Pancreatic Cancer-Derived Exosomes Cause Paraneoplastic β-cell Dysfunction

Naureen Javeed¹, Gunisha Sagar¹, Shamit K. Dutta¹, Thomas C. Smyrk², Julie S. Lau¹, Santana Bhattacharya¹, Mark Truty², Gloria M. Petersen⁴, Randal J. Kaufman⁵, Suresh T. Chari⁶, and Debabrata Mukhopadhyay¹

Abstract

**Purpose:** Pancreatic cancer frequently causes diabetes. We recently proposed adrenomedullin as a candidate mediator of pancreatic β-cell dysfunction in pancreatic cancer. How pancreatic cancer–derived adrenomedullin reaches β cells remote from the cancer to induce β-cell dysfunction is unknown. We tested a novel hypothesis that pancreatic cancer sheds adrenomedullin-containing exosomes into circulation, which are transported to β cells and impair insulin secretion.

**Experimental Methods:** We characterized exosomes from conditioned media of pancreatic cancer cell lines (n = 5) and portal/peripheral venous blood of patients with pancreatic cancer (n = 20). Western blot analysis showed the presence of adrenomedullin in pancreatic cancer-exosomes. We determined the effect of adrenomedullin-containing pancreatic cancer exosomes on insulin secretion from INS-1 β cells and human islets, and demonstrated the mechanism of exosome internalization into β cells. We studied the interaction between β-cell adrenomedullin receptors and adrenomedullin present in pancreatic cancer-exosomes. In addition, the effect of adrenomedullin on endoplasmic reticulum (ER) stress response genes and reactive oxygen/nitrogen species generation in β cells was shown.

**Results:** Exosomes were found to be the predominant extracellular vesicles secreted by pancreatic cancer into culture media and patient plasma. Pancreatic cancer-exosomes contained adrenomedullin and CA19-9, readily entered β cells through caveolin-mediated endocytosis or macropinocytosis, and inhibited insulin secretion. Adrenomedullin in pancreatic cancer exosomes interacted with its receptor on β cells. Adrenomedullin receptor blockade abrogated the inhibitory effect of exosomes on insulin secretion. β cells exposed to adrenomedullin or pancreatic cancer exosomes showed upregulation of ER stress genes and increased reactive oxygen/nitrogen species.

**Conclusions:** Pancreatic cancer causes paraneoplastic β-cell dysfunction by shedding adrenomedullin /CA19-9⁺ exosomes into circulation that inhibit insulin secretion, likely through adrenomedullin-induced ER stress and failure of the unfolded protein response. Clin Cancer Res; 21(7): 1–12. ©2014 AACR.

See related commentary by Korc, p. 1508

Introduction

Nearly 85% of pancreatic cancer patients have hyperglycemia and the majority (45%-67%) have diabetes mellitus (1, 2), which is frequently new onset (75%), i.e., less than 36 months in duration (1, 3, 4). The mechanism of pancreatic cancer–induced diabetes mellitus (PC-DM) remains unclear. Understanding the pathogenesis and mediators of PC-DM could lead to identification of novel biomarkers for early diagnosis of the cancer (5, 6).

Although the American Diabetes Association classifies PC-DM as a form of pancreatogenous diabetes mellitus (7), its mechanism remains unclear. PC-DM and type II diabetes mellitus share a number of features including risk factors (1), presence of marked insulin resistance, and high plasma insulin levels (1, 2, 8, 10). These features also distinguish PC-DM from chronic pancreatitis-related diabetes mellitus which is associated with decreased insulin levels secondary to low β-cell mass. Unlike type II diabetes mellitus, which is ameliorated by weight loss, PC-DM develops in the face of ongoing, often profound, weight loss (11). Finally, in pancreatic cancer, new-onset diabetes mellitus paradoxically resolves in more than 50% of patients after pancreatico-duodenectomy (1). Amyloid deposits in islets, a histologic hallmark of type II diabetes mellitus and a major contributor to β-cell dysfunction and loss (12–14) in type II diabetes mellitus, are absent in PC-DM (15, 16).

Thus, PC-DM appears to be distinct from both type II diabetes mellitus and other forms of pancreatogenous diabetes mellitus.

The very high prevalence of new-onset diabetes mellitus in pancreatic cancer suggests a pancreatic cancer–specific mediator that dysregulates glucose homeostasis (6). We have previously shown that pancreatic cancer cell line–conditioned media induce
Translational Relevance

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and is projected to move to the second position by the year 2015. Pancreatic cancer has a dismal prognosis (~5% 5-year survival) mainly because 85% are diagnosed when the cancer is already at an advanced stage. New development of diabetes is the only harbinger of pancreatic cancer before cancer symptoms occur as it develops in nearly 50% of patients with pancreatic cancer an average of 12 months before the development of cancer symptoms. Our previous work strongly suggests that the new diabetes associated with pancreatic cancer is caused by cancer-secreted factors. In this current study, we looked at pancreatic cancer exosomes as mediators of β-cell dysfunction, which can help to understand the mechanism and mediators of pancreatic cancer-induced diabetes, leading to early diagnosis of the cancer.

