was analyzed by using a χ² test. The significant difference in the proliferation of PANC-1 cells treated with HPR1 inhibitors, FGF2, and HPR1 was conducted by using an unpaired Student’s t test. A p value less than 0.05 was considered statistically significant. All statistical calculations were conducted by using SigmaStat 3 software.

### Table 1

|                  | IF staining |                | IHC staining |                |
|------------------|-------------|----------------|-------------|----------------|
|                  | Primary     | Metastases     | Normal      | Primary        | Metastases     | Normal      |
| Total            | 36          | 6              | 10          | 52             | 5              | 10          |
| Positive         | 27 (75%)    | 5 (83%)        | 0 (0%)      | 31 (63%)       | 4 (80%)        | 0 (0%)      |

**RESULTS**

**HPR1 Expression in Pancreatic Adenocarcinomas**—In order to determine whether HPR1 expression leads to the degradation of HS in pancreatic cancer, we first analyzed HPR1 expression by using IF and IHC staining with two immunological probes. We conducted IF staining on the sections of 42 pancreatic adenocarcinomas with an anti-HPR1 mAb (clone 3–12). This antibody has been previously characterized (21); it can neutralize HPR1 enzymatic activity and recognize HPR1 in the sections of paraffin embedded tissues. As shown in Fig. 1, strong cytoplasmic staining indicating HPR1 expression in the cytoplasm as seen in (B) (×200) and (C) (×400) was observed. The stromal cells and vascular endothelial cells did not express HPR1. HPR1 was not expressed or was expressed at very low levels in the normal ductal epithelium and acinar cells in the vicinity of a pancreatic adenocarcinoma (Fig. 1A, ×200). Mouse IgM was included as a negative control (D).

![Image](https://example.com/image.png)

**FIGURE 1.** HPR1 expression in primary and metastatic pancreatic adenocarcinoma assayed by immunofluorescence staining. Sections of paraffin-embedded human pancreatic cancer specimens were analyzed for HPR1 expression by IF staining with anti-HPR1 mAb (3-12 clone, working concentration 50 μg/ml). Positive HPR1 expression was indicated by bright green fluorescence in the cytoplasm as seen in (B) (×200) and (C) (×400) but not in normal ductal and acinar cells (A). Normal mouse IgM was included as a negative control (D).
clonal antiserum (22). As shown in Fig. 2, HPR1 was not expressed in normal ductal cells and acinar cells derived from normal pancreatic tissues (A) nor in a cystic adenoma (B). However, HPR1 was abundantly expressed in the cytoplasm of duct cells of a primary adenocarcinoma (C) and a pancreatic tumor metastasis in the duodenum (D). E, a HPR1-negative adenocarcinoma is shown. Normal rabbit IgG that was included as a negative control (F) did not give any nonspecific staining.

Fifty-two primary adenocarcinomas and five metastases were assessed for HPR1 expression by using IHC staining (Table 1). Among them, 31 of 52 primary (63%) and 4 of 5 metastasis samples were HPR1-positive, which is defined as at least 10% of tumor cells with moderate or strong HPR1 signals in tumor cells. Thirty-seven samples were examined for HPR1 expression by using both IF and IHC staining. The overall concordance by these two methods was 73% (Table 2). The discrepancy in HPR1 expression detected by these two antibodies could result from their differential affinity or different sensitivity of the detection methods.

**HS Analysis in the BM of Pancreatic Adenocarcinomas**—We next analyzed the HS content in the BM of pancreatic cancer cells by using IF staining with a monoclonal antibody (clone HepSS) specific for HS. As shown in Fig. 3, HS deposition was abundantly present in the BM of a benign cyst adenoma (A) and two HPR1-negative pancreatic adenocarcinomas (C and E) but was absent in the BM of two HPR1-positive pancreatic adenocarcinomas (B and D). Normal mouse IgM was included as a negative control, showing that there was no nonspecific staining. Twenty-two of 31 (71%) primary pancreatic adenocarcinomas and four of four metastases lacked HSPG deposition in the BM of the tumor ducts. Nine pancreatic adenocarcinomas with moderate to high fluorescence signals specific for HS surrounding the BM of at least 20% ducts were graded as HS-positive (Table 3).

