Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer

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Masamune A, Kikuta K, Watanabe T, Satoh K, Hirota M, Shimosegawa T. Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer. Am J Physiol Gastrointest Liver Physiol 295: G709–G717, 2008. First published July 31, 2008; doi:10.1152/ajpgi.90356.2008.—Pancreatic cancer is characterized by excessive desmoplastic reaction and by a hypoxic microenvironment within the solid tumor mass. Chronic pancreatitis is also characterized by fibrosis and hypoxia. Fibroblasts in the area of fibrosis in these pathological settings are now recognized as activated pancreatic stellate cells (PSCs). Recent studies have suggested that a hypoxic environment concomitantly exists not only in pancreatic cancer cells but also in surrounding PSCs. This study aimed to clarify whether hypoxia affected the cell functions in PSCs. Human PSCs were isolated and cultured under normoxia (21% O$_2$) or hypoxia (1% O$_2$). We examined the effects of hypoxia and conditioned media of hypoxia-treated PSCs on cell functions in PSCs and in human umbilical vein endothelial cells. Hypoxia induced migration, type I collagen expression, and vascular endothelial growth factor (VEGF) production in PSCs. Conditioned media of hypoxia-treated PSCs induced migration of PSCs, which was inhibited by anti-VEGF antibody but not by antibody against hepatocyte growth factor. Conditioned media of hypoxia-treated PSCs induced migration of PSCs, which was inhibited by anti-VEGF antibody but not by antibody against hepatocyte growth factor. Conditioned media of hypoxia-treated PSCs induced endothelial cell proliferation, migration, and angiogenesis in vitro and in vivo. PSCs expressed several angiogenesis-regulating molecules including VEGF receptors, angiotensin-1, and Tie-2. In conclusion, hypoxia induced profibrogenic and proangiogenic responses in PSCs. In addition to their established profibrogenic roles, PSCs might play proangiogenic roles during the development of pancreatic fibrosis, where they are subjected to hypoxia.

pancreatitis; pancreatic fibrosis; hepatocyte growth factor; vascular endothelial growth factor

PANCREATIC CANCER IS CHARACTERIZED BY EXCESSIVE DESMOPLASTIC REACTION AND BY A HYPOXIC MICROENVIRONMENT WITHIN THE SOLID TUMOR MASS, AS PROVEN BY THE NEEDLE MEASUREMENT OF INTRATUMORAL O$_2$(17). CHRONIC PANCREATITIS (CP) IS ALSO CHARACTERIZED BY PANCREATIC FIBROSIS AND HYPOXIA (24). THE CELLULAR RESPONSE TO HYPOXIA IS MEDIATED BY THE TRANSCRIPTION FACTOR, HYPOXIA-INDUCIBLE FACTOR (HIF)-1, WHICH IS A HETERODIMER PROTEIN COMPOSED OF α AND β SUBUNITS (14, 27). WHEREAS HIF-1α PROTEIN IS CONSTITUTIVELY EXPRESSED UNDER NORMOXIA, HIF-1α IS UNSTABLE UNDER NORMOXIA BECAUSE OF AN O$_2$-DEPENDENT DEGRADATION INVOLVING AN UBIQUITIN-PROTEASOMAL PATHWAY. HIF-1α IS AN O$_2$-SENSITIVE PARTNER, AS IT IS ACCUMULATED UNDER HYPOXIA, TRANSLATES TO THE NUCLEUS, AND TRANSCRIPTS A VARIETY OF GENES INCLUDING VEGF AND PLASMINOGEN ACTIVATOR INHIBITOR-1 (14, 27). IT HAS BEEN ESTABLISHED THAT HYPOXIA IS A DRIVING FORCE IN THE PROGRESSION OF PANCREATIC CANCER. HYPOXIA IN TUMORS IS ASSOCIATED WITH POOR PROGNOSIS, RESISTANCE TO CHEMOTHERAPY AND RADIATION THERAPY, AND INCREASED METASTATIC POTENTIAL (7, 9). THESE EFFECTS ARE ATTRIBUTABLE, IN PART, TO THE HYPOXIA-INDUCED EXPRESSION OF PROANGIOGENIC FACTORS SUCH AS VEGF IN Pancreatic cancer cells (7, 9).