β-cell dysfunction in both the rat insulinoma β-cell line (INS-1) and mouse islets (17). In previous studies, glucose intolerance could be induced in mice homozygous for the scid mutation by repeated intraperitoneal injection of pancreatic cancer–conditioned media (18) or by xenografting human pancreatic cancer (17). The weight of epidemiologic, clinical, and laboratory evidence suggests that PC-DM is a paraneoplastic phenomenon caused by tumor-secreted factors (6).

In recent years, membrane-derived extracellular vesicles have emerged as important conduits for cell-to-cell communication (19). Both normal and malignant cells shed microparticles of varying sizes into the surrounding extracellular space (20). Extracellular vesicles contain components of the cells’ membrane and cytoplasm, which affect recipient cells by transferring their biologically active cargoes (20). Extracellular vesicles are produced by at least three distinct mechanisms: inward or reverse budding of multivesicular bodies leading to formation of nanoparticle-sized (30–100 nm) exosomes, outward membrane blebbing producing large (500–2,000 nm) apoptotic bodies (21). The morphologic and functional characteristics of extracellular vesicles shed by pancreatic cancer have not been previously delineated. Here, we show that the majority of extracellular vesicles isolated from pancreatic cancer cell line–conditioned media and in the plasma of patients with pancreatic cancer are exosomes. This finding led us to study the in vitro biologic action of exosomes shed by pancreatic cancer on insulin secretion. We hypothesize that β-cell dysfunction in pancreatic cancer is mediated by exosomes carrying β-cell toxic cargo that are shed into blood circulation.

Microarray analysis of commercially available cell lines that caused β-cell dysfunction led us to identify adrenomedullin as a candidate mediator of PC-DM (17). Adrenomedullin is the most highly expressed gene in pancreatic cancer cell lines grown under harsh conditions of low glucose and low oxygen (22). Although this ubiquitous polypeptide inhibits insulin secretion, adrenomedullin has no known physiologic role in glucose metabolism (23). We showed that inhibition of insulin secretion by conditioned media from pancreatic cancer cell lines is abrogated by inhibiting adrenomedullin expression using shRNA (17). However, how adrenomedullin is transported from pancreatic cancer to β cells and the molecular mechanism of adrenomedullin-induced β-cell dysfunction are unknown.

In this study, we show that the predominant extracellular vesicles shed by pancreatic cancer, not only in conditioned media but also in peripheral and portal venous blood of patients, are exosomes. We show that pancreatic cancer exosomes (PC-Exo), containing adrenomedullin and CA19-9, readily enter INS-1 β cells and human islets by both caveolin-mediated endocytosis and macropinocytosis, and inhibit insulin secretion. We demonstrate that pancreatic cancer exosomal adrenomedullin interacts with adrenomedullin receptors [ADMR; calcitonin receptor–like receptors (CRLRs)] on β cells with the inhibitory effect of PC-Exo on insulin secretion being reversed by adrenomedullin receptor blockade. Finally, we show that adrenomedullin causes upregulation of genes associated with endoplasmic reticular (ER) stress (Bip, Chop) which promotes increased ROS/RNS production, due to failure of the unfolded protein response (UPR) leading to increased β-cell death. Adrenomedullin receptor blockade abolished these effects. Taken together, our studies provide insight into a novel molecular mechanism of paraneoplastic diabetes in pancreatic cancer.

Materials and Methods

Studies pertaining to human subjects, including the study of exosomes in human plasma, were approved by the Mayo Clinic Institutional Review Board.

Cell culture

INS-1 cells were cultured in RPMI medium containing 10% FBS, 10 mmol/L HEPES, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, and 50 μmol/L β-mercaptoethanol. Primary pancreatic cancer patient–derived xenograft cell lines developed in the Mukhopadhyay laboratory were cultured in DMEM/F-12 medium supplemented with 10% FBS and 1% antibiotic–antimycotic solution (Life Technologies) on plates coated with rat tail collagen type I. Whole human islets purchased from Prodo Laboratories were cultured for 48 hours in islet complete medium (Prodo Laboratories) before studies on glucose responsiveness.

