Adrenomedullin Is Expressed in Pancreatic Cancer and Stimulates Cell Proliferation and Invasion in an Autocrine Manner via the Adrenomedullin Receptor, ADMR

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Abstract

The current study investigated adrenomedullin as a potential autocrine regulator of pancreatic cancer cell function. Adrenomedullin was localized in the neoplastic epithelium of 90% (43 of 48) of human pancreatic adenocarcinomas analyzed by immunohistochemistry and was expressed by 100% (8 of 8) of pancreatic cancer cell lines analyzed by reverse transcription-PCR. Pancreatic cancer cell lines also secreted adrenomedullin into the culture medium as determined by ELISA (5 of 5). Exogenous adrenomedullin treatment of Panc-1, BxPC3, and MPanc96 cells in vitro stimulated cell proliferation, invasion, and nuclear factor κB activity, indicating the ability of the cells to respond to adrenomedullin. Treatment of the cell cultures with an adrenomedullin antagonist inhibited basal levels of proliferation and nuclear factor κB activity, supporting the autocrine function of this molecule. Furthermore, increasing adrenomedullin levels by gene transfer to Panc-1 cells increased, whereas adrenomedullin small hairpin RNA silencing in MPanc96 cells inhibited tumor growth and metastasis in vivo. Adrenomedullin is able to act through at least two different receptors, adrenomedullin receptor (ADMR) and calcitonin receptor–like receptor (CRLR). Reverse transcription-PCR and Western blotting indicated that pancreatic cancer cells expressed only ADMR but not CRLR. In contrast, cells found in the tumor microenvironment, primary human pancreatic stellate and endothelial (HUVEC) cells, expressed both ADMR and CRLR. Small hairpin RNA silencing of ADMR in pancreatic cancer cells blocked adrenomedullin-induced growth and invasion, indicating that this receptor is involved in the autocrine actions of adrenomedullin. These data indicate that adrenomedullin acting via ADMR increases the aggressiveness of pancreatic cancer cells and suggests that these molecules may be useful therapeutic targets. [Cancer Res 2007;67(6):2666–75]

Introduction

Pancreatic ductal adenocarcinoma is an important cause of malignancy-related deaths. In the United States, it ranks fourth among the leading causes of cancer death, accounting for more than 32,000 deaths annually (1). The mechanisms behind the aggressiveness of this cancer are not fully understood. Using a variety of profiling technologies, numerous cancer-related genes have been identified in pancreatic ductal adenocarcinoma (2–5). However, the functional significance of the majority of these altered genes remains unclear. Our previous microarray analysis suggested that adrenomedullin is overexpressed in pancreatic cancer compared with normal pancreas or chronic pancreatitis (6). Considerable evidence suggests that adrenomedullin may play an important role in cancer.

Adrenomedullin is a 52–amino acid peptide originally isolated from a human pheochromocytoma (7) that has been found to be a multifunctional regulatory peptide (8). Adrenomedullin is physiologically expressed in normal pancreatic islet cells, with predominant expression in the F cells, which also contain pancreatic polypeptide (9). Pancreatic β-cells express receptors known to respond to adrenomedullin including the adrenomedullin receptor (ADMR, also known as L1-R) and the calcitonin receptor–like receptor (CRLR), supporting its role in endocrine function (9–11). Adrenomedullin has also been shown to have important effects in vascular cell biology in which it regulates vascular tone and permeability and promotes vasodilation (12–16). Adrenomedullin is also a potent angiogenic molecule (15). The expression of adrenomedullin is regulated, at least in part, by hypoxia, and adrenomedullin is partially responsible for the angiogenic effects of hypoxia (15). The angiogenic effects of adrenomedullin are likely mediated through direct stimulation of endothelial cell proliferation (17) and protection of endothelial cells from apoptosis (18). In cancer, adrenomedullin seems to have an important role in angiogenesis and may have an additional trophic effect directly on cancer cells (19–21).

Little is known about adrenomedullin in pancreatic cancer. The frequency and distribution of adrenomedullin expression in tumors has not been previously reported. Also, the role of adrenomedullin in pancreatic cancer is not completely understood. Recent studies have indicated that inhibition of adrenomedullin receptor activation by direct application of a partial adrenomedullin peptide that acts as an antagonist (adrenomedullin antagonist, AMA; ref. 22), or intratumoral or i.m. transfection with an AMA-expressing plasmid (23), reduced microvascular density and growth of pancreatic tumors. These studies provide strong evidence for the angiogenic function of adrenomedullin. However, these studies did not determine if adrenomedullin also has an autocrine role in pancreatic cancer.

In our present study, we investigated the expression and possible autocrine role of adrenomedullin in pancreatic cancer. We found that adrenomedullin was highly expressed in pancreatic cancer cells within tumor samples and in pancreatic cancer cell lines. Pancreatic cancer cells also secreted adrenomedullin into the...
Role of Adrenomedullin in Pancreatic Cancer

culture medium. Inclusion of AMA inhibited basal levels of pancreatic cancer cell proliferation, invasion, and nuclear factor κB (NF-κB) activity in vitro. Furthermore, levels of cellular adrenomedullin correlated with the rate of tumor growth and metastasis in vivo. We also investigated the presence and function of adrenomedullin receptors in pancreatic cancer. We observed that ADMR, but not CRLR, is present in pancreatic cancer cells, whereas both the receptors are present in cells in the surrounding tumor microenvironment. We found that the autocrine effects of adrenomedullin were mediated by the ADMR receptor, as silencing of ADMR using small hairpin RNA (shRNA) blocked the effects of adrenomedullin in vitro. Taken together with the previous studies, these data suggest that adrenomedullin has both autocrine and paracrine roles in pancreatic cancer. Therefore, interference with the function of either adrenomedullin or ADMR might be useful as a therapeutic approach to pancreatic adenocarcinoma.

