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doi: https://doi.org/10.1016/j.bbrc.2015.01.112

Biochemical and Biophysical Research Communications 458 (2015) 341–346

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Gemcitabine-induced CXCL8 expression counteracts its actions by inducing tumor neovascularization

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ARTICLE INFO

Article history:
Received 11 January 2015
Available online 31 January 2015

Keywords:
Gemcitabine
CXCL8
Pancreatic cancer
Chemo-resistance

ABSTRACT

Patients with pancreatic ductal adenocarcinoma (PDAC) are frequently complicated with metastatic disease or locally advanced tumors, and consequently need chemotherapy. Gemcitabine is commonly used for PDAC treatment, but with limited efficacy. The capacity of gemcitabine to generate reactive oxygen species (ROS) in human pancreatic cancer cells, prompted us to examine its effects on the expression of pro-inflammatory cytokines and chemokines. We observed that gemcitabine enhanced selectively the expression of CXCL8 in human pancreatic cancer cells through ROS generation and NF-κB activation. In vitro blocking of CXCL8 failed to modulate gemcitabine-mediated inhibition of cell proliferation in human pancreatic cancer cells. Gemcitabine also enhanced CXCL8 expression in pancreatic cancer cells in xenografted tumor tissues. Moreover, anti-CXCL8 antibody treatment in vivo attenuated tumor formation as well as intra-tumoral vascularity in nude mice, which were transplanted with Miapaca-2 cells and treated with gemcitabine. Thus, gemcitabine-induced CXCL8 may counteract the drug through inducing neovascularization.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a tumor within the exocrine compartment of pancreatic gland [1]. About 80% PDAC cases are diagnosed with metastatic disease or with locally invasive tumors, and are subjected to chemotherapy. The rest of the patients are eligible for curative resection, but frequently develop local recurrence or distant metastasis after surgery. As a consequence, they also need chemotherapy.

Gemcitabine, an analogue of cytosine arabinoside (Ara-C) [2], is used as a standard drug for PDAC treatment, alone or in combination with other chemotherapeutics [1,3]. Gemcitabine is transported into cells across the cell membrane through multiple active nucleoside transporters [4], and is phosphorylated by deoxycytidine kinase to produce gemcitabine monophosphate (dFdCMP), which is further converted to active drug metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphophage (dFdCTP) [5]. dFdCTP directly inhibits DNA polymerase [6], cytidine triphosphate synthetase [7] and deoxycytidylate deaminase [8], and is incorporated into DNA, thereby terminating chain elongation [9]. dFdCDP potently inhibits ribonucleotide reductase (RR), thereby decreasing deoxynucleotide pools [10]. Despite its multiple intracellular targets, the resistance to gemcitabine often ensues [1].

Evidence is accumulating to indicate that gemcitabine induced reactive oxygen species (ROS) generation [11]. Moreover, the resultant ROS activated a transcription factor NF-κB [11], which has a crucial role in the activation of various pro-inflammatory genes, including cytokine and chemokine genes [12]. Consistently, we observed that gemcitabine induced ROS generation in a human pancreatic cancer cell line, Miapaca-2. Hence, we evaluated the effects of gemcitabine on chemokine and cytokine expression in human pancreatic cancer cell lines. Indeed, gemcitabine induced abundant expression of CXCL8 in human pancreatic cancer cells in vivo as well as in vitro. Moreover, endogenously-produced CXCL8

Abbreviations: ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; PDAC, pancreatic ductal adenocarcinoma; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.bbrc.2015.01.112
0006-291X/

has few effects on gemcitabine-mediated inhibition of in vitro cell proliferation of pancreatic cancer cells. However, CXCL8 can counteract the in vivo action of gemcitabine by promoting neovascularization.

2. Materials and methods

2.1. Cell lines and reagents

Miapaca-2 and Panc-1 cells were obtained from ATCC and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Mouse anti-human CXCL8 monoclonal and rabbit anti-human CXCL8 polyclonal antibodies were prepared as described previously [13]. The following antibodies were obtained commercially: rabbit anti-NF-κB/p65 polyclonal antibodies (Cell Signaling Technology, Beverly, MA); mouse IgG_1 (Sigma–Aldrich, St. Louis, MO); rabbit immunoglobulin fraction (Dako, Glostrup, Denmark). N-acetyl cysteine (NAC) and gemcitabine were obtained from Sigma Aldrich.