Isolation of exosomes

Exosomes were isolated by differential centrifugation of conditioned media collected from human umbilical vein endothelial cells (HUVEC), human pancreatic ductal epithelial (HPDE), Panc-1, primary patient-derived xenograft cell lines, or human plasma. Cells were grown in their respective media to 70% to 80% confluency. The medium was then replaced with medium containing FBS deprived of microparticles by differential centrifugation (60 minutes at 100,000 × g). After a 72-hour incubation, the conditioned media were initially cleared of cellular debris/dead cells with two sequential spins at 3,000 rpm for 10 minutes at 4°C. The resulting supernatants were then spun at 100,000 × g for 70 minutes at 4°C. The exosome pellet was washed with 1× PBS solution and centrifuged again at 100,000 × g for 70 minutes. The final exosome pellet was resuspended in 1× PBS. To isolate exosomes from peripheral or portal blood, samples were centrifuged at 3,000 rpm for 10 minutes to separate the plasma from the red blood cells. The collected plasma was centrifuged again at 3,000 rpm for 10 minutes and then isolated with high-speed centrifugation, as described above. The resulting exosome pellet was washed and resuspended as described. Protein quantification of exosome preparations was determined by the Bicinchoninic Acid Assay (Pierce).
NanoTracker analysis

Isolated exosome fractions from pancreatic cancer cell lines and pancreatic cancer patient blood plasma were analyzed on the NanoSight NS300. Fractions were diluted accordingly and 5 60-second movies were taken for each sample. Analysis of the data was done using the software supplied with the machine. Graphical analysis shows particle size distribution of the microparticles in each fraction, and a concentration was reported as particles per milliliter.

Western blot analysis

Exosomes were isolated by the procedure mentioned above from HUVEC, HPDE, PANC-1, four primary pancreatic cancer cell lines, and peripheral and portal venous pancreatic cancer blood samples. After isolation, exosomes were quantified by Bicinchoninic Acid Assay (Pierce), and tergitol-type NP-40 (Boston BioProducts) was added to 20 μg of exosomes from each preparation. Samples were run on a Western blot according to standard protocols, and probed with CA19-9 antibody (Abcam), or adrenomedullin antibody (Phoenix Pharmaceuticals) and either Alix (Cell Signaling Technology) or TSG101 (Abcam) to detect exosomes in the fractions.

For insulin and proinsulin Western blotting, INS-1 cells were grown in a 6-well plate to approximately 70% confluency. The media were replaced with media containing varying concentrations of adrenomedullin peptide and incubated for 48 hours. Cells were glucose stimulated for 4 to 6 hours and cell lysates were isolated using Tergitol-type NP-40 (Boston BioProducts). Twenty micrograms of protein was loaded and blots were probed with the insulin antibody (Santa Cruz Biotechnology Inc.) or proinsulin antibody (Novus Biologicals) with β-actin (Sigma-Aldrich) as the loading control.

Electron microscopic imaging of exosomes

Exosomes suspended in water from the PANC-1 cell line were resuspended in Trump fixative solution [4% formaldehyde: 1% glutaraldehyde: 0.1 M cacodylate buffer: 1% NaN₃] and 0.5% uranyl acetate. Samples were stained with 5% uranyl acetate. Stained samples were imaged using a Zeiss Libra 200 microscope (100 kV). Scale bar, 200 nm.
glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2). Multiple rinses occurred in this sequence: 0.1 mol/L sodium phosphate buffer (pH 7.2), 1% osmium tetroxide in 0.1 mol/L sodium phosphate buffer, distilled water, 2% uranyl acetate, distilled water, ethanol, and 100% acetone. Exosomes were then placed on a transmission electron microscopy grid, and images were taken using a Philips Tecnai T12 transmission electron microscope.

Exosome internalization and confocal analysis
Exosomes were isolated from the PANC-1 cell line and dyed with green fluorescent linker PKH67 (Sigma-Aldrich) according to the manufacturer’s protocol. Fifty micrograms of PKH67-dyed exosomes were incubated with cultured human islets in a 35-mm culture dish (culture conditions as described above). Confocal microscopy images were obtained after 24 and 48 hours using a Zeiss LSM 780 confocal microscope.

Insulin secretion assay
INS-1 or human islet medium was replaced with microparticle-free medium containing no glucose. Fifty micrograms of exosomes were incubated with the cells for 24 hours to allow uptake. Cells were glucose stimulated with 15 mmol/L glucose (INS-1) or 16.7 mmol/L glucose (islets) for 4 to 6 hours. For the dose–response curve, varying amounts of PC-Exo were incubated with INS-1 cells for 24 hours. Cells were stimulated with 15 mmol/L glucose for 4 to 6 hours. The supernatants were collected and assayed using a rat insulin ELISA Kit (Crystal Chem), and insulin values were normalized to total protein content. Protein samples were quantified using the Bicinchoninic Acid Assay (Pierce).