Fibroblasts in the stromal tissues in pancreatic cancer and in CP are now recognized as activated pancreatic stellate cells (PSCs) (3, 23, 30). In normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed (“activated”) from their quiescent phenotype into myofibroblast-like cells, which actively proliferate, migrate, and produce extracellular matrix components including type I collagen (1, 2, 20, 23). Recent studies have shown the expression of hypoxia-regulated markers HIF-1α and carbonic anhydrase 9 in stromal cells within the pancreatic tumors (6, 11, 16), suggesting that a hypoxic environment concomitantly exists not only in cancer cells but also in surrounding PSCs. Although the effects of hypoxia on pancreatic cancer cells have been extensively studied, little is known about the effects on PSCs. We here show that hypoxia induces profibrogenic and proangiogenic responses in PSCs.

MATERIALS AND METHODS

Materials and Animals

Mouse anti-HIF-α antibody was from Novus Biologicals (Littleton, CO). PDGF-BB and mouse neutralizing antibodies against VEGF and hepatocyte growth factor (HGF) were from R&D Systems (Minneapolis, MN). Goat anti-type I collagen antibody was from Southern Biotechnology (Birmingham, AL). Rabbit antibodies against ERK and Akt were from Cell Signaling Technology (Beverly, MA). Rabbit antibody against GAPDH was from Trevigen (Gaithersburg, MD). U0126, an inhibitor of ERK pathway, and wortmannin, an inhibitor of phosphatidylinositol-3-kinase-Akt pathway, were from Calbiochem-Novabiochem (San Diego, CA). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless specifically described.

Cell Culture

Human PSCs were isolated from the resected pancreas tissues of patients undergoing operation for pancreatic cancer as previously described (3) under the approval by the Ethics Committee of Tohoku University School of Medicine. Experiments were performed using human PSCs from at least three independent preparations and those between passages 3 and 7 after isolation. Cells were maintained in Ham’s F-12/DMEM (1:1) supplemented with 10% heat-inactivated FBS (MP Biomedicals, Irvine, CA), penicillin sodium, and streptomycin sulfate.

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Hypoxic Treatment and Preparation of the Conditioned Media

PSCs were serum starved overnight and incubated in a hypoxic incubator with 1% O_2, 5% CO_2, and 94% N_2. The oxygen level in the culture chambers was continuously monitored. Control experiments included parallel cultures in which cells were exposed to normoxia (21% O_2, 5% CO_2, and 74% N_2). Culture medium was preconditioned to the correct O_2 level in hypoxic experiments.

The serum-free conditioned media of PSCs was harvested following 48-h incubation under normoxia or hypoxia, centrifuged at 3,000 revolution/min for 10 min, filtered through 0.22-μm filters, and stored at −80°C until use. The supernatants were used as normoxia-conditioned media (NCM) or HCM. For in vivo angiogenesis assay, HCM was concentrated by 10-fold through 3,000 molecular weight cut-off filters (Centricron-YM3; Millipore, Bedford, MA) according to the manufacturer’s instruction.

Evaluation of Hypoxia

Detection of pimonidazole adducts. PSCs were treated with pimonidazole hydrochloride (Hypoxyprobe-1; NPL, Burlington, MA; at 200 μM) during hypoxic incubation for 6 h. Pimonidazole adducts were subsequently detected using the FITC-conjugated mouse antibody according to the manufacturer’s instruction. After being washed, cells were analyzed for fluorescence with the use of a confocal laser scanning microscope (ECLIPSE TE2000-U; Nikon, Tokyo, Japan). Nuclear counterstaining was performed with propidium iodide.

Nuclear extracts preparation and Western blotting. Nuclear extracts were prepared using CellLytic NuCLEAR extraction kit according to the manufacturer’s instruction. Nuclear extracts (~10 μg) were fractionated on a 10% SDS-polyacrylamide gel. They were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membrane was incubated with mouse anti-HIF-1α antibody overnight at 4°C. After incubation with peroxidase-conjugated goat anti-mouse IgG antibody, proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences UK, Buckinghamshire, UK).

Cell Proliferation Assay

Cell proliferation was assessed using a commercial kit (Roche Applied Science, Mannheim, Germany). This is a colorimetric immunoassay based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis. After 24-h incubation under normoxia, hypoxia, or with PDGF-BB (which served as a positive control), cells were labeled with BrdU for 3 h at 37°C. Cells were fixed and incubated with peroxidase-conjugated anti-BrdU antibody. Then the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine was added, and BrdU incorporation was quantitated by optical density (OD) 370-OD 492. Proliferation of HUVECs in response to HCM was examined in a similar manner.