2.2. Determination of intracellular ROS concentration by a flow cytometry

Intracellular ROS concentration was determined by using total ROS/superoxide detection kit (Enzo Life Science, San Diego, CA) according to the manufacturer's instructions. In brief, after Miapaca-2 cells were pre-treated with or without NAC (30 mM) for 1 h, the cells were further incubated in the absence or the presence of gemcitabine (10 μM) for 24 h. Then, the cells were collected and incubated with ROS/superoxide detection solution for 10 min at 37 °C. At least 50,000 stained cells were analyzed on a FACSCanto II system (Becton Dickinson, Bedford, MA) by using 490 nm and 525 nm wave lengths as excitation and emission wave lengths, respectively, for each determination.

2.3. In vitro cell proliferation

Cell suspensions (2×10^3 cells/100 μl) were added to a well of 96-multi-well plates and incubated at 37 °C for 24 h. Then, the cells were treated with the indicated concentrations of gemcitabine for the indicated time intervals. The cell viability was determined by using the cell counting kit-8 (Dojindo Co. Ltd., Kumamoto, Japan). The ratios of cell numbers were determined by comparing the cell numbers at day 0.

2.4. Assays of secreted CXCL8

Culture supernatants were collected from Miapaca-2 or Panc-1 cells at the indicated time intervals after gemcitabine treatment, to determine CXCL8 contents by using enzyme-linked immunosorbent assay (ELISA) for human CXCL8 (R&D Systems, Minneapolis, MN).

2.5. Transfection of CXCL8 siRNA

Miapaca-2 or Panc-1 cells were seeded at a density of 1×10^5 cells/well in 2 ml in a 6-well plate. After overnight incubation, CXCL8 or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was transduced into Miapaca-2 and Panc-1 cells using a JetPRIME DNA & siRNA transfection reagent (Polyplus Transfections, Illkirch, France) and siRNA transfection reagent (Santa Cruz Biotechnology), respectively. The cells were then incubated with or without gemcitabine (10 μM) for 48 h and were subjected to proliferation assay or total RNA extraction.

2.6. RNA isolation for RT-PCR

Total RNA was isolated and was reverse transcribed to cDNA as previously described [14]. The resultant cDNA was amplified to detect human CXCR1 and CXCR2 mRNA by using the specific sets of the primers, 5'-CCCCTGTATGCTAGAAACTGAGAC-3' (CXCR1 forward), 5'-CCAGCAGCCAAGACAAACAAAC-3' (CXCR1 reverse), 5'-CATGGGCAACAATACAGCAA-3' (CXCR2 forward) and 5'-TGAGGACGACAGCAAAGATG-3' (CXCR2 reverse) with 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min, and with a final extension at 68 °C for 5 min. Amplified DNA fragments were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining under ultraviolet light transillumination. GAPDH was used as a positive control. Quantitative (q)RT-PCR for CXCL8 was performed using the sets of the primers, 5'-TGGCAGCCTTCCTGATTTCT-3' (forward) and 5'-TTTCTGTGTTGGCGCAGTGT-3' (reverse) and mRNA amounts were normalized to the amounts of GAPDH as relative expression values as described previously [14].