Materials and Methods

Pancreatic tissues, cell lines, and adrenomedullin peptides. Paraffin-embedded tissue slides of normal pancreas, pancreatic adenocarcinoma, chronic pancreatitis, and a pancreatic cancer tissue microarray slide with 48 tumor samples were obtained from the Department of Pathology, University of Michigan. Pancreatic cancer cell lines BxPC3, MiaPaCa-2, CFPAC-1, HPAC, MPanc96, Panc-1, Aspc-1, and SU.86.86, as well as HUVEC cell lines were obtained from the American Type Culture Collection (Manassas, VA). BxPC3 cells were grown on 10% RPMI whereas all other pancreatic cancer cell lines were grown on 10% DMEM, and endothelial cells (HUVEC) were grown on 15% MEM. All media contained 1% antibiotic. Human pancreatic stellate cells (HPSC) were isolated using the outgrowth method for pancreatic adenocarcinoma samples from patients undergoing surgical resection. All human tissue samples were obtained in accordance with the policies and practices of the Institutional Review Board of the University of Michigan or the University of Texas M. D. Anderson Cancer Center. Briefly, tumor samples were minced and seeded in six-well plates containing 15% DMEM. After ~5 days, cells were able to grow out from the tissue clumps. Medium was changed every 3 days. The cells were confirmed to be pancreatic stellate cells by immunohistochemical staining for α smooth muscle actin and vimentin. All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Adrenomedullin (AM 52) and AMA (AM 22-52) were purchased from Sigma (St. Louis, MO).

Immunohistochemical staining. Unstained 4 μm sections of clinical specimens or a tissue microarray were deparaffinized with xylene and rehydrated with ethanol. Immunohistochemistry was done using Biotin Vectorstain Elite ABC Universal kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Primary antibody against adrenomedullin (Phoenix Pharmaceuticals, Inc., Belmont, CA) diluted 1:250 in 2% bovine serum albumin/0.2% Triton in PBS was used. Finally, slides were developed with 3,3-diaminobenzidine substrate counterstained with hematoxylin, dehydrated with ethanol, fixed with xylene, and mounted.

Reverse transcription-PCR. Total RNA was isolated from normal pancreas, pancreatic cancer, and chronic pancreatitis tissues, pancreatic cancer cell lines, HUVEC, and HPSCs, and were used for reverse transcription-PCR (RT-PCR). DNase was used to remove contaminating genomic DNA after RNA purification. The quality of the RNA was confirmed by running on a denaturing gel, in which we observed clear 28S and 18S rRNA bands. A non–reverse-transcribed control was run to assure that no reverse transcription-PCR (RT-PCR) reactions were: forward, 5'GGG AGG TGA AGA ACT ATG AG-3' and reverse, 5'-ATC ATG GCC AGT AGT ACA TC-5'. Primers designed for human RAMP1 (Genbank, NM_005854) were: forward, 5'-TGG AAG TGG TGC AAC CTG TG-3' and reverse, 5'-CAC GTC GAT GCC TAC TTG GC-3'. Primers designed for human RAMP2 (Genbank, NM_005854) were: forward, 5'-GGG AGG TGA AGA ACT ATG AG-3' and reverse, 5'-ATC ATG GCC AGT AGT ACA TC-5'. Primers designed for human RAMP3 (Genbank, NM_005856) were: forward, 5'-TGG AAG TGG TGC AAC CTG TG-3' and reverse, 5'-CAC GTC GAT GCC TAC TTG GC-3'. Primers designed for human CRLR (Genbank, NM_014778) were: forward, 5'-GTC GAG GAT GCC TAC TTG GC-3' and reverse, 5'-GTA GAG AGG AGG AGG GCA GCA GA-3'. Primers designed for human ADMR (Genbank, NM_007264) were: forward, 5'-CAT GGC GGA CCT GGC CAT TGT-3' and reverse, 5'-TGAA GAG AGG AGG AGG GCA GCA AGC-3'. Primers designed for δ-actin (Genbank, BC_0160458), which was used as a loading control for the RT-PCR reactions, were: forward, 5'-ATG ATA TCG CGG TCG TCG TC-3' and reverse, 5'-CGC TGG GCG GGT GTG GTG AA-3'. Amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide.

Adrenomedullin ELISA. Adrenomedullin was detected in conditioned medium from pancreatic cancer cell lines using a commercial ELISA. For collection of conditioned medium, wild-type cell lines including BxPC3, SU.86.86, MPanc96, Panc-1, and Miapaca-2; Panc-1 cells stably transfected with a control vector or an adrenomedullin expression vector and MPanc-96 cells stably transfected with a control shRNA or an adrenomedullin shRNA vector; and MPanc96 cells stably transfected with shRNA against control vector and ADMR, were equally plated with serum-free medium and after overnight incubation, medium was collected and concentrated using Centricron YM-3 filter devices (Millipore Corporation, Chicago, IL). Protein concentrations were determined using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA). Competitive ELISA was then conducted using an adrenomedullin detection kit (Phoenix Pharmaceuticals) following the manufacturer’s suggested protocol. Concentrated serum-free DMEM served as a reagent control and these control absorbance values were subtracted from those of all other samples. Calculations of adrenomedullin concentration used the standard curve included in the kit and were done according to the manufacturer’s protocol.

