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CXC-chemokine/CXCR2 biological axis promotes angiogenesis *in vitro* and *in vivo* in pancreatic cancer

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Angiogenesis is essential for tumor growth and metastasis. Although ELR$^+$-CXC-chemokines and their corresponding receptor, CXC-receptor 2 (CXCR2), are known mediators of angiogenesis, little is known about their role in pancreatic cancer (PaCa). The aim of our study was to determine the role of ELR$^+$-CXC-chemokine/CXCR2 biological axis in promoting PaCa angiogenesis. We prospectively collected secretin-stimulated exocrine pancreatic secretions (SSEPS) from normal individuals (NP) and PaCa patients. We showed that summed concentrations of ELR$^+$-CXC-chemokines in SSEPS from PaCa patients were significantly higher than in those from NP ($p = 0.002$). We measured ELR$^+$-CXC-chemokine levels in supernatants from multiple PaCa cell lines and confirmed that BxPC-3, Colo-357 and Panc-28 had significantly higher expression compared with an immortalized human pancreatic ductal epithelial (HPDE) cell line. After confirming lack of autocrine effects of ELR$^+$-CXC-chemokines on PaCa cells (due to absence of CXCR2 expression), we investigated paracrine effects of these chemokines on human umbilical vein endothelial cells (HUVEC). Both recombinant ELR$^+$-CXC-chemokines and co-culturing with BxPC-3 significantly enhanced proliferation, invasion, and tube formation of HUVEC ($p < 0.05$). These biological effects were significantly inhibited by treatment with a neutralizing antibody against CXCR2 (anti-CXCR2 Ab) ($p < 0.05$). Finally, anti-CXCR2 Ab significantly reduced tumor volume ($p < 0.05$), Ki-67 proliferation index ($p = 0.043$) and Factor VIII$^+$ microvessel density ($p = 0.004$) in an orthotopic nude mouse PaCa model. Our results show that ELR$^+$-CXC-chemokines promote PaCa tumor-associated angiogenesis through CXCR2, suggesting that CXCR2 is an anti-angiogenic target in PaCa.

Key words: ELR$^+$-CXC-chemokines; CXCR2; angiogenesis; pancreatic cancer

Pancreatic cancer (PaCa) is the fourth leading cause of cancer-related death in the United States, with about 32,000 newly diagnosed cases and an equal number of deaths occurring annually.[1] Because strategies for early detection of PaCa have not been developed, most pancreatic carcinomas are already locally advanced or metastatic at the time of their diagnosis.[2] Chemotherapy and irradiation are largely ineffective, and metastatic disease frequently develops even after potentially curative surgery. Most patients with metastatic disease survive only 3–6 months.[3] Gemcitabine is currently considered to be the most active agent for PaCa. However, its effects are largely palliative, and most patients given this drug still die of their disease within 6 months.[4] New and more effective therapies are clearly needed.

As with other solid tumors, growth and metastasis of PaCa depend on angiogenesis, *i.e.*, the formation of new blood vessels from a pre-existing network of capillaries.[5] Therefore, angiogenesis is a potential therapeutic target in PaCa. A specific target is interleukin-8 (IL-8)/CXC8, one of the major proangiogenic factors.[6,7] Previous reports revealed the critical effect of CXCL8 on PaCa angiogenesis.[8–10] CXC receptor 2 (CXCR2), a G protein-coupled receptor (GPCR), is the corresponding receptor for CXCL8, which promotes PaCa angiogenesis *via* CXCR2.[11] The ELR$^+$ (Glu-Leu-Arg$^+$) CXC-chemokines, including CXCL8, are ligands of CXCR2. Growth-related oncogene-α (GRO-α)/CXCL1 and epithelial-neutrophil activating protein-78 (ENA-78)/CXCL5 are also major members of the ELR$^+$-CXC-chemokine family.[11] CXCL1, originally identified as a melanoma growth-stimulating factor, promotes diverse biological activities including HIV-1 multiplication[12] as well as tumor growth, angiogenesis, and metastasis of squamous cell carcinoma and melanoma.[13,14] Involvement of CXCL5 has also been reported in multiple neoplastic processes including non-small cell lung cancer (NSCLC), where CXCL5 was shown to be an important angiogenic factor.[15]

Levels of multiple cytokines and chemokines are elevated in pancreatic diseases. Patients with elevated levels of interleukin-6 (IL-6) and CXCL8/IL-8 had a worse prognosis, cancer cachexia, and overall poor performance status.[16] In acute pancreatitis, elevated IL-6 and IL-8 levels correlated with symptom severity.[16] Analysis of CXCL8 levels in secretin-stimulated endoscopically-collected exocrine pancreatic secretions (SSEPS) showed a significant difference between normal individuals and patients with diagnosed pancreatic cancer.[16] These results indicated that ELR$^+$-CXC-chemokines secreted in pancreatic juices mediate PaCa tu-

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ANOVA, analysis of variance; Ab, antibody; b-FGF, basic fibroblast growth factor; MTS-assay, Celltiter 96 aqueous one-solution cell proliferation assay; CT, computed tomography; CXCR2, CXCR receptor 2; ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound; EGM, endothelial growth medium; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; ENA-78, epithelial-neutrophil activating protein-78; FCS, fetal calf serum; FNA, fine needle aspiration; FFPE, formalin-fixed, paraffin-embedded; GPCR, G protein-coupled receptor; GFP, green fluorescent protein; GRO-α, growth-related oncogene-α; HPF, high-power field; HMEC-D, human dermal microvascular endothelial cells; HMEC-L, human lung microvascular endothelial cells; HPDE, human pancreatic ductal epithelium; HUVEC, human umbilical vein endothelial cells; IACUC, institutional animal care and use committee; IL, interleukin; KSF, keratinocyte serum-free; MRI, magnetic resonance imaging; NSCLC, non-small cell lung cancer; NP, normal individuals; NS, not significant; PaCa, pancreatic cancer; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviations; SNK, Student-Newman-Keuls; SPA, Substance P antagonist; SOI, surgical orthotopic implantation; VEGF, vascular endothelial growth factor.

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morigenesis. Recently, another report demonstrated that ELR$^+$-CXC-chemokines were significantly over-expressed in malignant human PaCa specimens, suggesting a potential contribution of these chemokines to the pathogenesis of PaCa.[17]

Previous reports have demonstrated that blockade of the ELR$^+$-CXC-chemokines/CXCR2 biological axis reduced tumorigenesis and angiogenesis in lung cancer,[18,19] esophageal cancer,[20] and renal cell carcinoma.[21] Earlier we showed, using a subcutaneous xenograft model in nude mice, that a broad-spectrum GPCR antagonist (Substance P antagonist or SPA) blocked PaCa angiogenesis.[22] Wente *et al*. using a corneal neovascularization assay, showed that the blockade of CXCR2 reduced PaCa angiogenesis.[23] Further, our recent data showed that ELR$^+$-CXC-chemokine production from PaCa cells was increased by co-culturing them with stromal cells.[24] Given the fact that the network of ELR$^+$-CXC-chemokines is modulated by the interaction between the tumor and its microenvironment, it is crucial to investigate the role of the ELR$^+$-CXC-chemokines/CXCR2 biological axis in an orthotopic metastatic PaCa model.

In the present study, we showed that the secretion levels of ELR$^+$-CXC-chemokines in PaCa were higher compared to the normal pancreas using both SSEPS and cell culture supernatants. We observed no effects of ELR$^+$-CXC-chemokines on PaCa growth *in vitro*. We showed that both recombinant and PaCa-derived ELR$^+$-CXC-chemokines promoted the proliferation, invasion, and tube formation of HUVEC, which were inhibited by treatment with anti-CXCR2 Ab. Finally, in an orthotopic PaCa model, we demonstrated that treatment with anti-CXCR2 Ab reduced tumor-associated angiogenesis. Collectively, our results suggest that ELR$^+$-CXC-chemokines through CXCR2 promote PaCa tumor-associated angiogenesis.

Material and methods

Patients

The Mayo Clinic Institutional Board Review (IRB) approved the study, and patients gave written consent before enrollment. In this pilot study, we have enrolled 25 consecutive patients from December 2007 to July 2008, of which seven are normal individuals (NP) and 18 are pancreatic cancer (PaCa) patients.