2.7. Luciferase assay

Miapaca-2 cells were cultured at a concentration of 1×10^5 cells/well in a 6-well plate for 24 h. Then, the cells were transfected with the luciferase expression vectors containing various deleted or mutated 5'-flanking region of the CXCL8 promoter [15] (1.5 μg) and SV40-renilla luciferase expression vector (37.5 ng) as a control by using a JetPRIME DNA & siRNA transfection reagent. Cells were incubated for additional 48 h with or without gemcitabine (10 μM). Thereafter, the cells were harvested to determine luciferase activities with the use of the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase values were normalized to Renilla luciferase activities.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Miapaca-2 cells were cross-linked with 1% formaldehyde for 10 min at 37 °C and harvested in sodium dodecyl sulfate lysis buffer. The resultant chromatin was sheared to generate fragments of about 500 bp by sonication. Then, each aliquot of chromatin received anti-p65 (Cell Signaling Technology) or control antibodies, and was incubated at 4 °C overnight. The mixtures received Protein A agarose (Life Technology, Carlsbad, CA) and were incubated for additional 1 h at 4 °C. DNA was recovered by phenol–chloroform extraction followed by ethanol precipitation. The resultant DNA underwent PCR using the sets of the primers, 5'-GGAACAAATAGGAAGTGTGATGAC-3' (forward) and 5'-AGAGAACTTATGCACCCTCATCT-3' (reverse), to amplify the 112 bp-long fragment, which contains the NF-κB/p65 binding site in the human CXCL8 promoter, under the conditions consisting of an initial denaturation at 94 °C for 5 min followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, with a final extension at 68 °C for 5 min. The resultant PCR products were fractionated on 12% polyacrylamide gel and visualized by ethidium bromide staining under ultraviolet light transillumination.

2.9. Animal experiments

Miapaca-2 cells were suspended in HBSS at a concentration of 3.5×10^7 cells/ml and 200 μl cell suspensions were injected subcutaneously into the back of BALB/c nu/nu mice (SLC, Shizuoka, Japan). After the tumor size reached 100 mm^3, gemcitabine (30 mg/kg body weight) was administered four times on day 1, 5, 8, and 12, while anti-human CXCL8 monoclonal antibody or control antibody

(100 μg/body) was given four times on day 0, 4, 7, and 11. Tumor sizes were determined every 3–4 days and its volumes were calculated as follows. Tumor volume (mm^3) = (the longest diameter) (mm) × (the shortest diameter)^2 (mm)/2. At the indicated time points after the injection, tumors were removed for immunohistochemical analysis using anti-human CXCL8, anti-mouse CD31 (Abcam, Cambridge, UK), or anti-mouse Ly6G antibody (BD Biosciences) as previously described [14]. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

2.10. Statistical analysis

Data was analyzed statistically using methods indicated in each figure legend. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Gemcitabine-induced CXCL8 expression in pancreatic cancer cells

Consistent with the previous report [11], gemcitabine induced ROS generation in Miapaca-2 cells (Fig. 1A). Given the capacity of ROS to activate NF-κB [16] with a crucial role in the expression of pro-inflammatory cytokines and chemokines [12], we examined the effects of gemcitabine on the expression of pro-inflammatory cytokines and chemokines including interleukin (IL)-1, IL-6, CXCL8/IL-8, CXCL1, CCL2/monocyte chemoattractant protein (MCP)-1, CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/MIP-1β, CCL7/MCP-3, CCL11/eotaxin, and CX3CL1/fractalkine, in Miapaca-2 by using Mutiplex immunoassay system. Gemcitabine enhanced selectively IL-8/CXCL8 secretion among these molecules in Miapaca-2 cells (data not shown). Consistent with these observations, gemcitabine enhanced CXCL8 protein (Fig. 1B) and mRNA expression in a dose-dependent manner (Fig. 1C). Similar observations were obtained for another human pancreatic cancer cell line, Panc-1 (data not shown). We next examined the effect of a thiol-containing antioxidant, NAC, which is a precursor of reduced glutathione, with a capacity to scavenge ROS by interacting with OH· and H_2O_2 [17]. NAC reduced gemcitabine-induced ROS generation (Fig. 1A) and CXCL8 mRNA expression (Fig. 1D). Thus, gemcitabine induced CXCL8 expression in human pancreatic cancer cells through ROS generation.

3.2. Indispensable roles of NF-κB activation in gemcitabine-induced CXCL8 expression

Gemcitabine enhanced luciferase activities when Miapaca-2 cells were transduced with the reporter gene under the control of the 546, 272 or 133 bp CXCL8 promoter/enhancer region (Fig. 2A and B). The mutation in NF-κB binding site abrogated the gemcitabine-induced enhancement in luciferase activities, whereas the mutation in either AP-1 or NF-IL-6 binding site had minimal effects (Fig. 2C). Moreover, ChIP assay revealed that gemcitabine induced the binding of a component of NF-κB, p65, to CXCL8 promoter in Miapaca-2 cells (Fig. 2D). Thus, NF-κB activation is indispensable to gemcitabine-induced CXCL8 expression.