Cell growth studies. For all in vitro studies, adrenomedullin or AMA was added freshly to culture medium on a daily basis. For initial studies, wild-type BxPC3, MPanc96, and Panc-1 cells (1,000 cells) were plated on 96-well plates in 0.5% serum containing medium with and without adrenomedullin (200 nmol/L) or AMA (1 μmol/L), and cell numbers were estimated after 48 h by MTS assay. For a more detailed time course determination, wild-type Panc-1 cells (1,000 cells) were treated with adrenomedullin (200 nmol/L) or AMA (1 μmol/L) for 0, 12, 24, 48, and 72 h before cell number estimation. For dose-dependence studies, wild-type Panc-1 cells (5,000 cells) were treated with adrenomedullin (1–200 nmol/L) or AMA (1 μmol/L) for 48 h before cell number estimation. For small interfering RNA studies, wild-type BxPC3, MPanc96, and Panc-1 cells (5,000 cells) were plated on 96-well plates and transfected with control and siADMR oligos and cell numbers were estimated after 48 h. Cell growth was analyzed using the MTS assay (Promega, Madison, WI) added 1 h before taking the spectrophotometric reading, according to the manufacturer's directions.

NFκB activity (luciferase assay). Pancreatic cancer cell NFκB activity was analyzed using an NFκB luciferase reporter assay. The NFκB luciferase reporter was isolated from the pNFκB-Luc Vector (Clonetec, Mountain View, CA) and cloned into the lentiviral vector FG9, which drives gene expression with the cytomegalovirus/long terminal repeat and UbIC promoter (24) to form Lenti-NFκB-Luc. Lentiviral plasmid vectors were cotransfected with packaging vectors and lentivirus was produced in 293T cells by the calcium transfection method as has previously been described (24). Lenti-NFκB-Luc was titrated and pancreatic cancer cell lines were each infected using (25 μL of viral supernatant per milliliter of medium) mixed with polybrene (4 μg/mL medium). Functional validation of the NFκB reporter was conducted using tumor necrosis factor-α as a positive control. Adrenomedullin (200 nmol/L) and AMA (1 μmol/L) were examined for their effects on NFκB activity in BxPC3, MPanc96, and Panc-1 cells. After 4.5 h of treatment, 150 μg/mL of n-luciferin was added to cells and luciferase activity was measured using an IVIS bioluminescence system (Xenogen Corp., Alameda, CA).

Invasion assays. For studies of cell invasiveness, BIOCOAT Matrigel invasion chambers (BD Biosciences, Chicago, IL) were used. Briefly, 2 × 10⁵ human RAMP2 (Genbank, NM_005854) were: forward, 5'-GGG AGG TGA AGA ACT ATG AG-3' and reverse, 5'-ATC ATG GCC AGT AGT ACA TC-5'.


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cells in 100 μL of serum-free medium were added to the upper chamber and different concentrations of adrenomedullin (1–200 nmol/L) in 0.5% serum containing DMEM were added to the lower chamber. The cells were allowed to invade the Matrigel for 12 h at 37°C in a 5% CO₂ atmosphere. DMEM containing 0.5% serum was used as a control. The noninvading cells on the upper surface of the membrane were removed with a cotton swab and the invading cells on the lower surface of the membrane were estimated using a MTS reagent. The cells on the bottom surface were incubated for 1 h with MTS reagent and color development was read photometrically. For imaging, cells were fixed and stained with a Diff-Quick stain kit (BD Biosciences), washed twice with water and air-dried. Invading cells in three adjacent microscope fields for each membrane were imaged at 20× magnification.

Development of stable cell lines. We examined the effects of stable overexpression and shRNA silencing of adrenomedullin in pancreatic cancer cells. RT-PCR–amplified adrenomedullin products were cloned into the pcDNA3.1 vector (Invitrogen Corporation, Chicago, IL) using BamHI and EcoRI restriction enzymes and confirmed by DNA sequencing. Panc-1 cells, which have a low endogenous expression of adrenomedullin, were transfected using LipofectAMINE reagent (Invitrogen) with a control plasmid or a plasmid encoding the complete coding sequence of human adrenomedullin and selected for resistance to neomycin (3 mg/mL). MPanc96 cells, which express relatively high levels of adrenomedullin, were transfected with a shRNA designed for adrenomedullin or with a mismatch control shRNA. Initially, two different shRNAs were created using the Invitrogen BLOCK-IT RNAi Designer protocol (adrenomedullin target sequence 1, ggc aca cca gat cca gtt; adrenomedullin target sequence 2, gga ctc tgt ctt cta agc). The shRNAs were annealed and cloned into a BLOCK-IT U6 RNAi entry vector containing the human U6 pol III type promoter. Effective silencing of adrenomedullin was verified in transient transfection experiments in MPanc96 cells using LipofectAMINE and the shRNA for target 1 was selected for further study. For stable silencing, the adrenomedullin-silencing shRNA and control shRNA were cloned into DEST vectors according to the manufacturer’s protocols. Stable expression of both the adrenomedullin shRNA and the control shRNA were established in MPanc96 cells by selecting for neomycin resistance (1 mg/mL).