Inclusion criteria

Normal individuals (NP)—Adult patients (age >18 years) with no abdominal pain or personal history of pancreatic diseases with at least 1 normal imaging test of the pancreas (CT/MRI/EUS) were included in the study.

PaCa—Adult patients (age > 18 years) with clinical symptoms (jaundice, abdominal pain suggestive of pancreatic origin) in conjunction with the presence of a pancreatic mass on imaging studies (CT/MRI/EUS) were asked to participate in the study.

Exclusion criteria

Patients < 18 years, pregnant women, patients who had previous pancreatic or gastric surgery, or those who were unable to tolerate endoscopy were excluded from this study.

Secretin-stimulated exocrine pancreatic secretions (SSEPS) collection

Patients underwent upper gastrointestinal endoscopy after an overnight fast. Moderate conscious sedation was performed by using midazolam and/or meperidine given before the procedure. Synthetic human secretin (*ChiRho*Stim®, ChiRhoClin,Burtonsville, MD) at a dose of 0.2 μg/kg (or a total dose of 16 μg) was administered intravenously during a period of 1 min, immediately before endoscopy. Gastric fluid was aspirated before intubation of the pylorus to minimize contamination. With the endoscope positioned in the second portion of the duodenum, opposite the papilla of Vater (without cannulation), a 2.3-mm plastic aspirating catheter (Hobbs Medical, Stafford Springs, CT) was passed through the biopsy channel of the endoscope until the papilla was visible. Pancreatic juice exiting from the papilla was then suctioned through this plastic tube for 10 min. A 20-mL dry tube attached to the proximal end of the plastic catheter continuously collected the juice using suction. The collected fluid was rapidly placed in 2-mL vials, immediately snap-frozen in liquid nitrogen, and stored at −80°C until chemokine concentration assays were performed. A protease inhibitor cocktail pill (Roche Diagnostic Corporation, Indianapolis, IN) was added to the collected pancreatic juice to inhibit proteolytic digestion.

Determination of final diagnosis in SSEPS study

The gastroenterologist investigators (MR, TAW, and MBW), who were blinded to the results of the cytokine assays, determined the final diagnosis of the patients on the basis of clinical history, laboratory data, imaging (computed tomography [CT] or magnetic resonance imaging [MRI]) or endoscopic (EUS or ERCP) imaging, and cytology and histology, when available. Patients with normal pancreatic imaging procedures results (ERCP, EUS, CT, or MRI) were defined to have a normal pancreas and considered as the control group. For the 25 consecutive patients in this pilot study, final diagnosis was determined after obtaining measurements of ELR$^+$-CXC-chemokines in SSEPS by an independent investigator (KRG) and then further analyzed.

Cell culture

PaCa cell lines BxPC-3, AsPC-1, MIA PaCa-2, Panc-1, Capan-2 and SW 1990 were obtained from the American Type Culture Collection (Rockville, MD). PaCa cell line Colo-357 was kindly provided by Dr. Isaiah J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). PaCa cell line Panc-28 was kindly provided by Dr. Shrikanth Reddy (The University of Texas M. D. Anderson Cancer Center, Houston, TX). BxPC-3 and AsPC-1 cells were maintained in RPMI-1640 (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). MIA PaCa-2, Panc-1, Capan-2, SW 1990, Colo-357 and Panc-28 cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO) with high glucose and 10% FCS. Human umbilical vein endothelial cells (HUVEC), human dermal microvascular endothelial cells (HMEC-D), and human lung microvascular endothelial cells (HMEC-L) were obtained from Lonza Walkersville. (Walkerville, MD). HUVEC were maintained in Endothelial Growth Media-2 (EGM-2, Lonza) with EGM-2 singlequots (Lonza) which contain 2% FCS. HMEC-D and HMEC-L were maintained in Microvascular Endothelial Cell Growth Medium-2 (EGM-2MV, Lonza) with EGM-2 MV singlequots (Lonza) which contain 5% FCS. The human pancreatic duct epithelial (HPDE) cells were a generous gift from Dr. Ming-Sound Tsao (University of Toronto, Ontario, Canada). These cells were cultured in keratinocyte serum-free (KSF) medium supplied with 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (Invitrogen, Carlsbad, CA).

Reagents and antibodies

Recombinant CXCL1, CXCL5 and CXCL8 were obtained from PeproTech. (Rocky Hill, NJ). The following antibodies (Abs) against human CXCR2 (for *in vitro* study), mouse Factor VIII, and Ki-67 were obtained from R&D Systems (Minneapolis, MN), Santa Cruz Biotechnology (Santa Cruz, CA), and NeoMarkers (Fremont, CA), respectively. Normal goat serum IgG was obtained from R&D Systems (Minneapolis, MN). Polyclonal anti-mouse neutralizing CXCR2 antibody used in *in vivo* study was a kind gift from Dr. R. Strieter (University of Virginia, Charlottesville, VA).

Collection of supernatants from PaCa and HPDE cells

For enzyme-liked immunosorbent assays (ELISA), we collected supernatants from PaCa and HPDE cells. PaCa and HPDE cells were seeded at a density of 2×10^5 cells/mL into a 24-well plate and cultured overnight. Medium was then exchanged and cells

were cultured for a further 24, 48 or 72 hr. The culture media were then collected and microfuged at 1,500 rpm for 5 minutes to remove particles, and the supernatants frozen at −80°C until use in the ELISA.

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was performed for CXCL1, CXCL5 and CXCL8 in culture supernatants or SSEPS as per the manufacturer's instructions (R & D Systems, Minneapolis, MN). We also summed the concentrations of CXCL1, CXCL5 and CXCL8, which represent total ELR^+-CXC-chemokine production in culture supernatants or SSEPS.

RT-PCR analysis

Total RNA was prepared from HUVEC, HMEC-D, and HMEC-L using an RNeasy Mini Kit (Qiagen, Valencia, CA). RT-PCR was performed according to the manufacturer's instructions using a OneStep RT-PCR Kit (Qiagen). For RT-PCR of CXCR2, we used the following pair of forward and reverse primer sets: 5′- aacatggagagtgacagctttg -3′ and 5′- ttagagagtagtggaagtgtg -3′ (PCR product size is 1,071 bp). PCR was performed for 35 cycles with denaturation at 94°C for 60 sec, annealing at 54°C for 60 sec, and extension at 72°C for 60 sec. Amplified DNA fragments were resolved by electrophoresis on 1.2% agarose gels containing ethidium bromide. β-actin was used as a positive control.

Proliferation assay

To confirm the effect of ELR^+-CXC-chemokines on PaCa proliferation, we performed a proliferation assay using the Celltiter 96 Aqueous One Solution Cell Proliferation Assay (MTS-assay) (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, PaCa cells were seeded at a density of 5×10^3 cells/100 μL in 96-well plates and allowed to adhere overnight. Then, cultures were re-fed with serum-free fresh media with or without 50 ng/mL of CXCL1, CXCL5 or CXCL8. After 24, 48 and 72 hr incubation, 20 μL of CellTiter 96 Aqueous One Solution Reagent was added to each well and the trays were incubated for 3 hr at 37°C. The absorbance was then measured using a microplate reader with a test wavelength of 490 nm. The effect of ELR^+-CXC-chemokines on HUVEC proliferation was determined by MTS-assay after 48 hr incubation. To examine the effect of PaCa on HUVEC proliferation, conditioned medium was made as follows: BxPC-3 cells were seeded at 5×10^6 cells into 100-mm dishes containing medium with 10% FCS and then cultured overnight. Cells were next cultured for 48 hr with HUVEC medium. Using this conditioned medium, HUVEC proliferation was determined as described above. Because both recombinant CXCL8 and BxPC-3 conditioned medium significantly enhanced HUVEC proliferation ($p < 0.05$), we assessed the effect of anti-CXCR2 Ab on the enhanced HUVEC proliferation. HUVECs were pre-treated with anti-CXCR2 Ab (15 μg/mL) for 1 hr before stimulation with ELR^+-CXC-chemokines or BxPC-3 conditioned medium. HUVEC proliferation was then determined as described above after 48-h incubation.