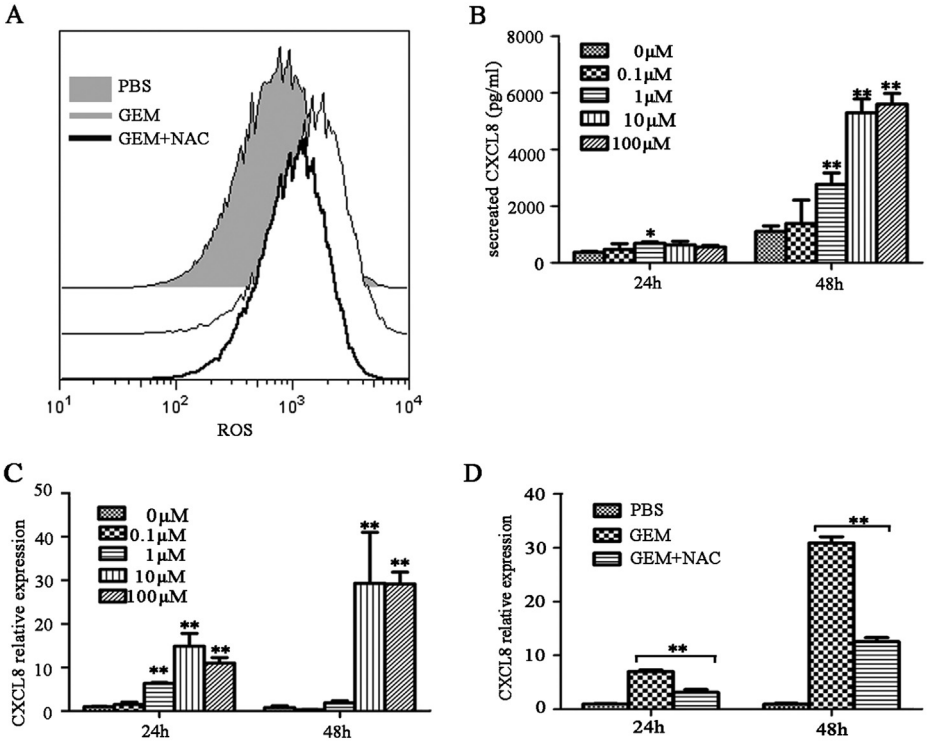

Fig. 1. Gemcitabine-induced CXCL8 expression in human pancreatic cancer cell lines. A. Miapaca-2 cells were incubated in the absence or the presence of gemcitabine for 24 h with 1 h pretreatment with NAC. Then, intracellular ROS levels were determined. Representative results from 3 independent experiments are shown here. B and C. Miapaca-2 cells were treated with the indicated concentrations of gemcitabine for 24 or 48 h. Supernatants were collected to determine CXCL8 contents by using a specific ELISA for human CXCL8 (B) while total RNA was extracted to determine CXCL8 mRNA levels by qRT-PCR (C). Mean and 1 SE were calculated for 3 independent experiments and are shown. *, $p < 0.05$; **, $p < 0.01$ using one-way ANOVA followed by the Dunnett test, compared with untreated. D. Miapaca-2 cells were incubated in the absence or the presence of gemcitabine for 48 h after 1 h pretreatment with NAC. Total RNA was extracted to conduct qRT-PCR to determine CXCL8 mRNA levels. Mean and 1 SE were calculated for 3 independent experiments and are shown here. **, $p < 0.01$ using Student's *t*-test.

