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INDUCTION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 BY NICOTINE IN PANCREATIC DUCTAL ADENOCARCINOMA CELLS: ROLE OF OSTEOPONTIN

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Abstract

Introduction—Cigarette smoke and nicotine are among the leading environmental risk factors for developing pancreatic ductal adenocarcinoma (PDA). We showed recently that nicotine induces osteopontin (OPN), a protein that plays critical roles in inflammation and tumor metastasis. We identified an OPN isoform, OPNc, that is selectively inducible by nicotine and highly expressed in PDA tissue from smokers. In this study, we explored the potential proinflammatory role of nicotine in PDA through studying its effect on the expression of monocyte chemoattractant protein- (MCP)-1 and evaluated the role of OPN in mediating these effects.

Methods—MCP-1 mRNA and protein in PDA cells treated with or without nicotine (3–300 nM) or OPN (0.15–15 nM) were analyzed by real time PCR and ELISA. Luciferase-labeled promoter studies evaluated the effects of nicotine and OPN on MCP-1 transcription. Intracellular and tissue colocalization of OPN and MCP-1 were examined by immunofluorescence and immunohistochemistry.

Results—Nicotine treatment significantly increased MCP-1 expression in PDA cells. Interestingly, blocking OPN with siRNA or OPN antibody abolished these effects. Transient transfection of the OPNc gene in PDA cells or their treatment with recombinant OPN protein significantly ($P < 0.05$) increased MCP-1 mRNA and protein and induced its promoter activity. MCP-1 was found in 60% of invasive PDA lesions, of which 66% were smokers. MCP-1 colocalized with OPN in PDA cells and in the malignant ducts, and correlated well with higher expression levels of OPN in the tissue from patients with invasive PDA.

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Conclusions—Our data suggest that cigarette smoking and nicotine may contribute to PDA inflammation through inducing MCP-1 and provide a novel insight into a unique role for OPN in mediating these effects.

Keywords

pancreatic cancer; nicotine; osteopontin; monocyte chemoattractant protein-1

INTRODUCTION

In 2009, it is estimated that there will be 42,470 new cases of pancreatic ductal adenocarcinoma (PDA) and 35,240 deaths from the disease in the United States (1). Because of the aggressive nature of PDA and the absence of methods for early detection, it is often diagnosed at an advanced stage. The overall 5-year survival rate is less than 5%, and more than 85% of patients with PDA are diagnosed after the tumor has infiltrated into adjacent organs or when distant metastases are present. Therefore, it is important to understand the basic molecular mechanisms which make the disease so aggressive so that more effective therapies can be designed.

Cigarette smoking is a well-established environmental risk factor for PDA. Smokers have a twofold increase in the risk of developing PDA (2) and it is estimated that 25–30% of all PDA cases are related to cigarette smoking (3). In a recent pooled analysis, it was shown that current cigarette smokers had a significantly increased risk of pancreatic cancer (OR = 1.77, 95% confidence interval (CI): 1.38, 2.26) compared to nonsmokers. Also for cigarette smokers, pancreatic cancer risk increased significantly with increasing intensity, duration, and pack-years of smoking (4). Nicotine, a major component of tobacco and cigarette smoke, is an addictive agent and has been characterized as a drug of abuse by the U.S. Surgeon General (5). Several studies have linked pancreatic cancer to nicotine through cigarette smoking (6,7). However, the mechanisms by which nicotine contributes to the onset or development of PDA are unclear.

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family (have two adjacent cysteine amino acids near their amino terminus), and is produced by many cells including fibroblasts, endothelial cells, smooth muscle cells, monocytes and macrophages (8,9). MCP-1 is a very potent macrophage recruiting factor and its expression is associated with monocyte recruitment in human glioma (10), breast (11,12), ovarian (13), and prostate (14) cancer. However, no studies have been conducted to examine the role of MCP-1 in PDA and its relationship to smoking.

In previous studies, we have shown that in PDA cells, nicotine induces the expression of osteopontin (OPN), a protein that plays important roles in inflammation and tumor metastasis (15). We identified an OPN isoform, OPNc, that is selectively inducible by nicotine and highly expressed in PDA tumor tissue from smokers (16). In this study, we examined the potential proinflammatory role of nicotine in PDA by studying its effect on the expression of MCP-1 and evaluated the role of OPN in mediating these effects. We also analyzed the expression of MCP-1 and OPN in invasive and premalignant PDA tissues from smokers and nonsmokers.