HUVEC invasion assay

HUVEC invasiveness was determined using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. HUVECs (5×10^4 cells) were suspended in growth medium and seeded into Matrigel pre-coated transwell chambers consisting of polycarbonate membranes with 8-μm pores. The transwell chambers were then placed into 24-well plates, to which basal medium only or basal medium containing various concentrations of CXCL1, CXCL5 or CXCL8 were added. After incubation of HUVECs for 16 hr, the upper surface of the transwell chambers was wiped with a cotton swab and the invading cells were fixed and stained with Diff-Quick stain. The number of invading cells was counted in 5 random microscopic fields (×200). Moreover, in order to examine alteration of HUVEC invasion potency by co-culturing with BxPC-3 or SW 1990 cells, we used a double-chamber method. HUVECs were seeded into Matrigel transwell chambers as mentioned above and then placed in 24-well plates where BsPC-3 or SW 1990 cells (1×10^5 cells) were pre-incubated. After co-culturing for 16 hr, invading cells were counted as described above. Furthermore, to confirm the effect of anti-CXCR2 Ab on enhanced HUVEC invasion potency by recombinant or PaCa-derived ELR^+-CXC-chemokines, HUVECs were pre-treated for 1 hr with anti-CXCR2 Ab (15 μg/mL) before being placed into the lower chamber (24-well plates).

Tube formation assay for angiogenesis

HUVEC tube formation potency was measured by an angiogenesis assay on Matrigel (BD Biosciences). For reconstitution of a basement membrane, Matrigel was diluted 2-fold with cold DMEM (without FCS) and added to the 24-well tissue culture plate (250 μL/well) at 4°C. The 24-well plate was incubated for 2 hr in a 37°C cell-culture incubator to allow the Matrigel to solidify. HUVECs were trypsinized, counted, resuspended in basal medium, and added on top of the reconstructed basement membrane (5×10^4 cells/well). Cells were incubated with or without 50 ng/mL of CXCL1, CXCL5 or CXCL8 for 16 hr to allow formation of capillary-like structures. Endotubes were quantified by counting 9 random fields/sample under the microscope (×40). Similarly, the effect of ELR^+-CXC-chemokines on HMEC-L tube formation was examined in the same way. In addition, in order to investigate the influence of PaCa on tube formation by HUVECs, we co-cultured HUVEC with BxPC-3 cells using a double-chamber method in 24-well plates. BxPC-3 cells (5×10^4 cells) were seeded into transwell chambers consisting of polycarbonate membranes with 0.4-μm pores (BD Biosciences) and allowed to adhere overnight. The chambers were then placed into the HUVEC tube-formation assay on Matrigel as described above. In addition, in order to confirm the effect of anti-CXCR2 Ab on the enhanced tube formation potency of HUVEC due to recombinant or PaCa-derived ELR^+-CXC-chemokines, HUVEC or HMEC-L were pre-treated for 1 hr with anti-CXCR2 Ab (15 μg/mL) before they were placed in the lower chambers of 24-well plates. Each condition was assessed in triplicate.

Animals

Male athymic NCr nude mice, between 5 and 6 weeks of age, were used in this study. The animals were bred and maintained in a HEPA-filtered environment with cages, food and bedding. Ampicillin (Sigma, St. Louis, MO) (5% [v/v]) was added to the autoclaved drinking water. Animal breeding and the entire animal study were approved by the Institutional Animal Care and Use Committee (IACUC) at AntiCancer Inc., and was carried out in accordance with the principles and procedures outlined in the NIH guide for the care and use of laboratory animals under assurance no. A3873-1.

Orthotopic PaCa model

BxPC-3 cells were stably transduced with green fluorescent protein (GFP) as previously described.[25] The animals were transplanted by surgical orthotopic implantation (SOI) using BxPC-3 GFP tumor fragments harvested from stock animals as previously described.[25] Briefly, the animals were anesthetized with isoflurane and the surgical area was sterilized using iodine and alcohol. An incision, approximately 1.5 cm long, was made in the left upper abdomen of the nude mouse using a pair of surgical scissors. The pancreas was exposed, and then 3 BxPC-3 GFP tumor fragments ($2\ mm^3$) were transplanted to the mouse pancreas with 8-0 surgical sutures (nylon). The abdomen was closed with 6-0 surgical sutures (silk). All procedures of the operation described above

were performed with a 7 × magnification microscope (Olympus) under HEPA-filtered laminar flow hoods.

Experimental protocol and imaging

Thirty days after tumor tissue implantation, mice were randomly divided into 2 groups with 5 mice assigned per group (based on a previous pilot study). Treatment with 500 μL/mouse of either normal goat serum IgG (vehicle group) or anti-CXCR2 Ab (CXCR2 Ab group) was performed intraperitoneally 3 days per week. The treatment was started 30 days after SOI and was administered for 26 days (11 doses in total). All mice were weighed on a weekly basis and observed 3 times per week for any adverse events during the entire duration of the study. Thirty days after tumor implantation, primary tumor size for each animal was followed on a weekly basis using a whole-body optical imaging system (FluorVivo, Indec Biosystems, Santa Clara, CA) as previously described.[25] Images were processed for tumor area with the help of FluorVivo software. Primary tumor sizes were also estimated by measuring the perpendicular minor dimension (W) and major dimension (L) using sliding calipers. Approximate tumor volume was calculated by the formula $(W^2 \times L) \times 1/2$. At the end of the study, open GFP imaging was conducted to examine the primary tumor in more detail as previously described.[25] The primary tumor was excised from the animal after euthanasia. Each tumor was weighed by an electronic balance as previously described.[25] The excised tumor was then processed for Ki-67 analysis and measurement of microvessel density.

Ki-67 analysis

Formalin-fixed, paraffin-embedded sections (5 μm) were stained with Ki-67 antibody as previously described.[22] Results were expressed as percent of Ki-67$^+$ cells ± SE per 40 × field. A total of 10 fields (×40) were examined and counted from each treatment group ($n = 5$ per group). The values were compared using the unpaired Student's *t* test.

Microvessel density

Formalin-fixed, paraffin-embedded sections (5 μm) were stained with rat anti-mouse Factor VIII monoclonal antibody as previously described.[22] Fields of greatest vessel density were examined under higher magnification (×100) and counted. Results were expressed as the mean number of vessels ± SE per high-power field (×100). A total of 20 high power fields were examined and counted in each treatment group ($n = 5$ per group). The values were compared using the unpaired Student's *t* test.

Statistical analysis

For the SSEPS study, statistical analyses were performed by SPSS (Statistical Packages for Social Sciences, Chicago, IL) version 13.0 software. Continuous variables were summarized by using medians as well as ranges. Because the data were not normally distributed, comparisons of patient characteristics between different groups were performed using nonparametric tests. The Kruskal-Wallis test was used (nonparametric analysis of variance) for this analysis. For other experiments, multiple group comparisons were performed using 1-way analysis of variance (ANOVA) with a post hoc test, the Student-Newman-Keuls (SNK) test, for subsequent individual group comparisons. Differences between the two groups were evaluated using the Student's *t* test. $p < 0.05$ was considered statistically significant. Mean values and standard deviations (SD) were calculated for experiments performed in triplicate (or more).

Results

SSEPS analysis

Seven patients had a normal pancreas determined by imaging and made up the control group. Four patients had irritable bowel syndrome; 2 had a positive family history of pancreatic cancer and 1 had a small gastric subepithelial tumor. Eighteen patients had pancreatic cancer. Of these, 9 underwent pancreatic resection. Only 1 patient in this group was deemed locally unresectable and underwent palliative biliary bypass. The other 9 patients had advanced pancreatic cancer diagnosed by EUS-FNA cytology. All patients underwent SSEPS with no complications. We were able to collect pancreatic juice from all patients, including patients with pancreatic head cancer complicated by atrophy and exocrine insufficiency of the gland (pancreas body-tail region). We collected at least 10 mL of pancreatic juice per patient in this group. Initially, we measured the secreted levels of ELR$^+$-CXC-chemokines in human pancreatic juice samples. The mean concentration (±SD) of CXCL5 (92.8 ± 40 pg/mL) and Σ ELR$^+$-CXC-chemokines (253 ± 86.4 pg/mL) of pancreatic juice from PaCa patients were significantly higher than from normal individuals (NP) [CXCL5 (29.7 ± 5.86 pg/mL) and Σ ELR$^+$-CXC-chemokines (43.4 ± 7.7 pg/mL)], ($p = 0.006$ and $p = 0.002$, respectively). The mean concentrations of CXCL1 and CXCL8 did not differ between the 2 groups in this pilot analysis (p = NS). Box and whisker plots of the ELR$^+$-CXC-chemokine concentration for NP and PaCa are displayed in Figure 1.