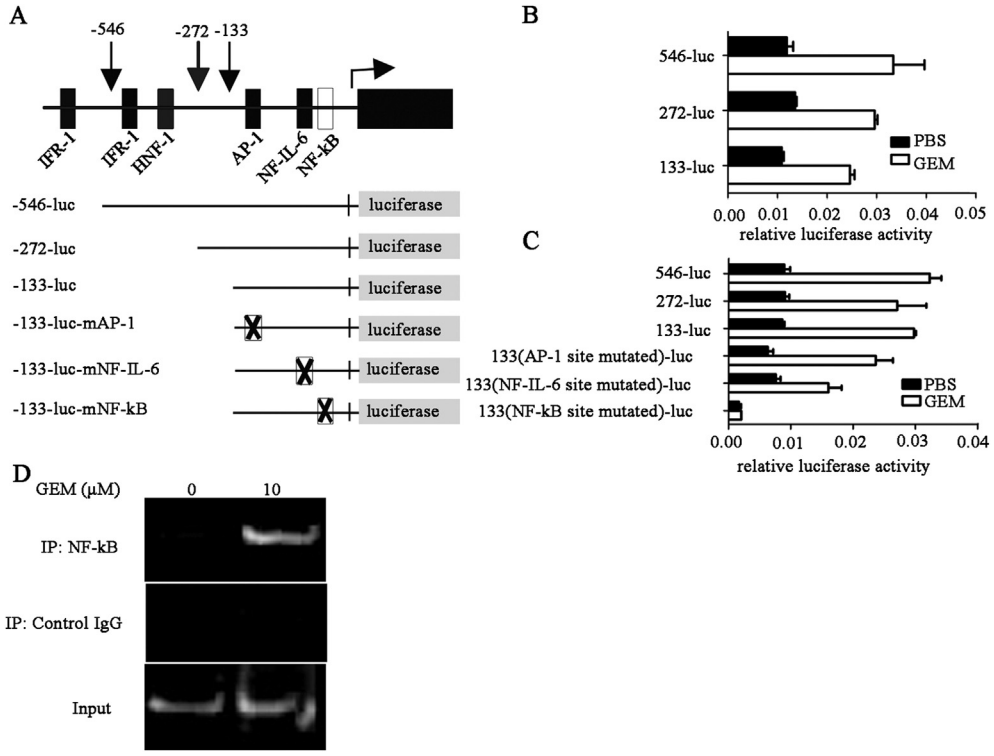

Fig. 2. Crucial roles of NF-κB activation in gemcitabine-induced CXCL8 expression. A. Schematic structure of 5′ promoter/enhancer region of human CXCL8 gene and luciferase expression linked with deleted or mutated 5′ promoter region of human CXCL8 gene. B and C. The cells were transiently transfected with CXCL8 promoter/enhancer-driven luciferase expression vectors together with SV40-renilla luciferase expression vector. After the cells were treated with or without gemcitabine for 48 h, they were harvested to determine their luciferase activities. Mean and 1 SE calculated for 3 independent experiments are shown. D. ChIP assay was conducted. Input indicates the results when total nuclear lysates were used without any immunoprecipitation. Representative results from 3 independent experiments are shown.

3.3. The roles of CXCL8 in gemcitabine-mediated inhibition of cellular proliferation

We could not detect expression of mRNA of specific receptors for CXCL8, CXCR1 and CXCR2, in untreated or gemcitabine-treated Miapaca-2 and Panc-1 cells (Fig. 3A). Consistently, flow cytometric analysis failed to detect CXCR1 and CXCR2 expression on untreated or gemcitabine-treated Miapaca-2 and Panc-1 cells (data not shown). CXCL8 siRNA efficiently inhibited gemcitabine-induced CXCL8 expression (Fig. 3B), but failed to modulate gemcitabine-induced reduction in cell proliferation in either cell line (Fig. 3C). Thus, gemcitabine-induced CXCL8 expression had few effects on cellular growth of human pancreatic cancer cells in an autocrine manner.