MATERIALS AND METHODS

Cell culture

The human PDA cell lines MiaPaca and AsPC-1 were purchased from the American Type Culture Collection (Manassas, VA). Cells were counted and cultured at 1×10^4 cells to near

confluence in 96-well plates and maintained in DMEM supplemented with 10% fetal bovine serum in a humid atmosphere of 5% CO₂/95% air. Cells were treated with nicotine (3–300 nM) for 3 hours and 24 hours, and were evaluated for the expression of MCP-1 mRNA by real-time polymerase chain reaction (PCR). Cells were also treated with OPN protein (0.15–15nM) in presence or absence of nicotine and were evaluated for MCP-1 mRNA. To block the action of OPN, MiaPaca cells were treated with (0.4µg/ml) rabbit polyclonal IgG OPN antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with or without nicotine and were evaluated for the expression of MCP-1 mRNA. The concentrations of nicotine and OPN used in these experiments were selected based on dose-response studies.

RNA extraction and real-time reverse transcription PCR

Total RNA was isolated from PDA cells or pancreata using Trizol reagent (Life Technologies, Gaithersburg, MD). RNAs were quantified and input amounts were optimized for each amplicon. MCP-1, OPN and beta-actin (internal control) primers and probes were designed with the help of Primer Express Software (Applied Biosystems, Foster City, CA). cDNA was prepared, diluted, and subjected to real-time PCR using the TaqMan technology (7500 Sequence Detector; Applied Biosystems). The relative mRNA levels were presented as unit values of $2^{[C_T(\text{MCP1/OPN}) - C_T(\text{beta-actin})]}$, where C_T is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Enzyme-linked immunosorbent assays

Cell culture supernatant or lysate were obtained from MiaPaca and AsPC-1 cells treated with or without nicotine. MCP-1 protein levels were measured using an enzyme-linked immunosorbent kit (R&D, Minneapolis, MN) according to the manufacturer's instructions. Spectrophotometric evaluation of MCP-1 levels was made by Synergy HT multi-detection Microplate reader (BioTek, Winooski, VT).

siRNA sequences and constructs

Using GenBank™ sequence AK315461 for human OPN cDNA and computer analysis software developed by Applied Biosystems/Ambion, candidate sequences in the OPN cDNA sequence for RNAi with no homology with other known human genes were selected and used during transient transfection experiments. Human mismatch or scrambled siRNA sequences (Applied Biosystems/Ambion; Austin, TX) possessing limited homology to human genes served as a negative control. Transfection was done with TransFast (Promega, Madison, WI) in AsPC-1 cells as directed by the manufacturer. Cells were examined for OPN expression by real time PCR.

Confocal microscopy

For confocal analysis, 0.5×10^6 AsPC-1 or MiaPaca cells were seeded on sterilized round glass cover slips and incubated overnight. Cells were rinsed three times with 0.1 mM CaCl₂ and 1mM MgCl₂ in phosphate buffered saline (PBS/CM), fixed with 2% paraformaldehyde in PBS/CM for 30 minutes, permeabilized with 0.1% Triton-X100 and 0.2% bovine albumin serum in PBS/CM (IF buffer) for 10 minutes, quenched with 50 mM NH₄Cl in PBS/CM for 10 minutes, then rinsed with IF buffer. For MCP-1/OPN staining, cells were incubated simultaneously with mouse anti-OPN antibody (1:200) (Santa Cruz, CA) and goat-anti-MCP-1 antibody (7.5ug/ml) (R&D, MN) for 1 hour at room temperature. After washing in IF buffer (3x 10 min), cells were incubated for 30 minutes with secondary antibodies: rhodamine red X goat anti-mouse IgG (for OPN) and FITC donkey anti-goat (for MCP-1), both from Jackson ImmunoResearch; PA. After repeating the washing steps, nuclei were stained with Hoechst 33342 for 2 minutes, followed by rinsing in PBS. Coverslips were

mounted with ProLong Gold anti-fade (Molecular Probes, OR) and left overnight in the dark. The laser confocal microscope LSM 510 microscope (Carl Zeiss GmbH, Thornwood, NY) was used to image the cells in the respective channels at a magnification of 60X.