Duolink assay system
INS-1 cells were treated with varying amounts of PKH67-dyed (as per the manufacturer’s protocol; Sigma-Aldrich) PC-Exo or PC-Exo with 10 nmol/L adrenomedullin inhibitor (AM 22-52; AnaSpec, Inc.) for 24 hours. Cells were washed with 1× PBS and fixed with 4% paraformaldehyde. Duolink in situ kits were purchased from Olink Biosciences. Antibodies for adrenomedullin (Phoenix Pharmaceuticals) and CRLR (Santa Cruz Biotechnology, Inc.) were used for assessing adrenomedullin/ADMR interactions. Antibodies for Bip (Abcam) and proinsulin (Novus Biologicals) were used to detect Bip/proinsulin in the presence of increasing PC-Exo. Conjugation of proximity ligation assay (PLA) probes, rolling circle amplification, and detection procedures were done according to the manufacturer’s instructions. Cells were mounted

Figure 2.
PC-Exo decrease insulin secretion with the effect abrogated by ADMR blockade. Glucose-stimulated insulin secretion in INS-1 cells (A, C–E) and human islets (B) coincubated with PC-Exo (+Exo) compared with control culture medium (−Exo), PC-Exo with ADMR inhibitor (+Exo + adrenomedullin (AM) inhib) and pancreatic cancer–conditioned medium depleted of exosomes (Exo-free medium). Exosomes were isolated from a pancreatic cancer patient–derived cell line (A and B), PANC-1(C), and plasma from two pancreatic cancer patients (D and E). F, INS-1 cell response to insulin secretion by PC-Exo in a dose-dependent manner.

Adapted from: Javeed et al. 2015, Clinical Cancer Research.
using 4',6-diamidino-2-phenylindole (DAPI)-containing mounting media (Vector Labs). Images were taken on a Zeiss LSM 780 confocal microscope.

Pharmacologic inhibition and quantification of exosome internalization
INS-1 cells were grown in 4-well chamber slides (800,000 cells/well) with optimal treatment concentrations and timings adapted from Bhattacharya and colleagues (ref. 24; Supplementary Table S1). After treatment, cells were washed three times with 1× PBS and MV-free media were added to the cells with the addition of 5 μL PKH67-dyed PANC-1 exosomes. Exosomes were incubated with cells for 5 hours to allow internalization, then washed with 1× PBS two times, fixed with 4% PFA, and mounted with DAPI-containing mounting media (Vector Labs). Images were taken using a Zeiss LSM 780 confocal microscope. Quantification of green fluorescence (exosome) internalization was performed using KS400 Image Analysis software (Zeiss). For each image, the nuclei were counted and the total green fluorescence for the entire image was divided by the nuclei (cell) count to obtain green fluorescence per cell.

Real-time PCR
INS-1 cells were treated with varying concentrations of adrenomedullin peptide (Phoenix Pharmaceuticals) or PC-Exo (50 μg) for 24 or 48 hours. Total RNA was isolated using the RNeasy Kit (Qiagen), and cDNA was reverse transcribed from 1 μg of RNA using the iScript cDNA kit (Bio-Rad). RT-PCR analysis was performed using the ABI 7500 Real-Time PCR System and the SYBR Green Master Mix (Applied Biosystems). Primers for insulin and ER stress markers (Bip, Chop, and Xbp-1s) were adapted from Lipson and colleagues (25).

Annexin V staining
INS-1 cells were plated on a 6-well plate (4 × 10^6 cells/well) in microparticle-free medium. Fifty micrograms of PC-Exo were incubated with the cells for 48 hours. Cells were trypsinized, collected, and resuspended in 1× binding buffer supplied with the Annexin V-FITC Apoptosis kit (BioVision, Inc). Annexin V-propidium iodide staining was completed according to the manufacturer's protocol. Flow cytometry analysis was completed to detect early and late apoptotic cells.

Cellular ROS/superoxide detection
INS-1 cells were seeded in 4-well chamber slides (800,000 cells/well). Cells were pretreated with 1 or 20 pmol/L adrenomedullin peptide for 24 hours, then glucose stimulated for 4 hours with 15 mmol/L glucose. Cellular ROS/Superoxide Detection Assay Kit (Abcam) was used for ROS/RNS detection. Treatment of cells for detection was adapted from this protocol including for the positive and negative controls. Cells were then washed in 1× wash buffer (supplied with kit), and mounted with DAPI-containing mounting media. Images were obtained using a Zeiss LSM 780 confocal microscope.

Statistical analysis
Statistical comparisons between 2 groups were performed using a two-sample t test. Comparisons across more than two groups were performed using ANOVA with a Dunnett posttest to compare all columns to the control. All analyses were conducted
using GraphPad software. *P* values less than 0.05 were considered statistically significant.

**Results**

**Extracellular vesicles in pancreatic cancer are predominantly exosomes**

We isolated all microparticles (microvesicles and exosomes) shed by pancreatic cancer using differential centrifugation. With the NANO-SIGHT NS300 system (Malvern Instruments Ltd), we determined that the predominant microparticles in pancreatic cancer cell lines and patient plasma were of exosomal size (modes, ~100 nm; Fig. 1A; Supplementary Fig. S1). However, this did not hold true for all pancreatic cancer cell lines, as exemplified in Supplementary Fig. S1B, where the majority of the microparticles were of microvesicular size. In addition, scanning electron microscopy revealed that microparticles from PANC-1–conditioned medium are of exosomal size and morphology, thereby confirming the NanoTracker results (Fig. 1E).