We next explored the relationship of HS deposition in the BM and HPR1 expression analyzed by IHC with an anti-HPR1 rabbit serum as described in the legend to Fig. 2. As shown in Table 3, 21 of 23 HPR1-positive primary adenocarcinomas and three of three HPR1-positive metastases did not have HS deposition in the BM, whereas seven of eight HPR1-negative primary adenocarcinomas had intact HS deposition. χ² analysis

**TABLE 2**

|                  | Number | HPR1-positive (IF) | Concordance |
|------------------|--------|--------------------|-------------|
| HPR1-positive (IHC) | 24     | 21                 | 88          |
| HPR1-negative (IHC) | 13     | 6                  | 54          |

**FIGURE 2. Immunohistochemical staining of HPR1 expression.** Sections of paraffin-embedded pancreatic tissues were processed for IHC staining with rabbit anti-HPR1 rabbit antiserum. HPR1 was not detected in a normal pancreas (A) and a surgical specimen of a cystadenoma (B), but it was detected in a primary adenocarcinoma (C) and a metastasis in the duodenum (D). E, a HPR1-negative adenocarcinoma is shown. Normal rabbit IgG that was included as a negative control (F) did not give any nonspecific staining.

**FIGURE 3. Immunodetection of heparan sulfate proteoglycan in pancreatic adenocarcinomas.** Sections of human specimens were dewaxed, rehydrated, and fixed with 1% paraformaldehyde. HS contents were detected by IF staining with anti-HS mAb (HepSS; working concentration at 5 μg/ml). A, an HS-positive benign cystadenoma; C and E, two HS-positive pancreatic adenocarcinomas; B and D, two HS-negative pancreatic adenocarcinomas; F, mouse IgM was used as a negative control for staining of a pancreatic adenocarcinoma.
revealed that HPR1 expression inversely correlated with the presence of HSPG in pancreatic adenocarcinomas (p < 0.05), suggesting that HPR1 plays a critical role in degrading heparan sulfate in the BM.

**Cell Surface HS Degradation by HPR1**—Previous studies have demonstrated that glypican-1, a heparan sulfate proteoglycan overexpressed in pancreatic adenocarcinoma and pancreatic tumor cell lines, contains little or no HS side chain (6). We postulate that the lack of HS in the glypican-1-overexpressed pancreatic cancer cell lines is due to the degradation of HS by increased HPR1 expression. To address this issue, we tested whether HPR1 expression inversely correlated with cell surface HS expression. We first analyzed HPR1 activity in the conditioned media and cell lysates of five pancreatic cancer cell lines. As shown in Fig. 4A, HPR1 activity was detected at relatively higher levels in COLO587, PANC-1, and MPANC-96 cells than in HPAF-II and CAPAN-1 cells in both conditioned media and cell lysates. We next conducted flow cytometric analysis to examine cell surface HS expression. As shown in Fig. 4B, cell surface HS levels were higher in CAPAN-1, HPAF-II, and MPANC96 cells than in COLO587 and PANC-1 cells. Cell surface HS levels inversely correlated with HPR1 activity, in particular, of the supernatants of pancreatic cancer cell lines. Similar results were obtained when cell surface HS levels were analyzed by a second anti-HS mAb (clone F58-10E4) (data not shown). Therefore, it is less likely that differential HS levels detected by anti-HS mAb are due to the different structures.

To determine whether HPR1 expressed in pancreatic cancer cell lines was responsible for the lack of cell surface HS, we tested whether two HPR1 inhibitors, heparin and PI-88, were able to block HPR1-mediated degradation of cell surface HS. As shown in Fig. 4C, HS was not detected in untreated PANC-1 cells but was increased in the cells treated either with PI-88 or heparin in a dose-dependent manner. HS levels in PANC-1 cells treated with PI-88 or heparin (80 μg/ml each) were significantly higher than PI-88 or heparin at concentrations of 20 and 5 μg/ml.