ELISA for measurement of ELR$^+$-CXC-chemokines in PaCa supernatants

We next examined the concentrations of ELR$^+$-CXC-chemokines in 8 PaCa cell lines and an HPDE cell line by ELISA after culturing for 3 different time points (24, 48 and 72 hr). The secretion levels of these chemokines by BxPC-3 and Colo-357 cells lines were especially high (*e.g.*, the concentration of Σ ELR$^+$-CXC-chemokines after 72 hr culture of BxPC-3 and Colo-357 were: 8270.148 ± 101.513 pg/mL and 10104.88 ± 486.225 pg/mL, respectively). Seven of the 8 PaCa cell lines had higher expression of Σ ELR$^+$-CXC-chemokines compared to an immortalized HPDE cell line (Fig. 2).

RT-PCR analysis for CXCR2 mRNA in vascular endothelial cell lines

Our focus in this study was PaCa-induced angiogenesis and its correlation with ELR$^+$-CXC-chemokines. Earlier, we showed that the majority of PaCa cell lines examined did not express CXCR2.[24] Therefore, we initially confirmed CXCR2 expression in vascular endothelial cells by RT-PCR analysis, which demonstrated CXCR2 mRNA expression in HUVEC, HMEC-D, and HMEC-L (Fig. 3*a*).

Effects on PaCa cell proliferation by ELR$^+$-CXC-chemokines

To examine the autocrine effect of ELR$^+$-CXC-chemokines on PaCa, we performed the PaCa proliferation assay with ELR$^+$-CXC-chemokine treatment. ELR$^+$-CXC-chemokines did not affect the proliferation of either of the BxPC-3 or MIA PaCa-2 cell lines (Fig. 3*b*). We also performed PaCa proliferation assay with increasing concentrations of anti-CXCR2 Ab and observed no effect on proliferation of either of the BxPC-3 or MIA PaCa-2 cell lines (Supporting Fig. 1).

Effects on HUVEC proliferation by ELR$^+$-CXC-chemokines and PaCa supernatants

Because ELR$^+$-CXC-chemokines had no autocrine effect on PaCa proliferation, we next focused our attention on their paracrine effect on angiogenesis. We then performed the HUVEC proliferation assay. HUVEC were cultured for 48 hr after being treated with ELR$^+$-CXC-chemokines followed by the MTS-assay. Neither CXCL1 nor CXCL5 enhanced HUVEC proliferation. Both recombinant CXCL8 and BxPC-3-conditioned medium significantly enhanced HUVEC proliferation ($p < 0.05$). Moreover, treatment with anti-CXCR2 Ab significantly inhibited HUVEC

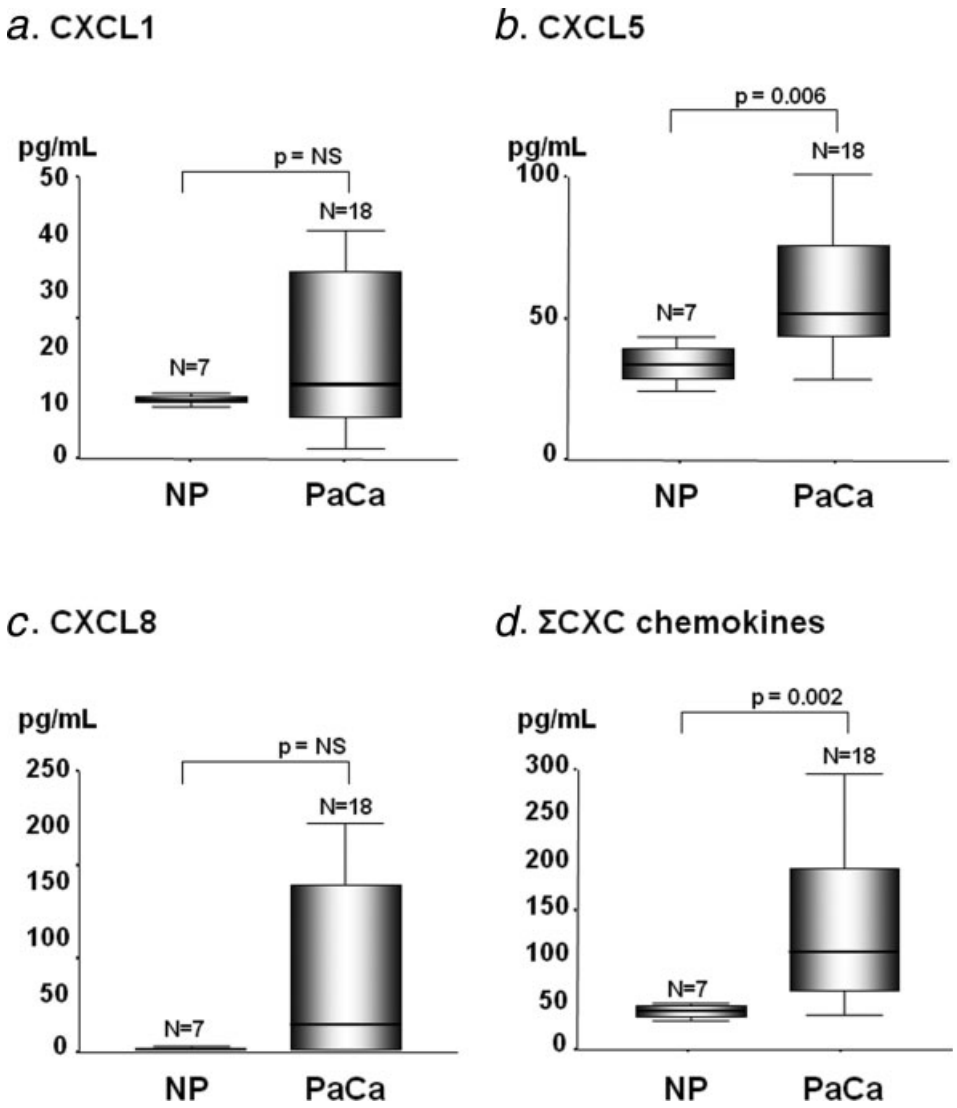

FIGURE 1 – Levels of ELR$^+$-CXC-chemokines in secretin-stimulated pancreatic exocrine secretions (SSEPS). Box and whisker plots show the concentrations of ELR$^+$-CXC-chemokines in pancreatic juice from normal individuals (NP) and pancreatic cancer (PaCa) patients. (*a*) CXCL1, (*b*) CXCL5, (*c*) CXCL8 and (*d*) Σ ELR$^+$-CXC-chemokines. Continuous variables were summarized by using the medians and ranges. Because the data were not normally distributed, comparisons of patient characteristics between groups were performed by a nonparametric test. The Kruskal-Wallis test was used (nonparametric analysis of variance) for the analysis.

proliferation enhanced by recombinant CXCL8 and BxPC-3 conditioned medium ($p < 0.05$) (Fig. 3*c*).

Effect on HUVEC invasion by ELR$^+$-CXC-chemokines

Since a previous report demonstrated the role of CXCL8 in invasiveness of vascular endothelial cells[26], we reconfirmed the effect of ELR$^+$-CXC-chemokines on HUVEC invasion potency. HUVEC invasion potency was significantly up-regulated by CXCL1 (100 ng/mL), CXCL5 ($\geq$ 10 ng/mL), or CXCL8 ($\geq$ 10 ng/mL). Moreover, treatment with anti-CXCR2 Ab significantly inhibited HUVEC invasion enhanced by ELR$^+$-CXC-chemokines (Fig. 4*a*). To determine the role of PaCa-derived ELR$^+$-CXC-chemokines on HUVEC invasive potency, we co-cultured HUVEC with PaCa cells using double chambers. HUVEC invasion was significantly enhanced by co-culturing with BxPC-3 or SW 1990 ($p < 0.01$). This enhancement was also significantly inhibited by treatment with anti-CXCR2 Ab ($p < 0.05$) (Fig. 4*b*).