**PC-Exo readily enter human islets**

Human islets were incubated with PKH67-dyed exosomes isolated from the PANC-1 cell line. Using confocal microscopy, we identified PKH67-dyed exosome internalization into islets within 48 hours of coincubation (Fig. 1F).

**Exosomes from pancreatic cancer–conditioned media and plasma of pancreatic cancer patients contain CA19-9 and adrenomedullin**

Western blot analysis revealed the presence of CA19-9 in all pancreatic cancer patient exosome samples (*n* = 11) with TSG101 serving as a marker of exosomes (Fig. 1B; five representative samples). In contrast, exosomes isolated from normal pancreatic cell lines HPDE and HUVEC showed no CA19-9 and TSG101 expression (Fig. 1B). This suggests that the content and amount of normal exosomes being produced differ in comparison with tumor-derived exosomes and that isolated exosome fractions from pancreatic cancer patient plasma are in fact derived from the pancreatic tumor. We next looked for the presence of adrenomedullin through Western blotting in exosomes shed by HPDE (control cell line), PANC-1, and 4 pancreatic cancer patient–derived xenograft cell lines. All revealed the presence of adrenomedullin; however, exosomes from the HPDE cell line may be produced less because of the lack of TSG101 expression found in 20 μg of exosomes (Fig. 1C). Western blotting of exosomes isolated from peripheral (*n* = 18) and portal veins (*n* = 2) blood of 20 patients with pancreatic cancer showed the presence of adrenomedullin (Fig. 1D; representative samples shown). Although exosome bioavailability and *t*½/2 studies were not conducted to determine the rate of PC-Exo uptake in peripheral organs, this result suggests that a steady production of PC-Exo is secreted from the tumor into circulation which contains metabolically active cargoes.

**PC-Exo inhibit insulin secretion**

In a recent study, we reported that conditioned media from various pancreatic cancer cell lines decreased insulin secretion in both a rat INS-1 β-cell line and mouse islets (17). We hypothesized that the inhibitory effect of conditioned media resided in exosomes. The addition of PC-Exo from a patient–derived pancreatic cancer cell line showed a decrease in insulin secretion in both INS-1 cells and human islets (Fig. 2A and B). In addition, PC-Exo isolated from the PANC-1 cell line and 2 pancreatic cancer patient plasma samples showed a similar effect of decreasing insulin secretion from β cells (Fig. 2C–E). Adrenomedullin receptor abrogation blocked the insulin inhibitory effect of PC-Exo with values exceeding past the baseline due to the blocking of endogenous (free) adrenomedullin to ADMRs (Fig. 2A–E). In addition, exosome-depleted media had no effect on insulin secretion, confirming that the inhibitory effect is caused by exosomes and not any other soluble factor(s) secreted into the media (Fig. 2A–C). In addition, this insulin inhibitory effect was seen in PC-Exo isolated from additional pancreatic cancer cell lines and plasma of patients with pancreatic cancer (Supplementary Fig. S2). Finally, increasing concentrations of PC-Exo isolated from pancreatic cancer patient plasma were incubated with INS-1 cells for 48 hours. The results showed that PC-Exo can inhibit insulin secretion in a dose-dependent manner (Fig. 2F).

**PC-Exosomal adrenomedullin binds to cell surface receptors on β cells to inhibit insulin secretion**

The Duolink Assay System (Olink Biosciences) was used to assess the interaction between exosomal adrenomedullin and its receptor ADMR. This method allows for determination of protein–protein interactions by using specialized fluorescent PLA probes that attach to the primary antibodies for adrenomedullin and the ADMR. ADMRs require activation of both co-receptors CRLR and either RAMP 2 or 3; therefore, we chose to use a primary antibody for CRLR as this co-receptor is consistently activated due to the blocking of endogenous (free) adrenomedullin and the ADMR. ADMRs require activation of both co-receptors CRLR and either RAMP 2 or 3; therefore, we chose to use a primary antibody for CRLR as this co-receptor is consistently activated each time there is an adrenomedullin/ADMR interaction. Rolling circle amplification amplifies this region, and punctate dots indicate ligand–receptor interactions. INS-1 cells were incubated for 24 hours with increasing PKH67-dyed PC-Exo. Confocal microscopy was used to visualize adrenomedullin/ADMR interactions with increasing PC-Exo as indicated by the diffuse cytoplasmic red dots (Fig. 3A). In addition, quantification of the interactions revealed an overall increase in adrenomedullin/ADMR interactions with increasing addition of PC-Exo (data not shown). Conversely, ADMR inhibition with AM 22-52 in the presence of PC-Exo decreased adrenomedullin/ADMR interactions, as indicated by less punctate red staining (Fig. 3B). These data indicate that PC-Exo containing adrenomedullin are able to shuttle the peptide efficiently to recipient cells and that competitively inhibiting adrenomedullin in the presence of PC-Exo results in less adrenomedullin/ADMR interaction.