To further confirm the role of HPR1 in degrading cell surface HS, we tested whether modulation of HPR1 expression by a genetic approach affected HPR1 expression. Our laboratory has previously generated several HT1080 transfectant cell lines in which HPR1 is overexpressed or suppressed by HPR1 sense or antisense gene expression, respectively (17). Western blot was not able to detect basal HPR1 expression in pcDNA3.1 empty vector-transfected HT1080 cells, probably due to the low affinity of anti-HPR1 antibody (a band present between 50- and 65-kDa HPR1 isoforms was nonspecific). HPR1 down-regulation in HPR1 antisense-transfected HT1080 cells could not be confirmed by Western blot. In contrast, Western blot analysis revealed HPR1 overexpression in HPR1-transfected cells (Fig. 5A). Consistently, FACS analysis revealed that HPR1 expression was detected on the cell surface by a monoclonal (Fig. 5B, top) and polyclonal anti-HPR1 antibody (Fig. 5B, bottom). Again, cell surface HPR1 expression was not detected by these two anti-HPR1 antibodies either in pcDNA- and pcDNA/HPR1-AS-transfected cells. We next tested whether modulation of HPR1 expression affected cell surface HS levels in these HT1080 transfectant cell lines. As shown in Fig. 5C, pcDNA3.1-transfected cells moderately expressed HS on the cell surface. However, cell surface HS levels were much higher in HPR1 antisense-transfected cells and dramatically reduced in HPR1-transfected cells.

**Effect of HPR1 Expression on Pancreatic Cancer Cell Proliferation**—Previous studies have shown that HSPG expression on the cell surface of pancreatic cancer cell lines can enhance FGF2-mediated cell proliferation (6). Here we tested whether HPR1 inhibitors that increased cell surface HS levels also increased the proliferation of PANC-1 cells mediated by endogenous and exogenous FGF2. As shown in Fig. 6A, FGF2 (10 ng/ml), PI-88, and heparin (50 μg/ml each) alone were able to increase HPR1-positive PANC-1 cell proliferation by 8, 40, and 22%, respectively. PI-88 and heparin in combination with exogenous FGF2 were able to additively stimulate the proliferation of PANC-1 cells by 52 and 68%, respectively. Statistical analysis revealed that cell proliferation rate was significantly higher in PANC-1 cells treated with PI-88 and heparin alone but not in the cells treated with FGF2, compared with the untreated control. The cell proliferation rate was also significantly higher in the cells treated with FGF2 plus heparin, but not in those treated with FGF2 plus PI-88, compared with the rate for cells treated with HPR1 inhibitor alone. In contrast, the addition of purified platelet HPR1 was able to inhibit the proliferation of PANC-1 cells by 21% (p < 0.05). We next showed that PI-88, heparin, and FGF2 increased PANC-1 cell proliferation in a dose-dependent manner, whereas HPR1 decreased PANC-1 cell proliferation in a dose-dependent manner (Fig. 6B). We next tested whether increased cell surface HS levels by various concentrations of PI-88 and heparin as shown in Fig. 4C led to a corresponding increase of cell proliferation stimulated by FGF2. As shown in Fig. 6C (left), FGF2 in combination with the increasing concentrations of PI-88 led to steadily increased PANC-1 cell proliferation. In contrast, heparin used at a low concentration (5 μg/ml) was able to boost FGF2-stimulated PANC-1 cell proliferation (Fig. 6C, right). Further increase of heparin concentration to 20 and 80 μg/ml did not result in a higher proliferation rate. The difference of FGF2-stimulated cell proliferation in the presence of PI-88 and heparin may lie on the differential ability of these two inhibitors to assist FGF2 binding to their receptors.