Effects on HUVEC tube formation by ELR$^+$-CXC-chemokines

We then examined the effect of ELR$^+$-CXC-chemokines on tube formation by vascular endothelial cells. Fifty nanogram per microliter of either of CXCL1, CXCL5, or CXCL8 significantly increased tube formation by HUVEC. Anti-CXCR2 Ab significantly inhibited the HUVEC tube formation by enhanced CXCL5 and CXCL8 (Fig. 5*a*). To understand the effect of ELR$^+$-CXC-chemokines on tube formation by vascular endothelial cells in detail, we performed the HMEC-L tube formation assay. Fifty nanograms per microliter of each of the ELR$^+$-CXC-chemokines significantly enhanced tube formation by HMEC-L. Furthermore, this enhanced tube formation was significantly inhibited by treatment with 15 μg/mL anti-CXCR2 Ab (Fig. 5*b*). We then examined the effect of PaCa-derived ELR$^+$-CXC-chemokines on tube formation by vascular endothelial cells. We performed the HUVEC tube formation assay on Matrigel using double chambers. Using

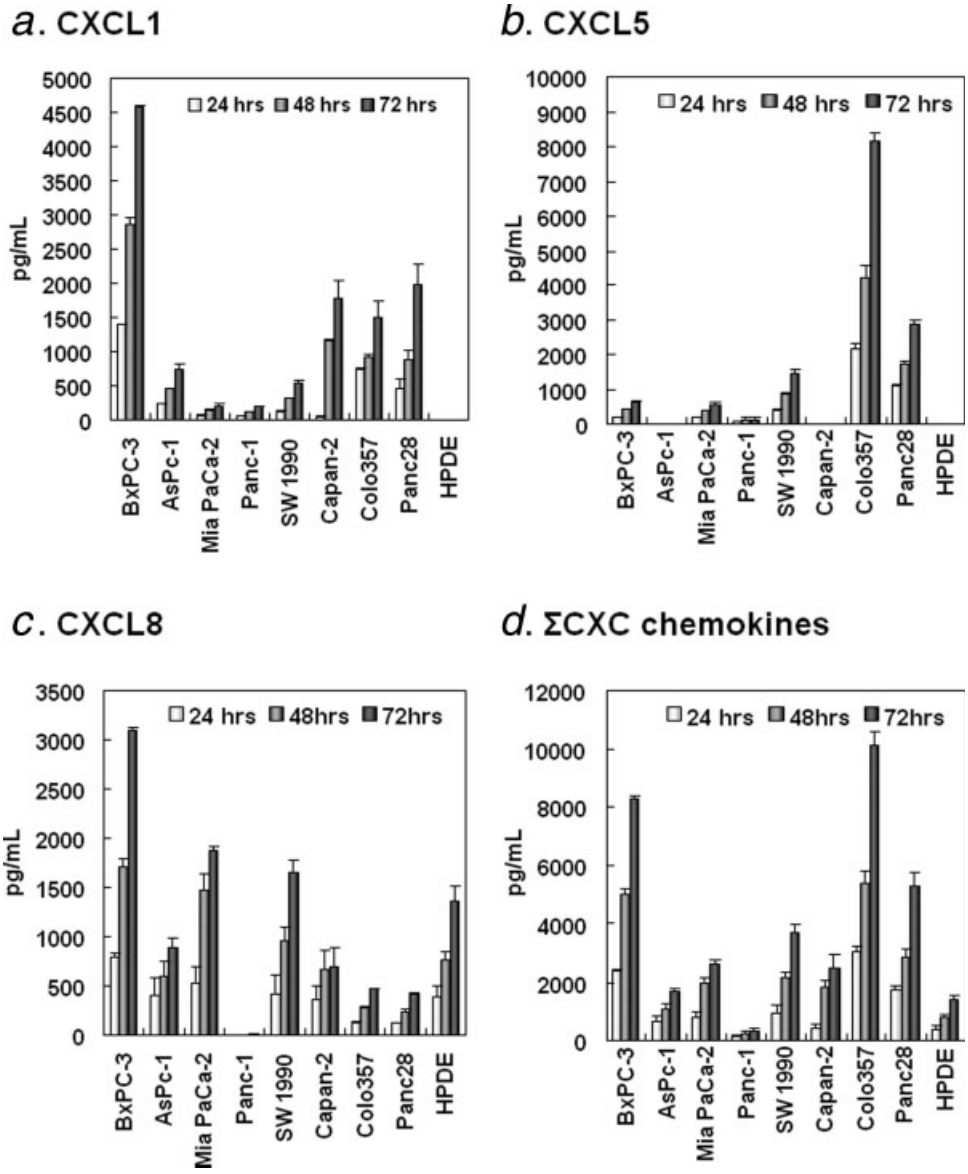

FIGURE 2 – Quantification of ELR$^+$-CXC-chemokines in culture medium from multiple PaCa and HPDE cell types. All cell types were cultured for 24, 48 and 72 hr, and then the concentrations of ELR$^+$-CXC-chemokines were measured by ELISA as described in the Material and methods. (*a*) CXCL1, (*b*) CXCL5, (*c*) CXCL8 and (*d*) Σ ELR$^+$-CXC-chemokines. Values are expressed as mean ± SD.

this co-culture system, we could evaluate HUVEC tube formation potency due to the interaction between vascular endothelial cells and PaCa cells. By co-culturing HUVEC with BxPC-3, HUVEC tube formation was significantly enhanced compared to control (HUVEC only) ($p < 0.01$). Although anti-CXCR2 Ab did not affect tube formation by HUVEC in monoculture, enhanced tube formation by co-culture of HUVEC with BxPC-3 was significantly inhibited by treatment with anit-CXCR2 Ab ($p < 0.01$) (Fig. 5*c* and 5*d*).

Effects of anti-CXCR2 Ab in an orthotopic PaCa model

We then examined whether anti-CXCR2 Ab could inhibit PaCa growth and angiogenesis *in vivo*. BxPC-3 GFP cells were orthotopically implanted into the pancreas of nude mice. Treatment with anti-goat IgG (Vehicle group) or anti-CXCR2 Ab (CXCR2 Ab group) began 30 days after implantation. Tumor volume was measured weekly. Tumor volume in the CXCR2 Ab group was significantly reduced compared to the vehicle group on days 14, 21 and 28 ($p < 0.05$) (Fig. 6*a*). Representative open GFP imaging, performed at the end of the study, showed that tumor growth in the CXCR2 Ab group was significantly reduced compared to the vehicle group (Fig. 6*b*1 and 6*b*2). Furthermore, treatment with anti-CXCR2 Ab significantly reduced final tumor weight compared to vehicle treatment ($p < 0.05$) (Fig. 6*b* left panel). We then showed that the proliferation index, determined by Ki-67 expression, in the CXCR2 Ab group was significantly lower compared to the vehicle group ($p = 0.043$) (Fig. 6*c*). There was a decrease in the microvessel density in the CXCR2 Ab group compared with the vehicle group ($p = 0.004$) (Fig. 6*d*). Collectively, our data indicated that treatment with anti-CXCR2 Ab reduced tumor volume and angiogenesis *in vivo*.