Cell Migration Assay

Nonoriented migration (chemokinesis) and chemotaxis were evaluated by the wound-healing assay and the modified Boyden chamber assay, respectively.

Wound-healing assay. PSCs were grown to confluent in 12-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ). After overnight serum starvation, cell monolayer was mechanically scarred with a sterile 200-μl pipette tip and subjected to normoxia or hypoxia for 24 h.

Chemotaxis assay. Chemotaxis was examined by the modified Boyden chamber assay using a commercial kit (QCM chemotaxis colorimetric cell migration assays; Millipore). Briefly, serum-starved PSCs were detached, washed, and resuspended in serum-free medium. Cell suspension (2 × 10^5 cells/well) was added to the culture inserts with 8-μm pore in a 24-well companion plate. The lower chamber included serum-free medium, NCM, or HCM in the absence or presence of antibodies against VEGF and HGF. After 24-h incubation, cell suspension in the upper chamber was aspirated, and the upper surface of the filter was carefully cleaned with cotton plugs. Cells that migrated through the polycarbonate membrane were stained, extracted, and quantified on a standard microplate at 560 nm. Chemotaxis of HUVECs in response to HCM was examined in a similar manner.

Collagen Assay

Type I collagen derives from a larger protein, type I procollagen, which has propeptide extensions at both ends of the molecule. Specific enzymes remove these propeptides before the collagen molecules are assembled into fibers. The sequence removed from the carboxy terminus, procollagen type I C-peptide (PICP), is secreted by cells, and its level reflects the amount of synthesis of type I collagen. Therefore, PICP can be used as an index for type I collagen synthesis. Human PSCs were plated in 24-well plates and grown to confluent. After 48-h incubation under hypoxia or normoxia, culture supernatants were harvested and stored at −80°C until use. PICP level in cell culture supernatant was determined by ELISA (Takara Bio) according to the manufacturer’s instruction. Type I collagen expression in cellular lysates was assessed by Western blotting.

ELISA

Cell culture supernatants were harvested, and the levels of VEGF, IL-8, and HGF were determined by ELISA (VEGF assay from Pierce Chemical, Rockford, IL; IL-8 and HGF assays from R&D Systems) according to the manufacturers’ instruction.

Assessment of ERK and Akt Activation

Activation of ERK and Akt was examined by Western blotting using anti-phosphospecific antibodies (Cell Signaling Technology) as previously described (22). These antibodies recognize only phosphorylated forms of ERK and Akt, thus allowing the assessment of the kinase activation. Cells were lysed, and total cell lysates (~100 μg) were fractionated on a 10% SDS-polyacrylamide gel. They were transferred to a nitrocellulose membrane (Bio-Rad), and the membrane was incubated overnight at 4°C with rabbit antibodies against phosphorylated ERK and Akt. After incubation with peroxidase-conjugated goat anti-rabbit IgG antibody, proteins were visualized using an enhanced chemiluminescence kit. The levels of total ERK and total Akt were also determined.

Tube Formation Assay

Angiogenesis in vitro was assessed using the endothelial tube formation assay (Cell Biolabs, San Diego, CA). The tube formation assay is based on the ability of endothelial cells to form three-dimensional capillary-like tubular structures when cultured on a gel of basement membrane extract. Briefly, each well of prechilled 24-well cell culture plates (Becton Dickinson) was coated with a thin layer of the extracellular matrix gel prepared from Engelbreth-Holm-Swarm tumor cells (200 μl/well), which was left to polymerize at 37°C for 1 h. HUVECs (5 × 10^4 cells/well) were added to each well onto...
solidified extracellular matrix gel in 600 µl of serum-free medium or of HCM. After 18-h incubation, endothelial cell tube formation was assessed under light microscopy.

**In Vivo Angiogenesis Assay**

We examined whether HCM induced angiogenesis in vivo by the directed in vivo angiogenesis assay (Trengien) according to the manufacturer’s instruction. All animal procedures were performed in accordance with the National Institutes of Health Animal Care and Use Guidelines. Briefly, prechilled, semi-closed silicone cylinders (“angioreactors”) were filled with 20 µl of the basement membrane extract premixed with serum-free medium only or 10-fold concentrated HCM. After 1-h incubation to promote gelling, angioreactors were implanted subcutaneously in the dorsal flanks of nude mice (Charles River Laboratories Japan, Yokohama, Japan). After 14 days, angioreactors were removed from mice, and vessel formation was visually assessed.