**Modulation of Cell Surface HS Levels by HPR1 and Its Inhibitors Affects the Activation of the MAPK Pathway**—We first tested whether modulation of cell surface HS expression by HPR1 inhibitors affected the activation of the MAPK pathway mediated by endogenous FGF2. As shown in Fig. 7A, ERK1/2 phosphorylation was increased in PANC-1 cells treated with...
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A

![Graph A](image)

HPR1 activity in conditioned medium

![Graph B](image)

HPR1 activity in cell lysate

B

![Graph C](image)

Cell counts

Fluorescence intensity

C

![Graph D](image)

None

Heparin 5 µg/ml

Heparin 20 µg/ml

Heparin 80 µg/ml

None

PI-88 5 µg/ml

PI-88 20 µg/ml

PI-88 80 µg/ml

Fluorescence Intensity
PI-88 and heparin (50 μg/ml) for 16 h. Heparin was more potent than PI-88 in inducing the phosphorylation of ERK1/2. FGF2 (20 ng/ml for 15 min) was slightly more effective in inducing ERK1/2 phosphorylation than heparin but much more effective than PI-88.

To confirm the specificity of PI-88 and heparin in activating the MAPK pathway, we showed that activation of ERK1/2 phosphorylation in PANC-1 cells by these two HPR1 inhibitors was dose- and time-dependent. Induction of ERK1/2 phosphorylation by FGF2, PI-88, and heparin was generally in a dose-dependent manner (Fig. 7B). Kinetics analysis of ERK1/2 phosphorylation by HPR1 inhibitors revealed that heparin induced a very weak and transient ERK1/2 phosphorylation between 30 and 60 min. However, the induction of ERK1/2 phosphorylation by heparin was remarkable at 16 h (Fig. 7C). The induction of ERK1/2 phosphorylation by PI-88 was detected in a time-dependent manner, with a maximal induction at 16 h.

HSPGs serve as the co-receptors for FGF2. Thus, we tested whether the alteration of cell surface HSPG levels by HPR1 inhibitors affected the induction of exogenous FGF2-mediated MAPK activation. As shown in Fig. 7D, induction of ERK1/2 phosphorylation by FGF2 (10 ng/ml) was much stronger in PANC-1 cells pretreated with PI-88 and heparin (50 μg/ml) than that in the cells without pretreatment with a HPR1 inhibitor. We next tested whether removal of cell surface HS on PANC-1 cells by exogenous HPR1 would suppress endogenous and exogenous FGF-mediated activation of ERK1/2. As shown in Fig. 7E, ERK1/2 phosphorylation was lower in HPR1-treated

**FIGURE 4.** HPR1 expression in pancreatic cancer cell lines and degradation of cell surface HS. A, HPR1 activity in the supernatants (left) and lysates (right) of five pancreatic cancer cell lines was measured by using ELISA. The data represent the mean ± S.D. of three independent experiments. B, FACS analysis of cell surface HS expression. Single cell suspensions were prepared and analyzed for surface HS expression by staining with a HS-specific monoclonal antibody. C, inhibition of cell surface HS degradation by HPR1 inhibitors. PANC-1 cells were incubated in the serum-free medium in the absence or presence of the indicated concentrations of PI-88 or heparin (5, 20, and 80 μg/ml each) at 37 °C overnight. Cells were analyzed for cell surface HS expression by FACS.
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FIGURE 6. Effect of HPR1 on the proliferation of pancreatic cancer cells. A, PANC-1 cells were seeded in 96-well plates in the serum-free medium with the indicated treatment. After incubation for 48 h, cell proliferation was analyzed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based proliferation assay kit. The results are the mean ± S.D. of three independent experiments. B, dose-dependent response of PANC-1 cell proliferation. PANC-1 cells were seeded in 96-well plates in the serum-free medium in the absence or presence of various concentrations of FGF2, PI-88, heparin, and HPR1. Cells were incubated for 48 h followed by a cell proliferation assay as described in A. C, the ability of PI-88 and heparin to enhance FGF2-mediated cell proliferation. PANC-1 cells seeded in a 96-well plate were left unstimulated or stimulated with FGF2 (20 ng/ml) in the absence or presence of the indicated concentrations of PI-88 (left) or heparin (right). After incubation for 48 h, cell proliferation was analyzed as in A. The data in Fig. 6, B and C, are the mean ± S.D. of the triplicate in one representative experiment. The experiment was repeated at least twice with similar results. *, statistical difference (p < 0.05) of cell proliferation in the treated group versus the control group; **, statistical difference (p < 0.05) in the proliferation of the cells treated with an HPR1 inhibitor plus FGF2 versus the cells treated with FGF2 alone.