Transient Transfection of OPNc

pDest-490 vector containing a truncated splice variant OPN-c (base pairs 1–93, 175–942) was a generous gift from Dr. X Wang, Center for Cancer Research, NCI, Bethesda, MD (17). MiaPaca cells were transfected with 0.5 µg OPNc plasmid DNA using TransFast (Promega, Madison, WI), and lysates were harvested after 24 h for initial real time PCR testing of expression of the OPNc. For subsequent experiments to determine the levels of MCP-1 by real time PCR, MiaPaca cells (1×10^6) were transfected with 0.5 µg OPNc plasmid DNA using optimized nucleofection conditions (60–80% efficiency by pGEM4/enhanced green fluorescent protein (EGFP) visualization). We determined the levels of MCP-1 by real time PCR after 3 and 24 h of transfection.

ELISA

Human OPN concentration in the cell culture media were measured using human-specific ELISA kit (Assay Design, Ann Arbor, MI). Spectrophotometric evaluation of OPN levels were made by Synergy HT multi-detection microplate reader (BioTeck, Winooski, VT).

Promoter studies

To evaluate the effect of nicotine and OPN on MCP-1 transcription, we used the MCP-1 gene promoter (GenBankTM accession number AF079313) in luciferase expression vector pGL₃ basic (Promega), kindly provided by Dr. Decio Ezirik, Free University Brussels, Belgium (18). Cells were seeded into 24-well culture plates (10^5). At ~80% confluence they were co transfected by TransFast reagent (Promega) and 0.5 µg of pGL₃ vectors containing the luciferase-labeled MCP-1 promoter and 0.1 µg of green fluorescence protein (GFP) as transfection control. Two hours later, serum-containing medium was overlaid and the cells were incubated for an additional 24 h. The cells then were incubated with serum free medium for 18 h, after which nicotine or OPN were added for 3h. Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega) in a Veritas Microplate Luminometer (Turner Designs, Sunnyvale, Calif., USA). Transfection efficiency was normalized using the total protein concentration of the cell lysates. The results for nicotine-treated cells were expressed as a fold-induction of the luciferase activity of the same construct in the control condition, taking the control (no treatment) value as 100.

Human Tissue Acquisition and Analysis

Human PDA (n=73) and premalignant specimens (n=6) and normal pancreatic tissue were obtained from patients who underwent surgical resection at Thomas Jefferson University Hospital between 2005 and 2007. All patients signed an appropriate consent for tissue acquisition and study. The study was approved by the Institutional Review Board of Thomas Jefferson University. Patients' smoking history was examined and correlated with MCP-1 and OPN expression levels.

Tissue samples were stored in *RNA Later* for RNA analysis or fixed in neutral formaline for histological processing. Sections at 5 µm were stained with H&E. To localize MCP-1 and OPN, sections from the different tissues were analyzed by immunohistochemistry using the OPN and MCP-1 antibodies. A vectastain universal elite ABC kit and 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (Vector Laboratories Inc.) was used according to the manufacturer protocol to visualize the tissue reaction. Antibody specificity was validated

with nonimmune isotype serum. Negative control sections, where the primary or secondary antibodies were omitted were also prepared.

Statistical analyses

All experiments were performed 3 to 5 times. Data were analyzed for statistical significance by ANOVA with post-hoc Student *t* test analysis. Data are presented as mean $\pm$ SEM. Continuous, normally distributed variables were analyzed by Student-*t*-test. Spearman's rank correlation test was performed to analyze the correlation between OPN, and MCP-1 mRNAs expression. Analyses were performed with the assistance of a computer program (JMP 5 Software SAS Campus Drive, Cary, NC). Differences were considered significant at $P \leq 0.05$.