### Table 1. Sequences of PCR primer sets used to amplify angiogenesis-regulating molecules

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flk-1</td>
<td>CACCATACCTCCTGGAAACCT</td>
<td>CCCACTTGCTGGCATCATAAGG</td>
<td>483</td>
</tr>
<tr>
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<td>ACCCGAGACATGGAATCACCA</td>
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<tr>
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<td>GTATCTGGGGGATCTGCCGC</td>
<td>339</td>
</tr>
<tr>
<td>Ang-2</td>
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<tr>
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<td>CACGTCACATCGCAGCTGA</td>
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<tr>
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<td>CCCAGCTTCACCTTCTGGA</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>GGAGGAAGTGGAAGGTCGAGA</td>
<td>GAGGGCGAGAGATGATGA</td>
<td>371</td>
</tr>
</tbody>
</table>

Ang, angiopoietin.

**Expression of Angiogenesis-related Molecules in PSCs**

Total RNA was prepared from PSCs using RNeasy total RNA preparation kit (Qiagen, Valencia, CA). Total RNA (200 ng) was reverse transcribed, and the resultant cDNA was subjected to PCR in a volume of 30 µl. Primer sequences and expected size of the PCR products are shown in Table 1. Cycle condition was as follows: preheating at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and then a final extension at 72°C for 10 min. A sample (5 out of the 30 µl of the PCR products was separated by 1.5% agarose gel electrophoresis and visualized under ultraviolet after staining with ethidium bromide.

**Immunofluorescent Staining**

The pancreas tissues were removed from patients undergoing operation for pancreatic cancer and fixed by immersing in 4% paraformaldehyde overnight at 4°C. The specimens were embedded in...
regular paraffin wax and cut into 4-μm sections. Immunofluorescent staining for VEGF and α-smooth muscle actin (SMA) was performed as previously described (21). Briefly, tissue sections were deparaffinized and rehydrated in PBS. After the incubation with 10% normal goat serum for 1 h, the slides were incubated with rabbit antibody against VEGF (at 1:100 dilution; Abcam, Cambridge, UK) and mouse antibody against α-SMA (at 1:200 dilution) overnight at 4°C. The slides were incubated with FITC-conjugated goat anti-rabbit IgG antibody (at 1:200 dilution; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 546-labeled goat anti-mouse IgG antibody (at 1:200 dilution; Molecular Probes, Eugene, OR) for 45 min. After washes, the slides were analyzed for fluorescence using a confocal laser scanning microscopy. Expression of HIF-1α in PSCs in vivo was assessed in a similar manner using mouse antibody against HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-α-SMA antibody (Abcam). FITC-conjugated goat anti-mouse IgG antibody (at 1:200 dilution; Jackson ImmunoResearch) and Alexa Fluor 546-labeled goat anti-rabbit IgG antibody (at 1:200 dilution; Molecular Probes) were used as secondary antibodies.

Statistical Analysis

The results were expressed as means ± SD. Experiments were performed at least three times, and similar results were obtained. Representative luminograms are shown. Differences between the groups were evaluated by ANOVA, followed by Fisher's test for post hoc analysis. A P value <0.05 was considered statistically significant.

RESULTS

PSCs were hypoxic in our experimental settings. To initially confirm that PSCs were indeed hypoxic, we used the hypoxia marker pimonidazole, a nitroheterocyclic drug whose hypoxia-dependent activation by cellular nitroreductases leads to the formation of covalent intracellular adducts between cellular macromolecules and the drug itself (19). PSCs treated under hypoxia but not normoxia stained for the adducts (Fig. 1A). Hypoxia induced nuclear expression of HIF-1α as assessed by Western blotting (Fig. 1B) and immunofluorescent staining (Fig. 1C), further supporting that PSCs responded to hypoxia in vitro in our experimental settings. In addition, we performed immunofluorescent staining in the resected pancreas tissues of patients with pancreatic cancer. In agreement with previous reports (6, 16), nuclear HIF-1α expression was observed in α-SMA-positive PSCs in the area of pancreatic fibrosis (Fig. 1D), suggesting that PSCs were under hypoxic conditions in vivo, too.