**Exosome internalization is mediated through caveolin-dependent endocytosis and macropinocytosis**

The results from the Duolink Assay suggest that adrenomedullin/ADMR interactions occur intracellularly as indicated by the diffuse red cytoplasmic dots (Fig. 3). We sought to gain an understanding of the mechanism of exosome entry and subsequent adrenomedullin delivery to the cell. Pharmacologic inhibition of various endocytic mechanisms was used to determine the exact method of exosome internalization. Chlorpromazine (clathrin inhibitor), nystatin (caveolin inhibitor), amiloride (inhibitor of macropinocytosis), and nocodazole (inhibitor of endosomal trafficking) were tested in INS-1 cells using the concentrations and incubation timings listed in Supplementary Table S1. The internalization of PKH67-dyed PC-Exo (green) was significantly inhibited with amiloride and nystatin treatments as indicated by less green fluorescence (Fig. 4A and D),
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Control

25 μmol/L amiloride

50 μmol/L amiloride

100 μmol/L amiloride

Control

25 μg/mL nystatin

50 μg/mL nystatin

Control 100 μmol/L amiloride

Control 50 μg/mL nystatin

Green fluorescence/cell

PKH67

DAPI

Merge

A

B

C

D

E

F

Green fluorescence/cell

Pancreatic Cancer Exosomes Cause Paraneoplastic Diabetes

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Quantiﬁcation of green ﬂuorescence/cell showed a 42.5% decrease in internalized exosomes with the addition of 100 μmol/L amiloride, and 20.5% and 59.5% decreases in exosome internalization with 25 μg/mL and 50 μg/mL nystatin, respectively (Fig. 4C and F). Conversely, varying treatment concentrations of chlorpromazine and nocodazole did not have an inhibitory effect on internalizing PC-Exo (Supplementary Fig. S3). Taken together, these data show that PC-Exo internalize into INS-1 cells through both caveolin-dependent endocytosis and macropinocytosis.

Adrenomedullin deregulates the UPR pathway leading to β-cell dysfunction

As in many other target cells, adrenomedullin has been reported to cause an increase in cAMP levels in islets (26) which is normally associated with increased insulin secretion (27–29). This is mediated through increases in insulin exocytosis as well as insulin production (30). Indeed, we found a modest increase in insulin mRNA in response to adrenomedullin (Supplementary Fig. S4). ER stress is a physiologic β-cell response to increased insulin production, while an efﬁcient UPR safely resolves ER stress resulting from a failed UPR. However, the question arises whether the paradoxical inhibition of insulin secretion by adrenomedullin, despite an increase in cAMP, could be due to an independent adrenomedullin-induced unresolved ER stress resulting from a failed UPR.

Classical characteristics of a failed UPR response are increased Bip (ER chaperone protein) coupling to proinsulin in the ER, and activation of Chop (inducer of apoptosis; ref. 31). To assess whether the presence of adrenomedullin in the cell can activate these ER stress markers, we looked at Bip and Chop gene expressions in the presence of adrenomedullin. INS-1 cells were treated with varying concentrations of the adrenomedullin peptide for 48 hours and then glucose stimulated for 4 to 6 hours. Real-time (RT)–PCR analysis showed upregulation of ER stress genes Bip and Chop, with only modest upregulation of Xbp-1s (ER stress marker involved in the normal UPR) with the addition of increasing concentrations of adrenomedullin (Fig. 5A–C). The addition of the adrenomedullin inhibitor (AM 22-52) with varying concentrations of adrenomedullin peptide caused mRNA levels of all three genes to subside to endogenous levels (Fig. 5A–C). The modest increase in Xbp-1s suggests that normal UPR function may be activated slightly in the presence of adrenomedullin; however, the evident upregulation of Bip and Chop in the presence of adrenomedullin suggests failure of this response. In addition, 50 μg of PC-Exo increased both markers (Fig. 5D and E). As Chop mRNA levels were elevated, annexin V staining was done to determine cellular apoptosis. Fifty micrometers of PC-Exo were incubated with INS-1 cells for 48 hours; a higher percentage of cells were observed undergoing early apoptosis compared with the control (untreated) cells (Fig. 5F).

Another deﬁning characteristic of a failed UPR in β cells is the increased coupling of proinsulin to Bip in the ER (31). Proinsulin protein levels were assessed in INS-1 cells with the addition of varying concentrations of adrenomedullin. Our results showed increased proinsulin in the presence of increasing adrenomedullin (Fig. 5G). In addition, the DuoLink Assay System (as previously noted) was utilized to assess the Bip/proinsulin interactions in situ with increasing amounts of PC-Exo. Indeed, INS-1 cells incubated with increasing amounts of adrenomedullin-containing PC-Exo showed increasing Bip/proinsulin interactions as indicated by increased red punctate dots (Fig. 6A).