RESULTS

Nicotine stimulates MCP-1 mRNA accumulation and protein secretion in cultured PDA cells

PDA cells express different basal levels of OPN (15,16). To investigate whether nicotine can directly increase MCP-1 mRNA accumulation in PDA cells, we used MiaPaca and AsPC-1 cells, which express low and high levels of basal OPN, respectively. Cells were treated with or without nicotine (3–300 nM) for 3 and 24 h. Significant induction of MCP-1 mRNA expression was seen with a maximum increase at 24 h in MiaPaca cells. (Fig 1A). In AsPC-1 cells, only the higher doses of nicotine (30 and 300 nM) significantly increased MCP-1 mRNA levels after 3 h of nicotine stimulation. To examine whether the increase in MCP-1 mRNA levels in response to nicotine is associated with MCP-1 production, MCP-1 protein levels in the media were determined by ELISA. MCP-1 levels were increased by almost 3-fold after 48h of nicotine stimulation in MiaPaca cells (Fig 2A). In AsPC-1 cells, MCP-1 protein concentration increased by more than 2-fold after 24 hours then levels returned to basal levels by 48 hours after nicotine treatment (Fig 2B). These data indicate that MCP-1 induction by nicotine is a general phenomenon seen in the tested PDA cells lines.

Nicotine does not induce MCP-1 promoter activity

However, nicotine did not induce a significant increase in MCP-1 promoter activity when it was added (3–300 nM) to PDA cells that were transfected with luciferase-labeled MCP-1 promoter for 1, 3, 6, 12, 24 and 48h (data not shown). This suggested that MCP-1 promoter does not respond directly to nicotine. Since we showed previously that nicotine directly induces OPN transcription in PDA cells (15,16), and since OPN was shown to increase MCP-1 expression in other cells (19), we tested the hypothesis that OPN mediates the upregulation of MCP-1 mRNA by nicotine.

RNAi decreases OPN expression and reduce MCP-1-mediated upregulation of nicotine

AsPC-1 cells are highly invasive and normally constitutively express OPN at high levels. To suppress OPN expression in AsPC-1 cells, we selected two 21-nt targets within the OPN cDNA for RNAi. Based on these targets, double-stranded 21-nt siRNA constructs were designed encoding sense and antisense siRNA, and the OPN levels were measured using real time PCR. As shown in Fig 3A, OPN mRNA expression level was significantly inhibited by ~ 80%. We added nicotine (3–300 nM) to these cells and evaluated MCP-1 expression by real time PCR. As seen in Fig 3B, addition of nicotine was unable to elicit an increase in MCP-1 mRNA. It also was apparent that cells lacking OPN also expressed less endogenous MCP-1 mRNA. These data indicate that there is a relationship between the presence of intracellular OPN and the cell response to nicotine to produce MCP-1.

To confirm our data in MiaPaca cells, we blocked OPN function by adding OPN rabbit polyclonal antibody (0.4 µg/ml) 1 h before addition of nicotine (30 nM) for 24 h. As seen in figure 3C, blocking OPN resulted in complete prevention of the nicotine-mediated increase in MCP-1 mRNA. Cells that express normal levels of OPN expressed higher levels of basal MCP-1 when compared with cells that express no OPN.

This indicates that the presence of OPN in PDA cells is essential for the nicotine-mediated induction of MCP-1. Next, we asked whether OPN itself could induce MCP-1 transcription in PDA cells.

OPN induces MCP-1 transcription in PDA cells

Recombinant OPN protein (0.15–15 nM) was added for 3 and 6 h to MiaPaca cells that were transfected with MCP-1 luciferase-labeled promoter. OPN significantly and dose-dependently activated MCP-1 promoter (Fig 4A). In other experiments, OPNc plasmid was transiently transfected into MiaPaca cells. Real time PCR and ELISA confirmed overexpression of OPN (Fig 4B). Real time PCR analysis showed more than 3-fold increase in MCP-1 mRNA levels in the cells that overexpress OPNc vs. control cells (Fig 4C). Interestingly, addition of nicotine to OPNc overexpressing PDA cells induced further upregulation of MCP-1 mRNA (Fig 4C), suggesting that initial stimulation of OPN is necessary for further upregulation of MCP-1 by nicotine. Next, we analyzed the localization of OPN and MCP-1 in PDA cells in vitro

Colocalization of OPN and MCP-1 in PDA cells

Confocal microscopy after immunofluorescence staining of MiaPaca cells (Fig 5A) and ASPC-1 cells (Fig 5B) with antibodies against OPN and MCP-1 show colocalization of both proteins in the cytosol of PDA cells. OPN (red) was expressed in the form of granules that could be localized in the cytosol and cell membrane of PDA cells, whereas MCP-1 showed a more homogenous pattern and was present in the cytosol. We next examined the endogenous levels and localization of MCP-1 and OPN in premalignant and malignant PDA lesions.