3.4. Involvement of gemcitabine-induced CXCL8 expression in in vivo tumor growth

We finally examined the effects of gemcitabine on CXCL8 expression in xenotransplanted tumor tissues. Gemcitabine

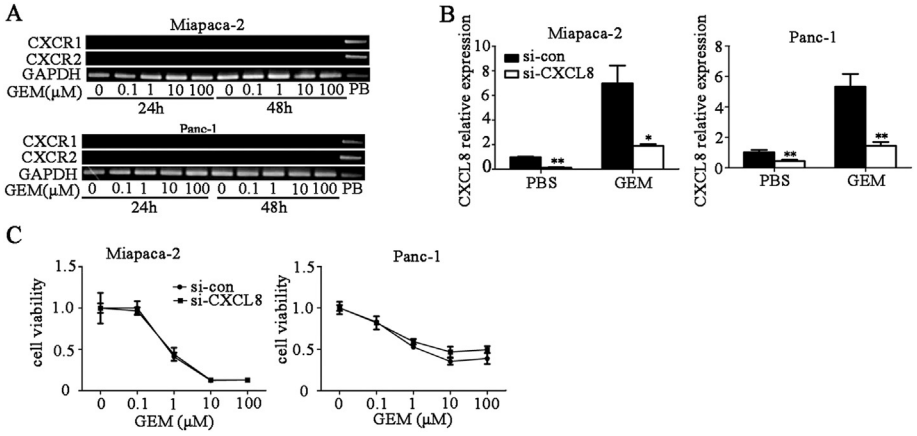

Fig. 3. Roles of CXCL8 in gemcitabine-induced inhibition of cell proliferation. A. Miapaca-2 cells (upper panels) and Panc-1 cells (lower panels) were treated with the indicated concentrations of gemcitabine for the indicated time intervals. Cells were then harvested and subjected to RT-PCR analysis to detect CXCR1 or CXCR2 mRNA expression. Representative results from 3 independent experiments are shown here. B and C. Miapaca-2 cells (left panel) or Panc-1 cells (right panel) were transfected with CXCL8 or control siRNA. CXCL8 mRNA expression was determined by using qRT-PCR (B). The transfected cells were incubated with the indicated concentrations of gemcitabine for 48 h. Cell viability was determined (C). Mean and 1 SE were calculated for 3 independent experiments and are shown here. *, $p < 0.05$; **, $p < 0.01$ using Student's *t*-test.