Hypoxia induced migration and collagen production. We examined the effects of hypoxia on key cell functions of PSCs. Hypoxia did not induce proliferation, whereas PDGF-BB, one of the most potent mitogens for PSCs in vitro (23), did (Fig. 2A). Hypoxia induced migration as assessed by the wound-healing assay (Fig. 2B). Hypoxia induced type I collagen production, as assessed by PICP production and Western blotting (Fig. 2, C and D). Hypoxia induced VEGF in a time-dependent manner but not IL-8 production (Fig. 3, A and B). Human PSCs constitutively expressed a significant amount of HGF, but the production was not increased by hypoxia (Fig. 3C).

HCM induced migration of PSCs in part through VEGF. We then hypothesized that some autocrine factor(s) produced by
PSCs under hypoxia induced migration of PSCs. To address this issue, PSCs were treated with HCM and NCM. HCM induced nonoriented and oriented (chemotaxis) migration of PSCs (Fig. 4, A and B). The effect of HCM on cell migration was abolished by boiling HCM for 10 min before the assay, suggesting the presence of labile growth factors (data not shown). NCM induced oriented migration as assessed by the Boyden chamber assay, but the effect was less evident compared with that of HCM (Fig. 4B). HCM-induced PSC migration was inhibited by the neutralizing antibody against VEGF but not by anti-HGF antibody, suggesting a role of VEGF in this process.

**HCM induced endothelial cell proliferation and migration.** Production of VEGF by hypoxia-treated PSCs prompted us to examine whether PSCs had angiogenic activities. Angiogenesis consists of multiple processes including endothelial cell proliferation, migration, alignment to form cord-like structures, and vessel maturation (26). HCM induced proliferation and migration in HUVECs (Fig. 5, A and B). HCM induced activation of ERK and Akt in time-dependent manners (Fig. 5C). HCM-induced proliferation was inhibited by U0126, an inhibitor of ERK pathway, and by wortmannin, an inhibitor of phosphatidylinositol-3-kinase-Akt pathway (Fig. 5D). Similarly, HCM-induced migration was inhibited by both inhibitors. Thus HCM induced endothelial cell proliferation and migration by the activation of ERK and phosphatidylinositol-3-kinase-Akt pathways.

**HCM induced angiogenesis in vitro and in vivo.** We examined whether HCM could support the alignment of endothelial cells and formation of lumen-like structures. HUVECs, when plated on extracellular matrix gels and incubated with HCM, aligned to form lumen-like structures and anastomosing tubes with multicentric junctions (Fig. 6A). By contrast, little endothelial cell morphogenesis occurred after the incubation of endothelial cells with the control serum-free medium.

In vivo angiogenesis was assessed by the directed in vivo angiogenesis assay. Semi-closed silicone cylinders, called angioreactors, were filled with the control serum-free medium only or with HCM. They were subcutaneously implanted into nude mice. After 14 days, HCM but not the control medium induced vessel formation (Fig. 6B). Collectively, HCM induced angiogenesis both in vitro and in vivo.

**PSCs expressed angiogenesis-regulating molecules.** Finally, we examined the expression of angiogenesis-related molecules in PSCs by reverse transcription-PCR. Human PSCs expressed mRNAs for VEGF receptors Flt-1 and Flk-1 (Fig. 7). PSCs also expressed angiopoietin-1 and its receptor Tie-2 but not angiopoietin-2. PSCs also expressed vasohibin-1, a recently-identified angiogenesis-regulating molecule (32). To examine whether PSCs expressed VEGF in vivo, we performed immunofluorescent staining in the resected pancreas tissues of patients with pancreatic cancer. Colocalized expression of α-SMA (an index of activated PSCs) and VEGF was observed in the area of pancreatic fibrosis (Fig. 8), suggesting that activated PSCs expressed VEGF in vivo. Thus PSCs expressed several angiogenesis-regulating molecules, further supporting their roles in angiogenesis.