Expression of OPN in human PDA

Using immunohistochemical staining, we found that MCP-1 was absent from the normal pancreatic ducts, whereas OPN was focally present, mostly on the apical surface of the ductal epithelium (15). In PDA (Fig 6A), OPN ductal epithelial staining was intensified and localized to the cell membrane and cytoplasm of the tumor cells. MCP-1 colocalized with OPN in the malignant ducts. Interestingly, both MCP-1 and OPN were also intensely stained in the metastatic lesions in the lymph nodes (Fig 6B).

Analysis of quantitative PCR data of MCP-1 mRNA corrected with GAPDH as an internal control, invasive PDA lesions showed significantly higher levels of MCP-1 mRNA when compared to IPMN premalignant lesions (Fig 6C). Interestingly, only 33% of the patients with premalignant lesions were smokers (2 of 6), whereas of the examined malignant lesions, 66% belonged to patients who were smokers (48 of 73) (Fig 6C).

To correlate the expression levels of MCP-1 and OPN in PDA tissue, the level of mRNA for each gene was recorded. Relative quantification (RQ) values of MCP-1 and OPN /GAPDH of >1 indicated high levels and were labeled (+++), values of 0.5 – 1 were labeled (++), of 0.1-0.5 were labeled (+), and of <0.1 was labeled (–). Expression of MCP-1 correlated well with OPN expression levels (Fig 6D). These data suggest that increased OPN expression in smokers is associated with the expression of MCP-1.

DISCUSSION

PDA is an aggressive disease that has a strong etiological association with cigarette smoking and high blood levels of nicotine (2–4). MCP-1 is the most frequently found CC chemokine in several tumors, including those of the pancreas (10–14,20,21). Although several studies investigated the role of MCP-1 in cancer progression, very few have investigated its relationship to smoking and the upstream factors involved in its regulation in PDA cells. In this study, we show for the first time that nicotine treatment both dose- and time-dependently increased MCP-1 expression in PDA cells. We also demonstrate a previously undescribed role for OPN as a mediator for these effects.

Our data show that nicotine induced MCP-1 accumulation rapidly in PDA cells and with significant magnitude. Dose-response studies demonstrated a significant induction of MCP-1 mRNA and protein levels at the physiological range of blood levels of nicotine in smokers (3–300 nM). The maximal effective concentration (at 30 nM) is similar to other nicotine actions that have been reported (22).

Our data show that the MCP-1 promoter was not activated by nicotine when it was added at (3–300 nM) to PDA cells at different time points. Since we showed previously that nicotine directly induces OPN transcription in PDA cells (15,16), and since OPN was shown to increase MCP-1 expression in other cells (19), we tested the hypothesis that OPN mediates the upregulation of MCP-1 by nicotine.

We conducted four sets of experiments to evaluate the role of OPN. First, we inhibited OPN synthesis by siRNA (Fig 3A,B) or blocked its function by a polyclonal antibody against human OPN (Fig 3C). In both studies, nicotine was unable to increase MCP-1 expression in PDA cells, suggesting that OPN may play a role in mediating the effects of nicotine. Next, we treated the PDA cells with recombinant human OPN protein (Fig 4A) or overexpressed an OPN isoform (OPNc) (Fig 4B), which has been shown to promote metastasis in cancer cells (23,24). In both experiments, we show that increased constitutive OPN or addition of exogenous OPN allowed the increase of MCP-1 expression by nicotine (Fig 4C). Furthermore, OPN also increased the basal levels of MCP-1 expression in PDA cells (Fig 4C) and increased its transcription by activating its promoter activity (Fig 4A). Previous studies have shown that nicotine induces the expression of total OPN (15) and selectively induce the expression of OPNc in PDA cells (16). This is the first report to demonstrate a relationship between nicotine, OPN/OPNc and MCP-1. Confocal microscopy analysis also revealed intracytoplasmic colocalization of OPN and MCP-1 in PDA cells (Fig 5), providing more evidence for the paracrine/autocrine relationship between the two molecules. Additional studies are now required to delineate the details of this relationship and the signaling pathways involved in mediating the increase of MCP-1 by OPN. Furthermore, the effect of the nicotine-mediated increase of OPNc on PDA cell inflammatory behavior and function is the subject of our currently ongoing studies in the laboratory.