Lentiviral silencing of ADMR. Silencing of ADMR was achieved in pancreatic cancer cell lines using lentivirus infection. Lentiviral plasmid vectors—control and ADMR shRNA (Open Biosystems, Huntsville, AL)—were cotransfected with packaging vectors and lentivirus was produced in 293T cells by the calcium transfection method as has previously been described (24). The lentiviral shRNA was transduced and pancreatic cancer cell lines were each infected using (25 L viral supernatant/mL of medium) mixed with polybrene (4 μg/mL medium). Cells were then selected for resistance to puromycin (1 mg/mL) and stable cell lines were developed thereafter.

Western blot. Cell lysates from MPanc96 cells stably silenced with ADMR shRNA and control shRNA were prepared and protein concentrations were measured by Bio-Rad reagent. Protein (50 μg) was loaded onto 10% SDS-PAGE gels and Western blotting was conducted using a primary antibody against ADMR (MBL International Corporation, Woburn, MA) at a 1:1,000 dilution. The same blot was re-probed for β-actin (Sigma; 1:2000 dilution), which served as a loading control for the experiment.

In vivo orthotopic tumor model. Growth of the adrenomedullin-modified pancreatic cancer cells were compared with wild-type control cells in orthotopic tumors formed in 4-week-old male CB-17 SCID mice. The MPanc96 cells stably transfected with shRNA against adrenomedullin and control shRNA were further modified to stably express the firefly luciferase gene by lentivirus transfection (25). These cells were then grown to 80% confluence, harvested by trypsinization, washed twice in PBS, and resuspended to a final concentration of 1 × 10⁶ cells/mL. Cell suspensions (0.1 mL) were injected into the pancreas of six mice per test group. Tumor growth was assessed by bioluminescence imaging at the end of 6 weeks. Bioluminescence imaging was conducted using a cryogenically cooled imaging system coupled to a data acquisition computer running LivingImage software (Xenogen). Before imaging, animals were anesthetized in an acrylic chamber with a 1.5% isoflurane/air mixture and injected i.p. with 40 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. A digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second. After the final tumor imaging, the pancreas was removed and the animals were re-imaged to visualize and count cancer cell dissemination and metastases. Tissues were also fixed with formaldehyde and histology was conducted to verify the accuracy of the bioluminescence data.

Statistical analysis. All experiments were conducted in triplicate and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments ±SE. Statistically significant differences were determined by two-tailed unpaired Student’s t test and were defined as *P < 0.05.

Results

Adrenomedullin is specifically expressed in pancreatic cancer tissues and cell lines. We previously reported that adrenomedullin mRNA levels were 4-fold higher in pancreatic cancer compared with normal pancreas and chronic pancreatitis based on microarray analysis (6). In the current study, adrenomedullin expression in human tissues was evaluated by immunohistochemistry. As expected, adrenomedullin was present in normal pancreatic islets (Fig. 1A). Adrenomedullin was also highly expressed specifically in pancreatic cancer cells but not in normal duct or acinar cells or in chronic pancreatitis tissues. A clinical pathologist examined a pancreatic cancer tissue array and observed that 90% of the tumors were positive for adrenomedullin (43 of 48). RT-PCR for adrenomedullin mRNA indicated its presence in eight of eight pancreatic cancer cell lines (Fig. 1B). An ELISA for adrenomedullin confirmed the presence of adrenomedullin in cell lysates (data not shown) and also indicated that all examined cell lines (5 of 5) secreted the protein into their tissue culture medium (Fig. 1C).

Exogenous adrenomedullin stimulates pancreatic cancer cell growth, invasion, and NF-κB activity. The effects of treatment with adrenomedullin were studied on pancreatic cancer cell lines in vitro. Adrenomedullin (200 nmol/L) significantly (P < 0.05) stimulated the proliferation of BxPC3, MPanc96, and Panc-1 pancreatic cancer cell lines by 156 ± 11.5%, 189 ± 14.0%, and 241 ± 24.3%, respectively, when compared with untreated cells after 48 h. Panc-1 cells were selected for more detailed studies of adrenomedullin time- (Fig. 2A) and concentration-dependence (Fig. 2B). Adrenomedullin treatment increased Panc-1 cell proliferation in a concentration-dependent manner with a significant effect observed...
with 10 nmol/L of adrenomedullin and a maximal effect observed with 200 nmol/L of adrenomedullin.

We next analyzed the effect of exogenous addition of adrenomedullin on Panc-1 cell invasion. The addition of adrenomedullin (1–200 nmol/L) to the bottom wells of the invasion chamber as a chemoattractant for 24 h increased the number of invading cells in a concentration-dependent manner (Fig. 2C). MTS assay showed that significant effects on invasion were noted with 100 nmol/L of adrenomedullin, and with 200 nmol/L of adrenomedullin, invasion was increased by 272% ± 15.6% compared with control. The same results were documented photographically (Fig. 2D).

Finally, as a potential intracellular signal generated by treatment with adrenomedullin, we investigated the level of NFκB activity in the pancreatic cancer cells expressing an NFκB reporter (Table 1). Exogenous addition of adrenomedullin (200 nmol/L) significantly induced NFκB reporter activity compared with untreated cells in all examined cell lines. Adrenomedullin also stimulated activation of NFκB examined in electrophoretic mobility shift assays (data not shown). These data indicate that pancreatic cancer cells are able to respond directly to adrenomedullin.