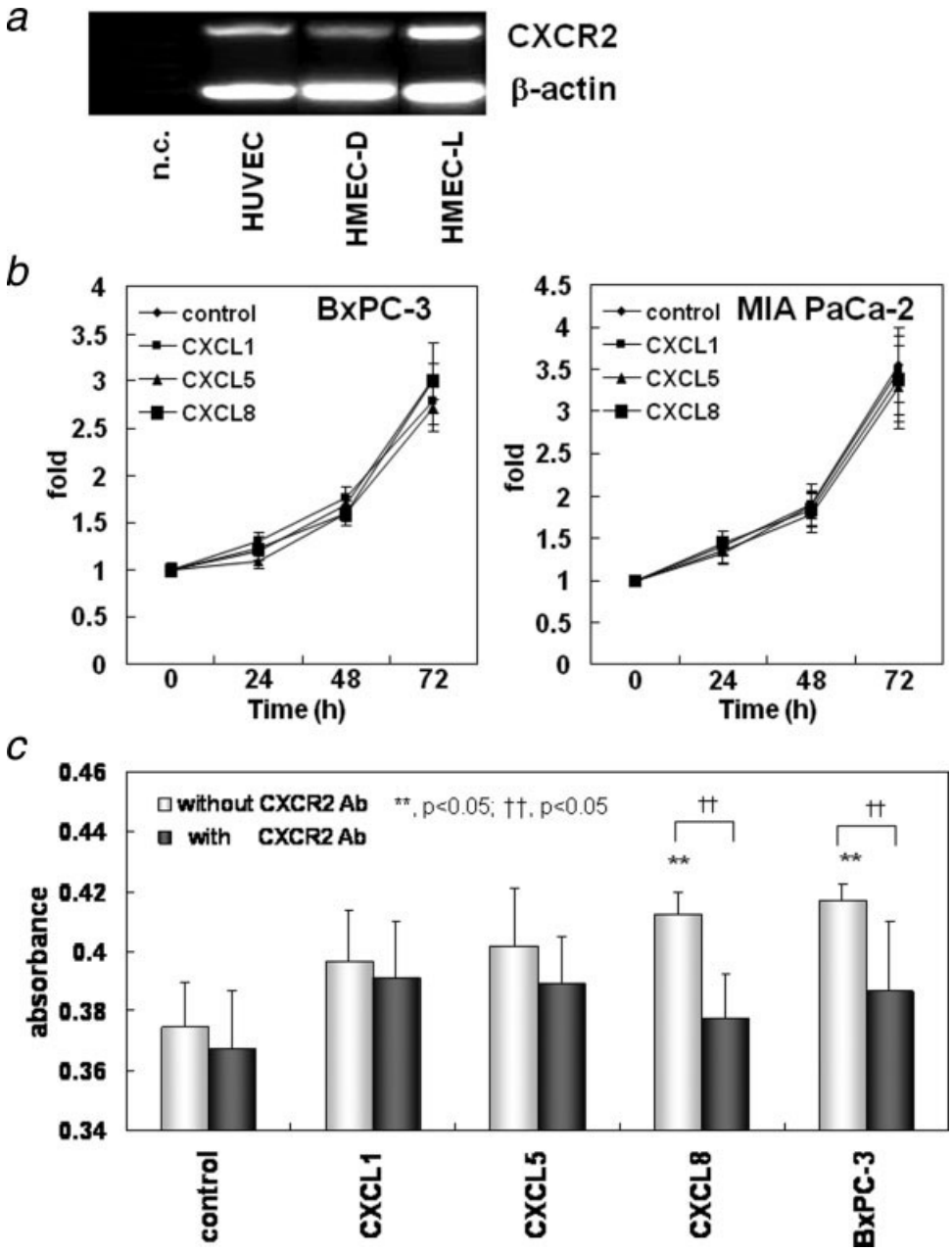

FIGURE 3 – (*a*) Expression of CXCR2 mRNA in vascular endothelial cells by RT-PCR. PCR amplification was performed using primer pairs designed from CXCR2 cDNA sequences. PCR products were separated by 1.2% agarose gel electrophoresis and stained with ethidium bromide as described in the Material and methods. (*b*) Effects on PaCa cell proliferation by ELR^+-CXC-chemokines. PaCa cells were seeded into 96-well plates and cultured for 24, 48 and 72 hr stimulated with or without 50 ng/mL ELR^+-CXC-chemokines. PaCa proliferation was determined by the MTS-assay as described in the Material and methods. Left panel, proliferation assay of BxPC-3; right panel, proliferation assay of MIA PaCa-2. Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Values are expressed as mean ± SD. (*c*) Effects on HUVEC proliferation by recombinant or PaCa-derived ELR^+-CXC-chemokines. HUVECs were seeded into 96-well plates and cultured for 48 hr with or without ELR^+-CXC-chemokines or BxPC-3 conditioned medium. Furthermore, to examine the effects of anti-CXCR2 Ab on HUVEC proliferation, enhanced by ELR^+-CXC-chemokines, HUVEC cells were pre-treated with anti-CXCR2 Ab (15 μg/mL) for 1 hr before stimulation with ELR^+-CXC-chemokines or BxPC-3 conditioned medium. After 48 hr incubation, HUVEC proliferation was determined by the MTS-assay as described in the Material and methods. Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Differences between the 2 groups were evaluated using the Student's *t* test. Values are expressed as mean ± SD. $^{**}p < 0.05$ compared with control without anti-CXCR2 Ab. $\dagger\dagger p < 0.05$ compared without anti-CXCR2 Ab.

Discussion

Although the biological effects of IL-8/CXCL8 on PaCa angiogenesis were previously studied,[9] little is known about the biological effects of other ELR^+-CXC-chemokines, such as GRO-α/CXCL1 and ENA-78/CXCL5, on PaCa angiogenesis. It is well known that all ELR^+-CXC-chemokines bind to CXCR2, a GPCR that promotes tumor-associated angiogenesis.[18] In the present study, we demonstrated that recombinant CXCL1 and CXCL5, in addition to IL-8/CXCL8, significantly enhanced invasion and tube formation by vascular endothelial cells. Given the fact that a ma-

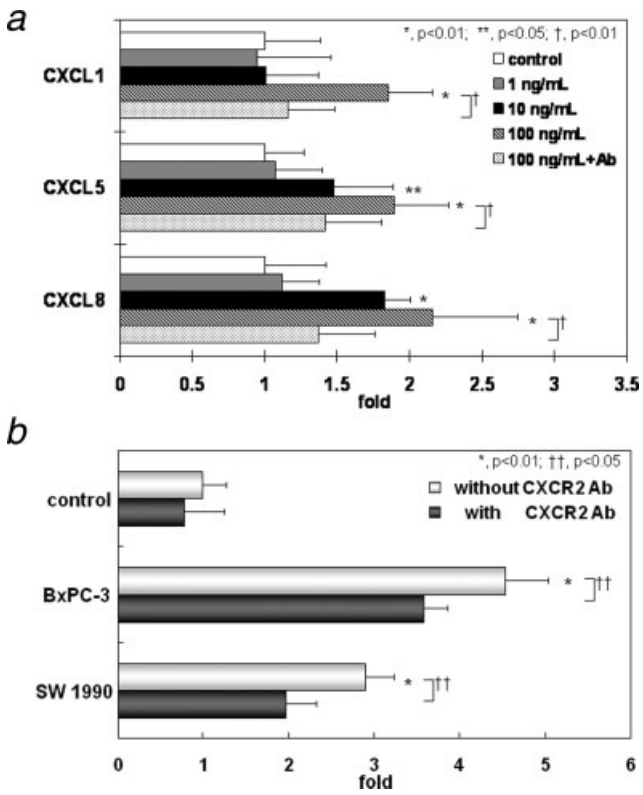

FIGURE 4 – Effects of ELR$^+$-CXC-chemokines on HUVEC invasion. (*a*) Effects on HUVEC invasion by recombinant ELR$^+$-CXC-chemokines. The HUVEC invasion assay was performed as described in the Material and methods. The effects of increasing concentrations (as noted in the figure) of CXCL1, CXCL5 or CXCL8 on HUVEC invasion were evaluated. To examine the effects of anti-CXCR2 Ab on HUVEC invasiveness, HUVECs were pre-treated with anti-CXCR2 Ab (15 μg/mL) and then challenged with maximal concentrations of CXCL1, CXCL5 or CXCL8 as noted in the figure. The number of invading cells was counted in 5 random microscopic fields (×200). Multiple comparisons were performed by one-way ANOVA followed by the SNK test. $*p < 0.01$, $**p < 0.05$, and $\dagger p < 0.01$. (*b*) Effects on HUVEC invasion by co-culture with PaCa cell lines. To determine the effects of interaction between PaCa cells and HUVECs, we performed an invasion assay using double chambers as described in the Material and methods. HUVECs were seeded into Matrigel precoated transwell chambers consisting of polycarbonate membranes with 8-μm pores. The inserts were then placed into the 24-well plates where BxPC-3 or SW 1990 cells were pre-seeded. The number of invading cells was counted as described in the Materials and Methods. Further, to evaluate the effects of anti-CXCR2 Ab on PaCa-induced HUVEC invasiveness, HUVECs were pre-treated with anti-CXCR2 Ab (15 μg/mL) and an invasion assay was performed as described in the Material and methods. Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Differences between the 2 groups were evaluated using the Student's *t* test. $*p < 0.01$ and $\dagger\dagger p < 0.05$.

jority of ELR$^+$-CXC-chemokines produced by PaCa cells promote HUVEC tube formation, we used a neutralizing anti-CXCR2 Ab to block the ELR$^+$-CXC-chemokines/CXCR2 biological axis in PaCa. We showed that treatments with anti-human CXCR2 Ab significantly reduced PaCa-induced HUVEC tube formation *in vitro* just as anti-mouse CXCR2 Ab reduced angiogenesis in an orthotopic PaCa model *in vivo*.