Numerous studies have correlated high levels of MCP-1 expression with tumor progression in many cancers, including pancreatic cancer (20,21). MCP-1 promotes cell survival (25) and induces the expression of vascular endothelial growth factor (26). These functions are similar to those reported to be mediated by OPN (27,28). Our in vitro data suggest that OPN might be acting upstream of MCP-1 to mediate these effects.

Studies in colorectal cancer have correlated higher levels of MCP-1 with tumor stage and high metastatic potential (26). Our analyses reported here have found that MCP-1 was found in 60% of invasive PDA lesions, of which 66% were smokers. This is the first report to examine the relationship between tumor MCP-1 and the status of smoking in PDA patients. Our analysis also reveals that higher levels of MCP-1 were seen in invasive PDA as

compared to premalignant lesions. Immunohistochemical analysis of PDA showed that MCP-1 colocalizes with OPN in the malignant ducts and its mRNA levels correlated well with higher expression levels of OPN in the tissue from patients with invasive PDA (Fig 6). It remains to be determined, however, whether MCP-1 levels correlate with pathologic stage, survival or recurrence. Furthermore, additional studies are required to determine whether similar findings could be obtained from Endoscopic Ultrasound and Fine Needle Aspiration (EUS FNA) samples.

Interestingly, our studies show that high levels of MCP-1 and OPN exist in invasive lesions from non-smokers. Factors such as second hand smoke could contribute to this. In addition, a previous history of pancreatic inflammation (pancreatitis), which has been linked to pancreatic carcinogenesis (29,30) could create a tumor microenvironment with higher levels of OPN and MCP-1. Additional studies addressing these possibilities are currently ongoing in our laboratory.

In summary, our study suggests that cigarette smoking and nicotine may contribute to PDA inflammation through inducing MCP-1 and provides a novel insight into a unique role for OPN in mediating these effects. Although the role of OPN-mediated induction of MCP-1 in pancreatic carcinogenesis remains to be defined, the existence of OPN as a downstream effector of nicotine, capable of mediating proinflammatory effects in PDA cells is novel and could provide a unique potential target to control pancreatic cancer aggressiveness, especially in the cigarette smoking population.

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Fig 1A **Fig 1B****Figure 1.**

Augmentation of MCP-1 expression by nicotine. (A) MiaPaca, and (B) AsPC-1 cells were treated with nicotine (3–300 nM) for 3 and 24 h. Significant induction of MCP-1 mRNA expression is seen with the maximum induction after 24 h at 30 nM in both cell line. Values are expressed as mean $\pm$ SEM of three experiments. Values are expressed as mean $\pm$ SEM of three experiments. * $p < 0.05$ # $p < 0.02$ vs. control untreated cells using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student *t*-test.

Fig 2A **Fig 2B****Figure 2.**

MCP-1 protein in culture media was measured using human-specific ELISA kit. Significant induction of MCP-1 protein secretion is seen in MiaPaca (**A**) and AsPC-1 cells (**B**) with a maximum at 48 and 24 h, respectively. Each experiment was repeated three times for reproducibility. Values are expressed as mean $\pm$ SEM of three experiments. # $p < 0.005$ vs. control levels, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student *t*-test.