**Adrenomedullin acts in an autocrine manner to stimulate pancreatic cancer cell growth and NFκB activity.** To examine the possibility that adrenomedullin influences pancreatic cancer cells in an autocrine manner, we examined the effects of AMA on basal pancreatic cancer cell functions. AMA (1 μmol/L) significantly (P < 0.05) reduced basal cell proliferation in BxPC3, MPanc-96, and Panc-1 pancreatic cancer cell lines by 41 ± 4.0%, 57 ± 5.5%, and 64 ± 9.8%, respectively, when compared with controls after 48 h. The effects of AMA treatment on Panc-1 cell proliferation were significant within 48 h and were more pronounced after 72 h (Fig. 24). AMA (1 μmol/L) treatment also significantly reduced basal NFκB activity compared with untreated cells in each of the examined pancreatic cancer cell lines (Table 1). Basal levels of NFκB activity varied between the cell lines, but AMA reduced basal levels compared with controls by at least 50% in all cell lines examined.

**Adrenomedullin levels correlate with rates of pancreatic cancer growth and invasion in vitro and in vivo.** To further investigate the influence of adrenomedullin on pancreatic cancer cell function, pancreatic cancer cell lines were genetically modified to alter adrenomedullin levels. MPanc-96 cells, with a relatively high level of endogenous adrenomedullin, were stably transfected with a shRNA to silence adrenomedullin. Panc-1 cells, with a relatively low level of endogenous adrenomedullin, were stably transfected with an adrenomedullin expression vector to increase adrenomedullin levels. ELISA was used to verify alterations in cellular secretion of adrenomedullin after transfections (Fig. 3). Genetic transfer of adrenomedullin to Panc-1 cells resulted in an increase in secreted levels of adrenomedullin. The level of adrenomedullin secretion in the adrenomedullin-transfected Panc-1 cells was similar to the level observed in wild-type MPanc-96 cells. Adrenomedullin levels in MPanc-96 cells were significantly reduced after shRNA silencing.

The effects of these alterations in adrenomedullin expression levels were evaluated on cell proliferation and invasion in vitro. Panc-1 cells overexpressing adrenomedullin showed an increased proliferation rate compared with empty vector–transfected cells (263 ± 19.5%, P < 0.05; Fig. 4A). In the complementary experiment, MPanc-96 cells showed a significant decrease in cell proliferation after stable silencing of adrenomedullin expression (52 ± 7.1%, P < 0.05) when compared with that of MPanc-96 cells stably expressing control shRNA. Overexpression of adrenomedullin also stimulated Panc-1 cell invasion (457 ± 52.3%, P < 0.05) when compared with empty vector (Fig. 4B and C). Furthermore, silencing of adrenomedullin in MPanc-96 cells decreased cell
invasion (43 ± 2.2%, \( P < 0.05 \)) when compared with control shRNA–transfected cells.

Based on our observations on the effects of adrenomedullin on pancreatic cancer cell growth and invasion \textit{in vitro}, we further wished to analyze its effect on tumors developed \textit{in vivo} in immunodeficient mice. In one set of experiments, orthotopic tumor formation following pancreatic injection of Panc-1 cells expressing adrenomedullin and control vector was assessed. Tumor growth measured after 4 weeks indicated that Panc-1 cells overexpressing adrenomedullin showed a 2.5-fold increase in tumor size when compared with vector control–transfected cells (Fig. 5A). Also, tumor invasion to the peritoneum was increased by 53% in adrenomedullin-overexpressing animals (4.0 ± 1.3 per animal in the control group and 6.1 ± 0.8 per animal in the adrenomedullin-overexpressing group).

In another set of experiments, the effect of adrenomedullin on pancreatic tumor growth \textit{in vivo} was assessed in the orthotopic pancreatic tumor model by injection of MPanc96 cells expressing shRNA for adrenomedullin or control shRNA using bioluminescence imaging. MPanc96 cells with silenced adrenomedullin expression showed an 8-fold reduction in tumor volume compared with control shRNA–transfected cells (Fig. 5B and C). We also observed that the extent of disseminated cancer measured at the end of the 6 weeks after the resection of the pancreas was significantly lower in adrenomedullin-silenced animals (Fig. 5D). After removal of the primary tumor, cancer was found widely dispersed throughout the peritoneum and liver metastasis was observed. Adrenomedullin-silenced animals showed a 98% reduction in the total volume of disseminated cancer, and a 40% reduction in the number of metastatic foci (3.0 ± 0.9 foci per control animal versus 1.2 ± 0.4 foci per adrenomedullin-silenced animal).

Table 1. Effect of adrenomedullin on pancreatic cancer cells line NFκB activity \textit{in vitro}

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<th>Adrenomedullin NFκB-Luciferase (% control)</th>
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<tr>
<td>BxPC3</td>
<td>133 ± 7.9*</td>
<td>61 ± 5.4*</td>
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<tr>
<td>MPanc96</td>
<td>165 ± 10.6*</td>
<td>69 ± 2.7*</td>
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<tr>
<td>Panc-1</td>
<td>130 ± 9.1*</td>
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NOTE: Pancreatic cancer cells stably infected with NFκB-LUC reporter gene containing lentivirus (5,000 cells) were plated on 96-well plates and adrenomedullin (200 nmol/L) and AMA (1 μmol/L) were added to the cells; NFκB activity was determined at 4.5 h by bioluminescence imaging method. Data shown are mean ± SE for three experiments. *\( P < 0.05 \) versus control.