One of the salient features of our study is the prospective blinded measurement of ELR$^+$-CXC-chemokines in SSEPS from patients with pancreatic cancer and normal individuals. We were previously able to demonstrate for the first time that ELR$^+$-CXC-chemokines could be successfully measured in SSEPS. In an earlier study, we demonstrated that CXCL8 levels in SSEPS were significantly higher in PaCa patients than in normal individuals and thus measurement of CXCL8 would be important for diagnosis of PaCa.[16] Our results now extend the conclusion to include summed concentrations of ELR$^+$-CXC-chemokines for the diagnosis of PaCa. The rationale behind this approach is based on the fact that ELR$^+$-CXC-chemokines have similar binding affinities to CXCR2, their corresponding receptor expressed on vascular endothelial cells.[11] This is of great importance given the fact that CXCR2 mediates angiogenesis.[11] We also showed that PaCa patients secreted significant levels of CXCL5 when compared with normal individuals. Unlike our previous report,[16] we did not observe a significant difference in CXCL8 levels between PaCa patients and normal individuals. This could be due to small sample size, as the trend was more towards PaCa patients. Another recent report[17] demonstrated that both CXCL5 and CXCL8 levels were significantly higher in PaCa patients. These reports suggest that targeting CXCR2, which binds ELR$^+$-CXC-chemokines, may be more effective than targeting individual ELR$^+$-CXC-chemokines, including CXCL8.

The corresponding receptors for ELR$^+$-CXC-chemokines are CXCR1 and CXCR2. CXCR2 plays an important role in tumorigenesis and tumor angiogenesis.[11,26,27] Previous reports demonstrated expression of CXCR2 in different neoplastic epithelial cells including colon cancer,[28,29] lung cancer,[30] ovarian cancer,[31] stomach cancer,[32] and malignant melanoma.[33] CXCL8 produced from these neoplastic cells promoted tumor growth in an autocrine manner. However, little is known about the role of other ELR$^+$-CXC-chemokines including CXCL1 and CXCL5 in these cancers. In PaCa, there are a few reports that suggest possible autocrine effects of CXCL8 in proliferation using Capan-1 and SUIT-2 pancreatic cancer cell lines.[34–37] Conversely, our previous study demonstrated that the majority of PaCa cell lines examined did not express CXCR2.[24] Moreover, in this study, we showed that ELR$^+$-CXC-chemokines did not affect proliferation of commonly used PaCa cell lines including BxPC-3 and MIA PaCa-2. It is known that CXCR2 is expressed in tumor-associated endothelial cells and we confirmed that in 3 distinct human vascular endothelial cell lines (HUVEC, HMEC-D, and HMEC-L). Taken together, our results suggest that the paracrine effects of PaCa-cell-derived ELR$^+$-CXC-chemokines on CXCR2 expressed on endothelial cells are of critical importance with respect to tumor-associated angiogenesis.

Angiogenesis is a complex multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, and capillary tube formation.[38] In tumors, angiogenesis depends on the production of proangiogenic factors by both cancer and stromal cells.[39] Our results demonstrated that PaCa cell-derived ELR$^+$-CXC-chemokines play a significant role in each of these steps. In addition, these chemokines cooperatively function with growth factors including VEGF, b-FGF, and PDGF in promoting tumor-associated angiogenesis.[21] Furthermore, our recent data showed that ELR$^+$-CXC-chemokine production from PaCa cells was significantly enhanced by co-culturing with stromal cells including fibroblasts.[24]

Therefore, given the fact that the network of ELR$^+$-CXC-chemokines is modulated by the interaction between the tumor and its microenvironment, we investigated the role of the ELR$^+$-CXC-chemokines/CXCR2 biological axis in an orthotopic PaCa model. Our results showed that a neutralizing anti-mouse CXCR2 Ab reduced tumor volume and potently inhibited microvessel density. This reduction in tumor volume was modest compared to inhibition of angiogenesis. This is due to the fact that CXCR2 is expressed only in the stromal cells and reduction in tumor volume is a bystander effect of angiogenesis inhibition.