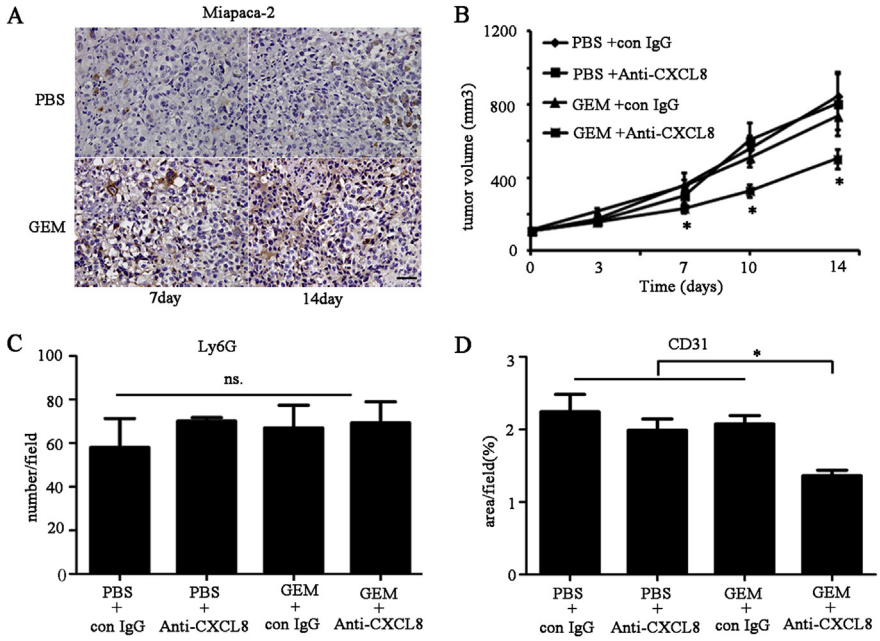

Fig. 4. Gemcitabine-induced CXCL8 expression in xenotransplanted tumor tissues. A. Miapaca-2 cells (7×10^6) were subcutaneously injected into nude mice. After tumor was formed, gemcitabine (30 mg/kg) or PBS was administered intraperitoneally as described in Materials and method. Tumor tissues were obtained 7 or 14 days after the start of gemcitabine treatment and were subjected to immunohistochemical analysis using anti-human CXCL8. Representative results from 3 independent animals are shown here with a scale bar of 100 μm. B. Tumor volumes were determined every 3–4 days after the initiation of gemcitabine and/or anti-CXCL8 antibody treatment. Each group consists of at least 10 tumors. Mean and 1 SE were calculated for 3 independent experiments and are shown here. *, $p < 0.05$, using one-way ANOVA, followed by the Tukey–Kramer test. C and D. At 14 days after the initiation of gemcitabine and/or anti-CXCL8 antibody treatment, tumors were removed and subjected to immunohistochemical analysis using anti-Ly6G (C) or anti-CD31 antibodies (D). Ly6G-postive cell numbers (C) or CD31-positive areas (D) were determined. Mean and 1 SE were calculated for 3 individual samples and are shown. *, $p < 0.05$ using one-way ANOVA, followed by the Tukey–Kramer test.

induced CXCL8 mRNA expression (data not shown) and CXCL8 protein expression in pancreatic cancer cells in xenografted tumor tissues (Fig. 4A). We next administered anti-CXCL8 antibody in combination with gemcitabine after xenografted Miapaca-2 cells formed tumor in mice. Tumor growth rates were reduced by the combined treatment of gemcitabine and anti-CXCL8 antibody but not with that of either single agent (Fig. 4B). Anti-CXCL8 antibody failed to reduce Ly6G-positive granulocytes (Fig. 4C) and F4/80-positive macrophages (data not shown). On the contrary, the treatment markedly reduced CD31-positive vascular areas (Fig. 4D). Thus, gemcitabine enhanced tumor cell-derived CXCL8 expression, which augmented intratumoral neovascularization, and eventually promoted in vivo tumor growth.

4. Discussion

CXC chemokines are divided into ELR-positive or ELR-negative CXC chemokines on the basis of the 3 amino acid sequence consisting of glutamic acid-leucine-arginine (the “ELR” motif) immediately preceding the first cysteine residue [18]. Most ELR-positive CXC chemokines utilize selectively CXCR2, except for CXCL6 and CXCL8 which utilize CXCR1 as well as CXCR2 [18–20]. CXCL8 is a potent chemotactic factor for neutrophils and has a crucial role in various types of neutrophil-mediated acute inflammation [21]. CXCR1 and CXCR2 are coordinately expressed by human neutrophils [22] and bind CXCL8 to induce a group of equipotent responses including chemotaxis and exocytosis [23]. However, they couple to distinct G proteins [24] to differentially generate other signals such as receptor internalization and phospholipase D activation [23] and these signals are required for full responsiveness of human neutrophils to CXCL8. Because mice do not possess the functional CXCR1 genes [25], human CXCL8 produced by gemcitabine-treated human cancer cells, could not fully induce mouse neutrophil responses and as a consequence, anti-CXCL8 antibody failed to reduce neutrophil infiltration under these conditions.

Tumor growth depends on the interaction with tumor microenvironment consisting of non-leukocytic cells including endothelial cells and fibroblasts as well as leukocytes [26]. Tumor microenvironment is influenced by various inflammatory molecules. Gemcitabine induced a robust production of CXCL8 in Miapaca-2 and Panc-1 cells. This chemokine can promote the migration and proliferation of endothelial cells, after binding mainly CXCR2 expressed by endothelial cells [27,28]. Moreover, accumulating evidence indicates the crucial roles of CXCL8 in tumor neovascularization in several animal tumor models, as evidenced by reduced tumor angiogenesis by CXCL8 blockade [29,30]. Likewise, we observed that anti-CXCL8 antibody treatment reduced intra-tumoral neovascularization as well as tumor sizes in mice treated with gemcitabine. Thus, gemcitabine treatment may counteract its anti-tumor effects by inducing a potent angiogenic factor, CXCL8, and CXCL8 blockade may be effective to enhance the efficacy of gemcitabine.

Conflict of interest

All of the authors have no financial conflicts of interests.

Acknowledgments

The authors express their sincere gratitude to Dr. Joost J. Oppenheim (NCI, Frederick, MD) for his critical review of the article.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.112.

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