Figure 2. Exogenous adrenomedullin stimulates pancreatic cancer cell proliferation and invasion \textit{in vitro}. \( A \), adrenomedullin stimulates pancreatic cancer cell growth in a time-dependent manner. Wild-type Panc-1 cells (1,000 cells) were plated on 96-well plates and adrenomedullin (200 nmol/L) or AMA (1 μmol/L) was added to the cells and cell numbers were estimated at 0, 12, 24, 48, and 72 h by MTS assay. Points, mean for three experiments; bars, SE; *, \( P < 0.05 \) versus control. \( B \), adrenomedullin stimulates pancreatic cancer cell growth in a concentration-dependent manner. Wild-type Panc-1 cells (5,000 cells) were plated on 96-well plates and adrenomedullin (1–500 nmol/L) or AMA (1 μmol/L) was added to the cells and cell numbers were estimated at 48 h by MTS assay. Columns, mean for three experiments; bars, SE; *, \( P < 0.05 \) versus control. \( C \), adrenomedullin stimulates pancreatic cancer cell invasion in a concentration-dependent manner. Wild-type Panc-1 cells (20,000 cells) were added into Biocoat Matrigel invasion upper chamber and different concentrations of adrenomedullin (1–200 nmol/L) were added into the lower chamber, and after 12 h, noninvaded cells in the upper membrane were removed and cells invading into the lower membrane were quantified photometrically by adding MTS reagent 1 h before taking the reading. DMEM containing 0.5% serum was used as control. Columns, mean for three experiments; bars, SE; *, \( P < 0.05 \) versus control. \( D \), invading cells in three adjacent microscope fields for each membrane (original magnification, \( \times 20 \)).
Pancreatic cancer cell lines respond to adrenomedullin via the adrenomedullin receptor ADMR. In order to identify the receptor responsible for the autocrine effects of adrenomedullin on pancreatic cancer cells, RT-PCR was conducted for known adrenomedullin receptor mRNAs. Surprisingly, the well-known adrenomedullin receptor, CRLR, was not amplified from mRNA prepared from any of the pancreatic cancer cell lines. However, CRLR mRNA was easily amplified from cell types similar to those found in the tumor microenvironment, including endothelial cells (HUVEC) or HPSC (Fig. 6A). Despite the lack of CRLR mRNA, the mRNAs for the CRLR-associated binding proteins RAMP1, RAMP2, and RCP, but not RAMP3, were present in pancreatic cancer cell lines. All three RAMPs and RCP were present in HUVEC and HPSCs. In contrast to the lack of CRLR, all pancreatic cancer cell lines expressed mRNA for ADMR. This receptor was also present in both HUVEC and pancreatic stellate cells. The presence of the ADMR was confirmed in all of the examined pancreatic cancer cell lines using Western blotting (Fig. 6B).

To determine whether ADMR might be responsible for the observed stimulatory effects of adrenomedullin on pancreatic cancer cells, RT-PCR was conducted for known adrenomedullin receptor mRNAs. Surprisingly, the well-known adrenomedullin receptor, CRLR, was not amplified from mRNA prepared from any of the pancreatic cancer cell lines. However, CRLR mRNA was easily amplified from cell types similar to those found in the tumor microenvironment, including endothelial cells (HUVEC) or HPSC (Fig. 6A). Despite the lack of CRLR mRNA, the mRNAs for the CRLR-associated binding proteins RAMP1, RAMP2, and RCP, but not RAMP3, were present in pancreatic cancer cell lines. All three RAMPs and RCP were present in HUVEC and HPSCs. In contrast to the lack of CRLR, all pancreatic cancer cell lines expressed mRNA for ADMR. This receptor was also present in both HUVEC and pancreatic stellate cells. The presence of the ADMR was confirmed in all of the examined pancreatic cancer cell lines using Western blotting (Fig. 6B).

To determine whether ADMR might be responsible for the observed stimulatory effects of adrenomedullin on pancreatic cancer cells, ADMR was silenced using a lentiviral shRNA technique. MPanc96 cells stably silenced with ADMR shRNA showed a 95% reduction in the expression of ADMR when compared with that of control shRNA–bearing cells (Fig. 6C). Silencing of ADMR did not influence the cellular levels of adrenomedullin measured by ELISA (data not shown). Control shRNA–bearing cells showed a significant increase in adrenomedullin-stimulated growth, whereas silencing of ADMR significantly reduced the basal growth as well as the adrenomedullin-stimulated growth of pancreatic cancer cell lines at 48 h when compared with control shRNA (Table 2). Also, ADMR-silenced cell lines showed a significant reduction on adrenomedullin stimulated invasion. MPanc96 cell lines stably silencing ADMR showed a significant...
reduction in basal- and adrenomedullin-stimulated invasion (Fig. 6D). Panc-1 cell lines also showed a significant reduction in invasion when ADMR was silenced (data not shown).

Discussion

Pancreatic cancer is an oncological challenge with <4% 5-year survival rates. The mechanisms responsible for the aggressive nature of this cancer are not well understood. We previously observed that the peptide regulatory factor adrenomedullin was expressed in pancreatic cancer (6). In the current study, we further investigated the expression of adrenomedullin in pancreatic cancer and the potential role of this molecule as an autocrine factor in the disease. We observed that adrenomedullin was present in nearly all pancreatic tumors and cell lines and that adrenomedullin stimulated pancreatic cancer cell growth and invasion both in vitro and in vivo. Furthermore, an AMA was able to reduce the basal levels of pancreatic cancer cell proliferation and NFκB signaling in vitro. Genetic manipulations to increase or decrease adrenomedullin expression in pancreatic cancer cells resulted in increased or decreased proliferation and invasion in vitro and tumor growth and dissemination in vivo. Thus, our observations strongly suggest an involvement of adrenomedullin in the regulation of pancreatic cancer progression and indicate the presence of an autocrine component to this effect. These data support the further investigation of adrenomedullin as a potential therapeutic target in this deadly cancer.