PANC-1 cells (lane 3) than in the untreated control (lane 1). The addition of exogenous HPR1 also weakened the ability of exogenous FGF2-induced ERK1/2 phosphorylation (compare lane 2 and lane 4).

DISCUSSION

Our earlier studies demonstrated that HPR1 expression in thyroid and breast cancers correlates with the lack of HS in the BM (17, 19). A recent study by Reiland et al. (26) demonstrated that exogenous HPR1 added to cell culture of a melanoma cell line is able to degrade cell surface HS. Our present study provides the following evidence suggesting that HPR1 expressed in pancreatic adenocarcinomas and cell lines is functional and capable of degrading HS in the BM and on the cell surface. 1) HPR1 expression correlated with the lack of HS in the BM of pancreatic cancer tissue; 2) in vitro studies showed that cell surface HS expression was low or undetectable in pancreatic cancer cell lines with high HPR1 activity but was detected in three pancreatic cancer cell lines with relatively low HPR1 activity; 3) heparin and PI-88, two HPR1 inhibitors, were able to increase cell surface HS levels; 4) HPR1 can be readily detected as a cell surface molecule on HPR1-transfected HT1080 cells, suggesting that HPR1 has access to cell surface HSPGs and cleaves the HS side chain; 5) overexpression of HPR1 led to the down-regulation of cell surface HS levels, whereas suppression of HPR1 expression by HPR1 antisense led to increased cell surface HS levels. Despite these corroborating data, caution should be taken when interpreting the lack of HS detection in the BM of pancreatic tumor ducts as loss of HS, since the binding of anti-HS antibodies also depends on the structure of HS.

Although our observations collectively suggest that HPR1 plays a critical role in degrading HSPGs in tumor tissues, one should keep in mind that the evidence for HPR1-mediated HS degradation in pancreatic tumor tissue is indirect. Loss of HSPGs in pancreatic cancer could be contributed by increased degradation of the core protein of HSPGs due to increased expression of metalloproteinases and other proteases. To address this, we conducted additional experiments using the broad inhibitors of two protease families (matrix metalloproteinase and serine proteases), matrix metalloproteinase inhibitor III (Calbiochem) and pefabloc SC (Roche Applied Science). We found that neither of them were able to increase cell surface HS levels. However, other proteases capable of shedding the cell surface HSPGs cannot be excluded. In addition, degradation of HS side chains by HPR1 may facilitate the access of proteases to degrade the protein cores of HSPGs. Careful structural analysis of HSPGs and the relationship between HSPG levels and the expression of HPR1 and proteases may provide direct evidence on how much HPR1 contributes to HSPG degradation in tumor tissues. Nevertheless, the reciprocal relationship between HPR1 expression in pancreatic cancer and HS deposition in the BM as well as in our in vitro studies strongly suggest that HPR1 is responsible for decreased HS levels in pancreatic tumor cells.

Our recent study demonstrated that serum HPR1 levels in pancreatic cancer patients are elevated, that serum HPR1 levels in pancreatic cancer patients with HPR1-positive tumors are significantly higher than in those with HPR1-negative tumors, and that HPR1 presented in the serum from pancreatic cancer patients is capable of shedding cell surface HS of pancreatic cancer cells (27). These findings strongly suggest that HRP1 expressed in pancreatic cancer is capable of being secreted as a soluble functional molecule. In support of this notion, our present study demonstrated that HPR1 activity can be detected in the supernatant of the pancreatic cancer cell line and that HPR1 activity in the supernatants was proportional to HPR1 activity.
receptors and inhibits the proliferative effect of FGF on tumor or heparitinase decreases the affinity of FGF binding to FGF. Down-regulation of cell surface HSPG expression by chlorate lack HS expression due to the mutation of HSPGs as their co-receptors. Chinese hamster ovary cells that presented on the cell surface or secreted as a soluble molecule. Lines and in the BM of tumor tissue can be degraded by HPR1 in the cytosols of five pancreatic cancer cell lines. Further studies using FACS analysis showed that HPR1 was present on the cell surface of a transfected cell line. It is likely that HPR1 may also be expressed on the cell surface of pancreatic cancer cells. Unfortunately, we were unable to detect it with several anti-HPR1 antibodies available in our laboratory. Nevertheless, our present study suggests that HS on the cell surface of tumor cell lines and in the BM of tumor tissue can be degraded by HPR1 presented on the cell surface or secreted as a soluble molecule. It is not clear whether HS degradation also occurs in the cytosol by HPR1 before they traffic to the cell surface.