Overwhelming ER stress can also induce oxidative stress (31). We therefore examined whether adrenomedullin can invoke reactive oxygen/nitrogen species (ROS/RNS) production in INS-1 cells. With the addition of 1 and 20 pmol/L adrenomedullin, ROS (green ﬁlter) and superoxide (orange ﬁlter) production increased compared with control (untreated) cells (Supplementary Fig. S5). This collective body of evidence suggests that adrenomedullin and PC-Exo containing adrenomedullin is capable of deregulating the UPR pathway, which inevitably leads to marked β-cell dysfunction/death.

**Discussion**

Compelling clinical, epidemiologic, and experimental evidence strongly supports the notion that new-onset diabetes mellitus in pancreatic cancer is a paraneoplastic phenomenon caused by tumor-secreted products (7). We observed that pancreatic cancer (PC) sheds nanoparticle-sized exosomes that are abundantly present in pancreatic cancer–conditioned media and in portal and peripheral venous blood of patients with pancreatic cancer. There is growing evidence that exosomes are important mediators of cell–cell communication (20). Their ability to inﬂuence adjacent cells and stroma is now increasingly recognized as a critical cellular function. We observed that the PC-Exo derived from in vitro and in vivo sources were morphologically and functionally indistinguishable and exosomes from both sources readily entered β cells and inhibited insulin secretion. We also show that the inhibitory effect of pancreatic cancer cell line conditioned media on insulin secretion is mediated by exosomes as exosome-free media had no effect on insulin secretion. Thus, our studies not only show that exosomes are the predominant extracellular vesicles shed by pancreatic cancer, both into culture media and plasma, but also demonstrate their ability to dysregulate insulin secretion.

We have previously proposed adrenomedullin as a candidate mediator of β-cell dysfunction in pancreatic cancer as it is overexpressed in pancreatic cancer and inhibits insulin secretion (17). We have also reported increased plasma adrenomedullin concentrations in patients with PC-DM (22.9 ± 10.7 fmol/L) compared with patients with pancreatic cancer but normal fasting glucose (18.3 ± 7.0 fmol/L), noncancer patients with new-onset type II diabetes (14.8 ± 10.7 fmol/L), and noncancer subjects with normal fasting glucose levels (12.9 ± 6.6 fmol/L; ref. 17). However, because plasma concentrations were only modestly elevated this raises the...
question of whether adrenomedullin is acting as a hormone. Our current work suggests that adrenomedullin is carried from pancreatic cancer to β cells as exosomal cargo. After Kitamura and colleagues first reported isolating adrenomedullin from human pheochromocytoma in 1993 (32), adrenomedullin was proposed to be a circulating hormone regulating systemic and pulmonary blood pressure (33). In healthy individuals, circulating adrenomedullin levels are in the low picomolar range. Physiologic increases in adrenomedullin levels are seen during pregnancy, with the source being the placenta (34). Pathologic conditions associated with increased adrenomedullin levels include diabetes, severe hypertension, chronic renal failure, heart failure, pulmonary hypertension, subarachnoid hemorrhage, sepsis, hyperthyroidism, and during cardiac surgery.

It has been debated whether these elevations simply reflect excess tissue adrenomedullin production or whether excess...
circulating adrenomedullin has systemic biologic effects. However, infusion of adrenomedullin into humans (35) at a rate that quadrupled physiologic levels of adrenomedullin had no effect on heart rate and blood pressure. Extremely high doses that achieved a concentration over 40 times normal circulating adrenomedullin levels did significantly decrease blood pressure (35). In another study, high dose adrenomedullin infusion affected hemodynamic parameters, but not low-dose adrenomedullin infusion (36). Thus, the circulating concentration of adrenomedullin necessary to affect blood pressure greatly exceeds that observed in healthy volunteers and in patients with a range of physiologic (notably pregnancy) and pathologic (notably cancer, heart failure, vasculopathies) conditions.

In relation to our work, neither low-dose nor high-dose adrenomedullin infusion had any effect on blood glucose (35). Thus, the elevation of plasma adrenomedullin is unlikely to be the cause of the biologic effects of adrenomedullin in pancreatic cancer. We are not aware of any mechanism for intrapancreatic transport of molecules through the length of the pancreas to explain global β-cell dysfunction leading to diabetes mellitus in pancreatic cancer. Therefore, we studied a novel hypothesis that adrenomedullin was being transported from pancreatic cancer to islets distant from the cancer in exosomes shed into portal circulation and eventually reaching the islets through systemic circulation.

In PC-DM, there are both β-cell dysfunction and increased insulin resistance that resolve with tumor resection (1, 4, 8, 18, 38). Insulin levels in pancreatic cancer are high due to insulin resistance. Our data suggest that β-cell dysfunction leads to inadequate insulin response to insulin resistance. We believe it is this dual hit that promotes a consistent destabilization of glucose homeostasis in pancreatic cancer, resulting in hyperglycemia. Thus far, the mechanism and mediators of insulin resistance in pancreatic cancer remain unknown, and the role of PC-Exo, adrenomedullin, and other factors contributing to insulin resistance needs further study.