We previously reported that adrenomedullin mRNA levels were ~4-fold higher in pancreatic adenocarcinoma compared with normal pancreas and chronic pancreatitis using microarray data analysis (6). In the current study, we extended this observation to a large number of samples and found the presence of adrenomedullin in the vast majority of pancreatic adenocarcinoma. Immunohistochemical assays localized adrenomedullin in pancreatic islets as expected. Adrenomedullin was also present in pancreatic cancer cells, but not in ductal or acinar cells from normal or chronic pancreatitis. We also detected adrenomedullin mRNA and protein in all pancreatic cancer cell lines that we examined. Furthermore, we were able to detect adrenomedullin in conditioned culture media from the pancreatic cancer cell lines, indicating that the pancreatic cancer cells secrete adrenomedullin. Thus, adrenomedullin is produced and secreted and therefore is able to interact with receptors in the extracellular space, including those on the cancer cells themselves as well as those on endothelial cells or stellate cells in the tumor microenvironment.

To examine the possibility of an autocrine effect of adrenomedullin on pancreatic cancer cells, we determined the effect of exogenous addition or genetic manipulation of adrenomedullin in vitro. We observed that adrenomedullin significantly increased pancreatic cancer cell proliferation, invasiveness, and NFκB activity. These data indicate that pancreatic cancer cells are able to respond to adrenomedullin in ways that would be expected to further the aggressiveness of pancreatic cancer. Importantly, we further found that inclusion of the adrenomedullin peptide antagonist AMA (AM 22-52) inhibited the basal levels of pancreatic cancer cell proliferation and invasion in vitro. These data indicate that inhibition of the interaction of pancreatic cancer cell–secreted adrenomedullin with cellular receptors reduces these cellular functions. Combined with the observation that the cells normally express and secrete adrenomedullin, these data strongly support the conclusion that adrenomedullin can act as an autocrine factor in pancreatic cancer.

Our conclusion that adrenomedullin can act as an autocrine factor in pancreatic cancer is in agreement with the observations...
made previously in other forms of cancer that adrenomedullin may act in this manner (19–21). However, our data showing that adrenomedullin is an autocrine regulator in pancreatic cancer is in disagreement with a previous report that did not find evidence for an autocrine role of adrenomedullin in pancreatic cancer cells (22). In that study, inclusion of AMA in tissue culture medium did not inhibit the proliferation of two pancreatic cancer cell lines, prompting the conclusion that it did not act in an autocrine manner. In contrast, we observed that AMA inhibited the proliferation, invasion, and basal NF-κB levels of several pancreatic cancer cell lines. The explanation for this difference may be a technical issue. We found that adrenomedullin and AMA needed to be added daily to the culture medium in order to observe significant effects, likely due to the short half-lives of these proteins in the tissue culture medium. Another possible explanation for the divergent results observed between the two studies is the use of different pancreatic cancer cell lines.

The current study did not directly address the role of adrenomedullin in the tumor microenvironment. The important role of adrenomedullin in angiogenesis in other cancers has been well established (26). Adrenomedullin signaling is of particular significance in endothelial cell biology because the peptide protects these cells from apoptosis (18), promotes angiogenesis (26), and affects vascular tone and permeability (27). Both adrenomedullin and its receptor (CRLR) are up-regulated under hypoxic conditions in microvascular endothelial cells (28). In pancreatic cancer, inhibition of adrenomedullin by direct intratumoral injection of AMA (22) or by intratumoral or i.m. injection of a vector expressing AMA (23) reduced tumor volume and vascularization. These data

<table>
<thead>
<tr>
<th>Control shRNA + adrenomedullin (% control)</th>
<th>ADMR shRNA (% control)</th>
<th>ADMR shRNA + adrenomedullin (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC3 143 ± 5.4* 76 ± 3.9* 72 ± 3.7*</td>
<td>MPanc96 175 ± 4.2* 80 ± 3.5* 80 ± 2.8*</td>
<td>Panc-1 223 ± 6.1* 71 ± 22* 70 ± 2.7*</td>
</tr>
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Table 2. Effect of silencing ADMR on basal- and adrenomedullin-stimulated pancreatic cancer cell proliferation in vitro

NOTE: Pancreatic cancer cells stably infected with control or ADMR shRNA (5,000 cells) were plated on 96-well plates and adrenomedullin (200 nmol/L) was added to the cells and cell growth was determined at 48 h by MTS assay. Data shown are mean ±SE for three experiments. *P < 0.05 versus control.