HSPGs have been implicated in playing a critical role in regulating the mitogenic activity of the growth factors that require HSPGs as their co-receptors. Chinese hamster ovary cells that lack HS expression due to the mutation of N-sulfotransferase respond poorly to FGF-stimulated cell proliferation (28). Down-regulation of cell surface HSPG expression by chlorate or heparitinase decreases the affinity of FGF binding to FGF receptors and inhibits the proliferative effect of FGF on tumor and endothelial cells. Korc and co-workers (5, 6) reported that removal of cell surface glypican-1 by phosphatidylinositol-phospholipase C inhibits FGF- and HB-EGF-induced proliferation but has no effect on epidermal growth factor- and insulin-like growth factor-induced proliferation in pancreatic and breast cancer cell lines. In the present study, we demonstrated that up-regulation of cell surface HS expression by HPR1 inhibitors alone led to increased PANC-1 cell proliferation in the absence of exogenous FGF. We speculate that heparin and PI-88 exert their mitogenic effect by increasing cell surface HS levels, subsequently facilitating the binding of endogenous FGF2 to the receptors.

The role of cell surface HSPGs in enhancing FGF2-mediated mitogenic effect was also supported by our observation showing that removal of cell surface HS by exogenous HPR1 led to the inhibition of the proliferation of PANC-1 cells. Since HPR1 cleaves HS with limited number of cleavage sites per HS chain, the innermost fragment of HS side chain is not cleaved from the core protein of HSPGs. However, the remaining stub of HS side chain is incapable of facilitating the binding of FGF2 to its receptors. Rather, exogenous HPR1-mediated suppression of cell proliferation is due to the further shedding of intact residual HS, which could be detected on glypican-1 after affinity purification of total cellular HSPGs (6). Taken together, our data suggest that modulation of cell surface HS levels by HPR1 can affect the proliferation of pancreatic cancer cells in response to endogenous or exogenous FGF.

Francis et al. (29) recently showed that PI-88 is able to inhibit the proliferation of rat aortic smooth muscle cells grown in 5% FBS and to inhibit neointima formation after balloon injury to rat and rabbit carotid arteries in vivo. The inhibitory effect of PI-88 on smooth muscle proliferation is mediated by blocking FGF2 binding to the FGF receptors and by blocking the release of FGF2 stored in the extracellular matrix of the arterial walls. Although PI-88 treatment blocked the degradation of HSPGs in the arterial wall, increased HS levels in the smooth muscle cells by PI-88 did not result in an increase of cell proliferation, probably due to the unavailability of free FGF or due to the binding of PI-88 to FGF. In contrast, our present study showed that PI-88 was able to stimulate the proliferation of PANC-1 cells grown in serum-free medium and that PI-88 did not block FGF2-stimulated PANC-1 cell proliferation but rather had an additive effect on FGF2-mediated cell proliferation. We speculate that the mitogenic activity of PI-88 on PANC-1 cells is mediated by strengthening the binding of FGF2 or other heparin-bound growth factor to their receptors. The inability of PI-88 to block FGF2-induced cell proliferation may be due to the high levels of endogenous FGF2 produced by pancreatic cancer cells that have already bound to the FGF receptors.