Through the use of the Duolink Assay System, we showed that adrenomedullin contained in PC-Exo can interact with ADMRs (CRLR) intracellularly with the absence of PC-Exo showing little to no interaction in INS-1 cells (Fig. 3). The exact mechanism of how adrenomedullin in PC-Exo interacts with ADMRs is not known. We hypothesize that PC-Exo internalize along with ADMRs in the early endosomes as this location is a potential site of adrenomedullin release from the exosomes, which leaves the peptide in close proximity to ADMRs internalized into early endosomes (Fig. 6B). Further studies are necessary to confirm this exact mechanism.

We show that exosomes inhibit insulin secretion and adrenomedullin receptor blockade abrogates the effect of exosomes on insulin secretion. On the basis of our studies, we propose a mechanism for β-cell dysfunction in PC-DM in that the effect of adrenomedullin and exosomal adrenomedullin is mediated via upregulation and eventual failure of the UPR due to hyperstimulation of the cAMP-dependent pathway. We found hallmark features of a failed UPR, including increased mRNA expression of ER stress markers Bip and Chop (Fig. 5). We observed increased

Figure 6. Adrenomedullin (AM)-containing PC-Exo can increase Bip/proinsulin interactions in β cells. A, the Duolink Assay System was used to assess Bip/proinsulin interactions with increasing amounts (0, 5, 10, or 15 μL) of PKH67-dyed PC-Exo. Scale bar represents 10 μm. B, the potential mechanism for exosomal adrenomedullin release and signaling differs from the conventional mechanism shown in (1) in which adrenomedullin binds to cell surface adrenomedullin receptors (ADMRs) and activates the cAMP-dependent pathway. Instead, PC-Exo internalize through either caveolin-mediated endocytosis or macropinocytosis thus fusing to the early endosome, which is also the site of endocytosed ADMRs (2). The early endosome/multivesicular body could be another site of adrenomedullin/ADMR interaction. Once the pathway is activated adenylyl cyclase activation of cAMP releases the catalytic subunits of PKA, which translocate to the nucleus and subsequently activates insulin gene transcription. Excessive exosomal adrenomedullin activates ER stress proteins that regulate degradation of misfolded or unfolded ER proteins. However, overproduction of insulin due to excessive adrenomedullin pathway activation eventually leads to failure of the UPR marked by increased Bip/proinsulin coupling in the ER, increased ROS/RNS production, and an increase in Chop, an inducer of apoptosis, leading to a decrease in insulin secretion due to β-cell death.
association of Bip with proinsulin in the presence of increasing amounts of PC-Exo, another classic feature of a failed UPR response in β cells (Fig. 6A). We also showed increased β-cell apoptosis after exposure to adrenomedullin, further confirming Chop-mediated apoptosis (Fig. 5F). The upregulation of Bip and Chop was abolished by adrenomedullin receptor blockade, suggesting that the effects seen were due to adrenomedullin—ADMR interactions and not a nonspecific toxic effect of adrenomedullin on β cells. We conclude from these data that adrenomedullin leads to excessive activation and subsequent failure of the UPR leading to eventual β-cell death (Fig. 6B). Further work will be needed to understand the molecular mechanism of the failed UPR in response to exosomal adrenomedullin.

Our studies thus far have focused specifically on the role that adrenomedullin plays on β-cell dysfunction in PC-DM. However, it is possible that adrenomedullin is not the only mediator of β-cell dysfunction, but rather it may be working in concert with other key factors, such as tumor necrosis factor α, to promote β-cell malfunction (38). Therefore, further studies will investigate whether these factors are contained in PC-Exo and their potential interaction with adrenomedullin. Understanding all the key modulators that cause exosome-mediated β-cell dysfunction, and the interplay between them, will give us a more in-depth understanding of the pathogenesis of PC-DM.

Our long-term programmatic goal is to develop a rational, evidence-based strategy to screen for sporadic pancreatic cancer. Our work has shown that new-onset diabetes mellitus is a harbinger of pancreatic cancer (39). We reported that subjects with new-onset diabetes mellitus are eight times more likely than the general population to be diagnosed with pancreatic cancer within 3 years of meeting the criteria for diabetes mellitus (40). On the basis of these observations, we are conducting the first clinical trial of screening for pancreatic cancer in new-onset diabetes mellitus (NCI02001337). However, it is urgently needed in the field is a reliable marker to distinguish PC-DM from the more common type II diabetes mellitus.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D. Mukhopadhyay, N. Javed, S. Bhattacharya, S.T. Chari
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Javed, S.K. Dutta, T. Smyrky, J.S. Lau, S. Bhattacharya, M.J. Truty, G.M. Petersen, R.T. Chari
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.T. Chari
writing, review, and/or revision of the manuscript: D. Mukhopadhyay, N. Javed, T. Smyrky, J.S. Lau, M.J. Truty, G.M. Petersen, R.T. Chari
other (conducted Western blot analyses for the manuscript): G. Sagar

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Naureen Javeed, Gunisha Sagar, Shamit K. Dutta, et al.

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