Figure 6. Different adrenomedullin receptors and binding proteins are expressed in pancreatic cancer cells and cells of the tumor microenvironment, and small interfering RNA can be used to silence ADMR in pancreatic cancer cells. A, RT-PCR expression of adrenomedullin receptors—ADMR and CRLR and its binding proteins RAMP1-3 and RCP in pancreatic cancer cell lines, in endothelial cells and in stellate cells. β-Actin served as a control. B, Western blot showing the expression of ADMR in pancreatic cancer cell lines. β-Actin served as control. C, cell lysates from MPanc96 cells stably silencing ADMR and control cells were prepared and Western blotting was conducted for ADMR. D, MPanc96 cells stably silencing ADMR and control cells were added into BioCoat Matrigel invasion upper chamber, whereas adrenomedullin (200 nmol/L) was added to the lower chamber and cells invading the lower chamber were estimated using MTS reagent. Columns, mean for three experiments; bars, SE; *, P < 0.05 versus control.
strongly indicate that adrenomedullin has an important role in pancreatic cancer angiogenesis. Adrenomedullin may also have an important action on cells of the tumor stroma. In human hepatic stellate cells, secretion of adrenomedullin and the existence of CRLR, RAMP1, and RAMP2 have been reported (29). The current study is the first to identify adrenomedullin receptors in pancreatic stellate cells. Thus, it is likely that adrenomedullin secretion by pancreatic cancer cells influences cancer cells themselves in an autocrine manner and the tumor microenvironment in a paracrine manner through effects on endothelial and stellate cells.

Among the biological actions of adrenomedullin on pancreatic cancer cells, we noted that adrenomedullin influenced the invasiveness of the pancreatic cancer cells in a concentration-dependent manner in vitro. Likewise, overexpression of adrenomedullin significantly increased, whereas silencing of adrenomedullin significantly decreased, invasion in vitro. Invasion is a critical component of metastasis (30). Our in vivo data further showed that adrenomedullin silencing reduced total pancreatic cancer dissemination, which included peritoneal invasion and metastasis to liver and lung. However, because adrenomedullin silencing had such profound effects on cancer cell proliferation, it is difficult to determine from the current studies whether there were independent effects on metastasis. We also observed a reduction in the numbers of metastatic foci after silencing adrenomedullin, which is less likely to be influenced by cell proliferation rates. Nevertheless, further studies will be necessary to fully understand the role of adrenomedullin in pancreatic cancer metastasis.

We also observed that adrenomedullin treatment of pancreatic cancer cells activated the transcription factor NFκB. NFκB is a key regulator of inflammation that also induces several antiapoptotic mechanisms (31). NFκB is constitutively active in pancreatic cancer (32) and has been considered an important component of resistance to cancer therapy in this disease (33, 34). Adrenomedullin has previously been reported to activate NFκB in some epithelial cells (35). In the current study, exogenous adrenomedullin stimulated pancreatic cancer cell NFκB activity. Furthermore, treatment of pancreatic cancer cells with an AMA led to a reduction of basal NFκB activity. These data suggest that an adrenomedullin autocrine loop contributes to basal NFκB activity in these cells. Thus, interference with adrenomedullin or adrenomedullin receptors in pancreatic cancer may reduce NFκB signaling and increase the sensitivity of pancreatic tumors to therapy. The potential role of adrenomedullin in the chemoresistance of pancreatic cancer remains to be explored.

Adrenomedullin acts through at least two subtypes of G protein–coupled receptors, CRLR (36) and ADMR (10). CRLR functions as a calcitonin gene–related peptide receptor or an adrenomedullin receptor depending on the expression of specific receptor activity–modifying proteins (RAMP). In combination with RAMP1, CRLR has the highest affinity for calcitonin gene–related peptides, and in combination with RAMP2 or RAMP3, it prefers adrenomedullin (37). The levels of various adrenomedullin receptors differ depending on the cell type. For example, in human adrenal cortex and aldosteronomas, ADMR mRNA is present in high levels when compared with CRLR and the three RAMPs (36). Previously, it has been suggested that CRLR is the main mediator of the effects of adrenomedullin on the vasculature (38). In the current study, we did not detect mRNA for CRLR in any of the pancreatic cancer cell lines despite the presence of mRNA for RAMP1/2 and another binding protein of CRLR, RCP (38). Therefore, it is unlikely that the autocrine effects of adrenomedullin in pancreatic cancer are mediated by CRLR. In contrast to the lack of CRLR, ADMR was present on all of the pancreatic cancer cell lines. Furthermore, silencing of ADMR inhibited basal as well as adrenomedullin-stimulated pancreatic cancer cell proliferation and invasion. These data strongly suggest that the autocrine effects of adrenomedullin are mediated through ADMR on pancreatic cancer cells.

Although not the focus of our study, we also evaluated the presence of adrenomedullin receptors on important cells in the tumor microenvironment. We confirmed findings from previous studies which indicated that endothelial cells express both CRLR and ADMR (28, 39). We also showed for the first time that HPSCs express these receptors. Thus, adrenomedullin could act via either receptor type on cells of the tumor microenvironment. The importance of CRLR for the effects of adrenomedullin on endothelial cells has previously been reported (28). It is not known whether adrenomedullin regulates pancreatic stellate cell function. However, adrenomedullin has been suggested as a regulator of the related hepatic stellate cell (29). Clearly, further studies to determine the effects of adrenomedullin and the relative contribution of ADMR and CRLR receptors on pancreatic stellate cells are warranted.

In summary, this study describes the presence and important functional roles of adrenomedullin as an autocrine factor that increases the aggressive characteristics of pancreatic cancer. Furthermore, the ADMR receptor was identified as being critical for these effects. These observations add significant new insight into the multifaceted role of adrenomedullin in pancreatic cancer. Combined with the known importance of adrenomedullin as an angiogenic factor, these data support the further investigation of adrenomedullin and/or its receptor (ADMR) as new and potentially important targets for pancreatic cancer therapy.

Acknowledgments

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References


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Adrenomedullin Is Expressed in Pancreatic Cancer and Stimulates Cell Proliferation and Invasion in an Autocrine Manner via the Adrenomedullin Receptor, ADMR

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