**DISCUSSION**

A hypoxic environment in tumor plays an important role in pancreatic cancer progression, and CP is also characterized by hypoxia. Recent studies have suggested that a hypoxic environment concomitantly exists not only in cancer cells but also in surrounding PSCs (6, 11, 16). Therefore, PSCs, cancer cells, endothelial cells, and other cells involved in the development of pancreatic fibrosis have to coordinately operate in a low-
oxygen microenvironment. We hypothesized that hypoxia had stimulatory rather than inhibitory effects on PSC functions because activation of PSCs is a prerequisite for the development of pancreatic fibrosis (1, 2, 23). We here showed that hypoxia induced migration, type I collagen, and VEGF production in human PSCs. To our knowledge, this is the first study showing that hypoxia directly stimulates cell functions in PSCs. Conditioned media of hypoxia-treated PSCs induced migration of PSCs, which was inhibited by the neutralizing antibody against VEGF. Thus the effect on cell migration

Fig. 4. Hypoxia-conditioned medium (HCM) induced migration of PSCs, which was inhibited by anti-VEGF neutralizing antibody. A: PSCs were grown to confluency on 12-well cell culture dishes. After overnight serum starvation, cell monolayer was mechanically scarred, and cells were allowed to migrate in the presence of normoxia-conditioned medium (NCM) or HCM for 24 h. Arrows denote the scraped margins. B: chemotaxis assay was performed using normoxic cells exposed to NCM or HCM, in the absence or presence of antibodies (Ab) against VEGF or HGF. Serum-free medium served as a control (Cont). Cells that migrated through the polycarbonate membrane were stained, extracted, and quantified on a standard microplate at OD 560. **P < 0.01 vs. HCM only.

Fig. 5. HCM induced endothelial cell proliferation and migration. A and B: human umbilical vein endothelial cells (HUVECs) were treated with HCM at the indicated concentrations. A: cell proliferation was assessed by BrdU incorporation ELISA, and the optical density (OD 370-OD 492) is shown. **P < 0.01 vs. HCM at 0%. B: chemotaxis of HUVECs was examined by the modified Boyden chamber assay. Cells that migrated through the polycarbonate membrane were stained, extracted, and quantified on a standard microplate at OD 560. **P < 0.01 vs. HCM at 0%. C: after the treatment with HCM for the indicated time, cell lysates were prepared from HUVECs, and the levels of ERK (phosphorylated and total) and Akt (phosphorylated at Ser473 and total) were determined by Western blotting. D: HUVECs were left untreated or treated with HCM (at 90%) in the absence or presence of U0126 (U, at 5 μM) or wortmannin (W, at 100 nM). Cell proliferation and migration were examined. **P < 0.01 vs. HCM only.
appeared to be mediated, in part, through hypoxia-induced production of VEGF in PSCs although other factors might be also involved. Contribution of PDGF, the most potent mitogen for PSCs in vitro (23), was unlikely because hypoxia did not induce proliferation. Ide et al. (11) examined the effects of HCM of pancreatic stromal cells on the invasion of pancreatic cancer cells. They showed that HCM increased invasion of pancreatic cancer cells through the hypoxia-induced production of HGF in pancreatic stromal cells. However, the role of HGF was unlikely in our study because hypoxia did not induce HGF, and HCM-induced PSC migration was not inhibited by anti-HGF antibody. The reason for the discrepancy between ours and the study by Ide et al. (11) remains unclear, but one explanation might be that Ide et al. employed human fetal lung
fibroblast cell line MRC5 but not PSCs as a model of stromal cells in the pancreas.