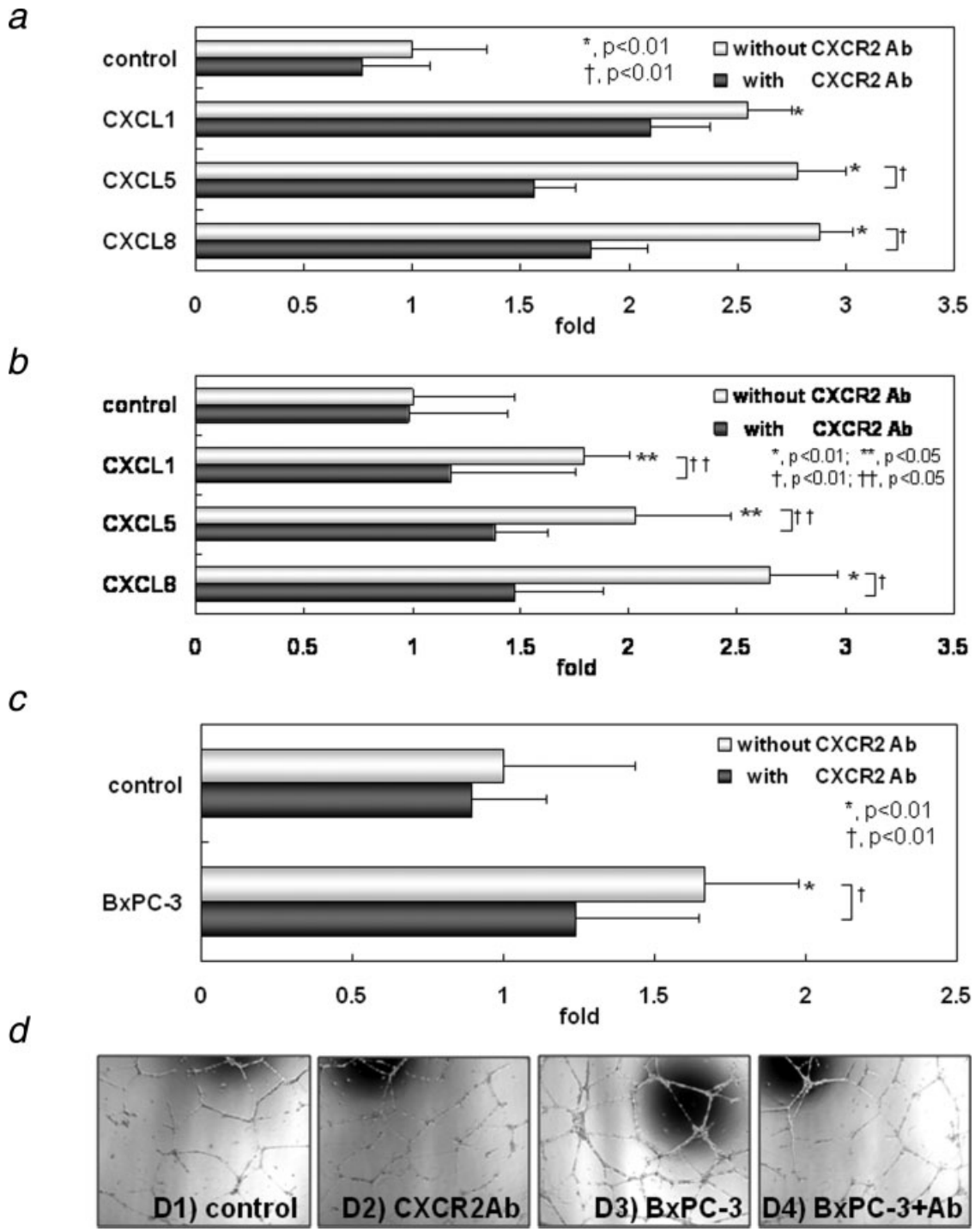

FIGURE 5 – Effects of ELR$^+$-CXC-chemokines on tube formation by vascular endothelial cells. (*a*) Effects on HUVEC tube formation by recombinant ELR$^+$-CXC-chemokines. The HUVEC tube formation assay on Matrigel was performed as described in the Material and methods. HUVECs were stimulated with 50 ng/mL of CXCL1, CXCL5 or CXCL8 and treated with or without 15 μg/mL anti-CXCR2 Ab. Endotubes were quantified by counting 9 random fields/sample under the microscope (×40). Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Differences between the 2 groups were evaluated using the Student's *t* test. Values are expressed as mean ± SD. $*p < 0.01$, $\dagger p < 0.01$. (*b*) Effects on HMEC-L tube formation potency by recombinant ELR$^+$-CXC-chemokines. HMEC-L tube formation assay on Matrigel was performed in the same way as described for HUVEC. $*p < 0.01$, $**p < 0.05$, $\dagger p < 0.01$ and $\dagger\dagger p < 0.05$. (*c*, *d*) Effects on HUVEC tube formation potency by co-culture with PaCa cells. To examine the effects of interaction between PaCa cells and vascular endothelial cells on tube formation, we performed the HUVEC tube formation assay on Matrigel by co-culture with BxPC-3 cells using double chambers as described in the Material and methods. To determine the effects of the anti-CXCR2 Ab on enhanced HUVEC tube formation by BxPC-3, HUVEC were pre-treated with anti-CXCR2 Ab (15 μg/mL). The tube formation assay was then performed as described in the Material and methods. $*p < 0.01$ and $\dagger p < 0.01$. D1: control, D2: treated with anti-CXCR2 Ab, D3: co-culture with BxPC-3, D4: co-culture with BxPC-3 treated with anti-CXCR2 Ab (×40).

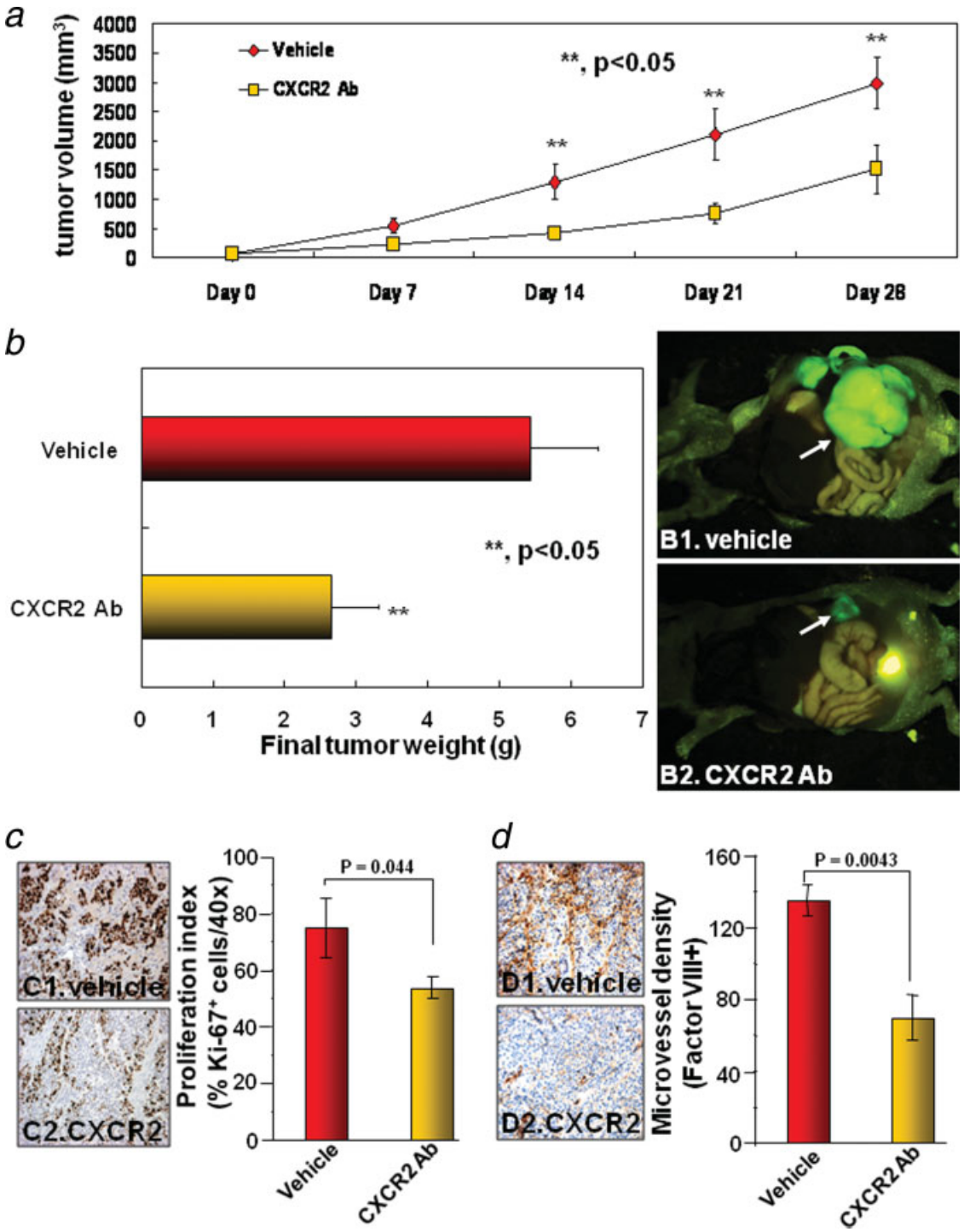

FIGURE 6 – Effects of anti-CXCR2 Ab on growth of PaCa tumor and angiogenesis *in vivo*. (*a*) Anti-CXCR2 Ab reduces tumor volume. Twenty-nine days after tumor implantation by surgical orthotopic implantation (SOI), the primary tumor size for each animal was followed on a weekly basis as described in the Material and methods. Primary tumor size was estimated by measuring the perpendicular minor dimension (W) and major dimension (L) using sliding calipers. Approximate tumor volume was calculated by the formula $(W^2 \times L) \times 1/2$. Values are expressed as mean ± SE. Differences between the 2 groups ($n = 5$ per group) were evaluated using one-way ANOVA (followed by Dunnett's two-sided test) with $\alpha = 0.05$. $^{**}p < 0.05$. (*b*) Anti-CXCR2 Ab reduces final tumor volume. At the end of the study, open GFP imaging was conducted as described in the Material and methods to examine primary tumor growth. B1: vehicle, B2: treated with anti-CXCR2 Ab, arrow indicates the tumor. The primary tumor was excised from the animal. Each tumor was weighed using an electronic balance (left panel). Values are expressed as mean ± SE. Differences between the 2 groups ($n = 5$) were evaluated using one-way ANOVA (followed by Dunnett's two-sided test). $^{**}p < 0.05$. (*c*) Quantification of Ki-67$^+$ cells. Representative Ki-67$^+$ IHC is shown in C1 (vehicle) and C2 (anti-CXCR2) (×100). The proliferation index was measured as described in the Material and methods. Values are expressed as mean ± SE. Differences between the 2 groups were evaluated using the Student's *t* test with $\alpha = 0.05$. (*d*) Quantification of Factor VIII$^+$ cells. Representative Factor VIII$^+$ IHC is shown in D1 (vehicle) and D2 (anti-CXCR2) (×100). Microvessel density was measured as described in the Material and methods. Values are expressed as mean ± SE. Differences between the 2 groups were evaluated using the Student's *t* test with $\alpha = 0.05$.

In conclusion, expression of ELR^+-CXC-chemokines is an important characteristic of the process of PaCa tumorigenesis. Blockade of CXCR2, the corresponding receptor for all ELR^+-CXC-chemokines, significantly attenuated PaCa tumor-associated angiogenesis. Thus, CXCR2 should be considered as a novel anti-angiogenic target in PaCa.

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