Similar to PI-88, heparin has the opposite effects on cell proliferation, depending on cell type. Heparin inhibits the proliferation of smooth muscle cells (30). The mechanisms of heparin-mediated antimitogenic activity remain to be fully understood. Recent studies showed that heparin is able to activate the double-stranded RNA-activated protein kinase (PKR) (31) and to block cell cycle progression (32). In contrast, the mechanism of heparin-mediated stimulatory effect on tumor cells is better understood. Heparin facilitates FGF2 binding to FGF recep-
tors, subsequently promoting the proliferation of tumor cells stimulated by FGF and other heparin-bound growth factors. Since heparin is also an inhibitor of HPR1 (33), and our present study demonstrated that heparin was able to increase cell surface HSPG expression by inhibiting HPR1 activity in pancreatic cancer cells, the stimulatory effect of heparin on FGF-mediated cell proliferation may be mediated in part by increasing the expression of cell surface HSPGs that function as the co-receptor of FGF.

Although both heparin and PI-88 can suppress or stimulate cell proliferation, it should be made clear that the underlying mechanisms may not be identical. 1) PI-88 is able to bind to FGF2 and to prevent its binding to FGF receptor, whereas heparin always enhances the binding of FGF2 to its receptor. 2) Heparin can bind PKR (31) and block cell cycle progression (32), whereas it is not clear whether PI-88 can do the same. 3) Heparin was able to induce a rapid, transient activation of the MAPK pathway at 0.5–1 h after the addition of heparin in Rama-27 fibroblast cells. In contrast, PI-88 induced ERK1/2 phosphorylation in a steady manner over a 16-h incubation period. 4) Heparin but not PI-88 at a low concentration was able to enhance FGF2-stimulated PANC-1 cell proliferation (Fig. 6C). Although heparin and PI-88 may have different mechanisms of action, it appears that both can enhance cell proliferation by inhibiting HPR1 activity and increasing cell surface HS levels, subsequently facilitating the binding of endogenous or exogenous FGF2 to their receptor.

The antimitogenic effect of HPR1 shown here in vitro seems to contradict the clinical and preclinical observations that HPR1 expression in a variety of malignancies correlates with a shorter survival. We believe that the in vivo effect of HPR1 is more complicated. First, HPR1 can promote tumor angiogenesis and metastasis by breaking down the BM and extracellular matrix and releasing the growth factors, such as FGF and vascular endothelial growth factor, trapped in the extracellular matrix and BM (11, 12). Tumor cells stably transfected with the HPR1 gene become more angiogenic and metastatic (35, 36), whereas the inhibition of HPR1 activity by pharmacologic agents or genetic approaches suppresses tumor metastasis and angiogenesis (33, 37, 38). Second, the contribution of the FGF receptor-mediated signaling pathway to the cell proliferation of pancreatic adenocarcinomas may be much weaker than the signals from mutant K-Ras, overexpression of HER2/Neu (39–41), sonic hedgehog and its receptor (42, 43). Finally, since FGF2 binding to its HSPG co-receptors can inhibit the degradation of HS mediated by HPR1 (44), it is possible that FGF binding to its receptor and co-receptor on pancreatic tumor cells may block HS cleavage by HPR1. For these reasons, the ultimate outcome of HPR1 expression in tumor cells will depend on the delicate balance between the levels of HPR1 and its substrates and on whether the heparin-bound growth factors are required for their proliferation.

In summary, our present study provides evidence that HPR1 expressed in pancreatic adenocarcinomas and tumor cell lines is capable of degrading HS in the BM and on the cell surface. Cell surface HS degradation by HPR1 led to a poor response of PANC-1 tumor cells to FGF2-mediated activation of the MAPK pathway. Our study suggests that HPR1 expression may have two opposite effects. It can promote tumor angiogenesis and metastasis by degrading the extracellular matrix and releasing the growth factors; at the same time, degradation of cell surface HSPGs also inhibits the mitogenic effect of FGF and possibly other growth factors that require HSPGs as their co-receptors. A recent study by Joyce et al. (45) using a mouse model of multistage pancreatic islet carcinogenesis showed that PI-88 is able to inhibit the growth of islet tumor cells by reducing angiogenesis and cell proliferation and promoting tumor cell apoptosis. However, the authors caution that inhibition of HPR1 activity under certain special situations may actually promote growth factor receptor signaling, which is exactly shown in our present study.

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