Although originally identified as a principal producer of extracellular matrix components, recent studies have revealed that PSCs exhibit a variety of cell functions. HIF-1α is a central factor in the modulation of cellular responses to hypoxia (14, 27). Hypoxia induced nuclear HIF-1α expression, implying that PSCs might serve as oxygen-sensing cells in the pancreas. PSCs were a significant source of VEGF; VEGF might operate as a hypoxia-dependent, autocrine and paracrine factor able to stimulate migration and recruit profibrogenic PSCs into the areas of cancer and inflammation. In addition, PSCs exhibited proangiogenic phenotype; HCM induced endothelial cell proliferation, migration, and angiogenesis both in vitro and in vivo. HCM-induced proliferation and migration in HUVECs were inhibited by inhibitors of ERK and phosphotyridinositol-3-kinase-Akt pathways, suggesting a role of these signaling pathways in these processes. Of course, VEGF is likely to play a role in HCM-induced endothelial cell activation and angiogenesis in agreement with the concept that VEGF production by stromal fibroblasts plays an important role in tumor angiogenesis (8, 13). We here showed the colocalization of VEGF and α-SMA (an index of activated PSCs) in the area of pancreatic fibrosis in patients with pancreatic cancer, suggesting that activated PSCs expressed VEGF also in vivo. In tumors, stromal fibroblasts (activated PSCs in the case of pancreatic cancer) as well as cancer cells are a significant source of VEGF-promoting angiogenesis (8, 13). Of note, previous studies have shown that conditioned media of fibroblasts failed to induce angiogenesis in vitro and in vivo (10, 13). One explanation might be that VEGF secreted from some fibroblasts is intrinsically inactive and that cancer cells reactivate this latent inactive form of VEGF, making it selectively available within the tumor microenvironment (13). Indeed, through the production of VEGF, human VA-13 fibroblasts promoted angiogenesis and growth of Capan-1 human pancreatic cancer xenograft tumors, whereas VA-13 fibroblasts alone did not show significant angiogenesis. In addition to VEGF and its receptors (Flt-1 and Flk-1), PSCs expressed mRNAs for angiopoietin-1 and its receptor Tie-2, suggesting another autocrine/paracrine loop in the regulation of proangiogenic functions in PSCs. PSCs also expressed mRNA for vasohibin-1, a recently identified angiogenesis regulator (32). In addition to predominant expression in endothelial cells, other cell types express vasohibin-1 including human aortic smooth muscle cells and fibroblasts (32). Of note, the present and previous studies have shown that PSCs constitutively produce and secrete an array of proangiogenic factors including VEGF, basic fibroblast growth factor, IL-8, and PDGF (reviewed in Ref. 23). PSCs also produce and secrete matrix metalloproteinase 9, which is implicated in the proteolysis of the basement membrane early during the invasion stage of angiogenesis (25). Many of these factors act additively or synergistically to promote neovascularization (10, 26).

Previous studies have shown that hypoxia directly contributes to an aggressive behavior of pancreatic cancer cells; hypoxia induced proliferation, migration, and resistance to gemcitabine-induced apoptosis in pancreatic cancer cells in vitro (7, 9, 29, 35). In vivo, hypoxia in tumors is associated with poor prognosis, resistance to chemotherapy and radiation therapy, and increased metastatic potential (7, 9). These effects are attributable, in part, to the expression of proangiogenic factors such as VEGF and IL-8 in pancreatic cancer cells (4, 7, 9, 28, 29). Our study adds a new insight as to the role of hypoxia on the progression of pancreatic cancer from the viewpoint of stromal-tumor interaction, which has received increasing attention in recent years. Although it is still controversial, recent studies have supported the concept that desmoplastic reaction created by the interaction between pancreatic cancer cells and PSCs favors the progression of pancreatic cancer (3, 30). Profibrogenic and proangiogenic responses in PSCs suggest a novel mechanism by which hypoxia contributes to the development of tumor-supportive microenvironments. In addition, VEGF, produced by PSCs under hypoxia, might affect the behavior of neighboring pancreatic cancer cells in a paracrine manner. VEGF and its receptors are overexpressed in human pancreatic cancer as well as in endothelial cells (12). It has been shown that VEGF induced migration, invasion, and epithelial-to-mesenchymal transition in pancreatic cancer cells (33, 34).

There is a concept that angiogenesis and fibrosis commonly occur together in many disease states where neovascularization is involved in the pathological cascade (15). For example, pathological angiogenesis is associated with the fibrogenic progression in the liver (5, 36). Neutralizing antibodies against VEGF receptors attenuated the development of liver fibrosis in association with the suppression of neovascularization in injured liver (36). Anti-angiogenesis factor TNP-470 suppressed the activation of hepatic stellate cells and subsequent vascularity in injured liver in vivo (31). Thus VEGF produced by activated hepatic stellate cells is now believed to play a key role in angiogenesis and fibrogenesis in the injured liver. In the pancreas, a significant association between fibrosis, angiogenesis, and higher VEGF expression has been reported (6). Increased angiogenesis has been described in pancreatic cancer and in CP (18). Our results suggest that PSCs play profibrogenic and proangiogenic roles during the development of pancreatic fibrosis, where they are subjected to hypoxia. Although further in vivo studies are needed for a more detailed characterization of this scenario, our results suggest a novel mechanism linking hypoxia, inflammatory responses, angiogenesis, and fibrogenesis in the pancreas.

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