Fig 3A **Fig 3B**

Fig 3C**Figure 3.**

OPN is required for nicotine's stimulatory effect on MCP-1. **A.** Real time PCR analysis demonstrating the specificity and level of OPN knockdown in AsPC-1 cells. Cells were transfected for 24 h with either scrambled siRNA or OPN siRNA. Forty-eight h after transfection, cells were harvested and RNA isolated. Data (OPN/GAPDH) represent mean $\pm$ SE from 3 independent experiments. # $p < 0.005$ vs. nontransfected cells, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student *t*-test. **B.** Expression of MCP-1 mRNA in AsPC-1 cells transfected with OPN siRNA or scrambled siRNA for 24 h and treated with or without nicotine (3–300 nM) for 24 h. Values are expressed as mean $\pm$ SEM of three experiments. ** $p < 0.002$ vs. scrambled siRNA-transfected cells levels, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student *t*-test. **C.** Expression of MCP-1 mRNA in MiaPaca cells treated with rabbit polyclonal OPN antibody for 1 h and treated with nicotine (30 nM) for 24 h. Values are expressed as mean $\pm$ SEM of three experiments. * $p < 0.05$ vs. control, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student *t*-test.

Fig 4A

Fig 4B **Fig 4C****Figure 4.**

A. OPN induces MCP-1 promoter activity in MiaPaca cells. After 24 h of transfection, the cells were incubated with OPN (0.15–15 nM) for 3 and 6 h. After incubation, the luciferase activity in the cell lysates was measured. OPN increased MCP-1 promoter activity with maximum induction at 6 h. Relative luciferase activity was calculated after deduction of the activity levels with pGL3 vector alone. Results represent mean $\pm$ SEM of triplicate determinations. All experiments were repeated at least three times to confirm the reproducibility of the observations. **B.** Transient transfection of OPNc in MiaPaca cells. Total RNA was extracted from control and transiently transfected MiaPaca cells. Real time PCR (left panel), using specific OPN and GAPDH primers shows > 11-fold increase in OPN

mRNA. ELISA analysis of culture media from these cells (right panel) shows significant increase in OPN protein expression in the media. * $p < 0.05$ vs. control cells. **C.** Nicotine induces higher levels of MCP-1 mRNA accumulation in OPNc expressing cells. OPNc expressing MiaPaca cells were treated with or without nicotine (3–300 nM) for 24 h. Significant increase in basal MCP-1 mRNA levels can also be seen. Values are expressed as mean $\pm$ SEM of three experiments. * $p < 0.05$ # $p < 0.005$ vs. control levels, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by student *t*-test.

Fig 5A

Fig 5B

Figure 5.

Double-immunofluorescence analysis of OPN and MCP-1 in PDA cells. Optical sections at 2- μ m intervals from the dorsal to ventral surface of MiaPaca cells (**A**) and AsPC-1 cells (**B**) were immunostained for both OPN (red) and MCP-1 (green) and examined by confocal microscopy. OPN shows a granular appearance and is localized in the cytosol and cell membrane of PDA cells, whereas MCP-1 shows a more homogenous pattern and was present in the cytosol. The merged images show colocalization of both proteins in the cytosol of PDA cells (X 600 original magnification).

Fig 6A

Fig 6B

Fig 6C

Fig 6D

Figure 6.

A. Representative immunohistochemical staining for OPN and MCP-1 in malignant PDA. Serial sections of paraffin embedded PDA sections were stained with OPN and MCP-1 antibodies. MCP-1 colocalized with OPN in the malignant ductal epithelial staining and in stromal cells. (X200 original magnification). **B.** Lymph node metastatic lesions showed intense OPN and MCP-1 staining in the ductal metastasis. (X 100 original magnification). Negative control (–ve C) sections where the primary antibody was not added did not show non-specific reaction. **C.** Expression of MCP-1 in human tissue. Real time PCR analysis of MCP-1 mRNA in premalignant lesions, and invasive PDA. Significantly higher MCP-1 mRNA levels are seen in invasive PDA. Analysis of patient history of the samples used for MCP-1 analysis shows that invasive PDA patients were mostly (66%) smokers, while a the majority (67%) of patients with premalignant lesions were non smokers* $p < 0.05$ vs. non-malignant levels using one-way repeated ANOVA with subsequent all pairwise comparison procedure by student *t*-test. **D.** Significant correlation ($p < 0.05$) between tissue OPN and MCP-1 mRNAs. High MCP-1 (+++) was found in 65% of the invasive PDA samples that expressed high OPN (+++). No MCP-1 was found in IPMN lesions that expressed very low levels of OPN.