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Role of parathyroid hormone-related protein in the pro-inflammatory and pro-fibrogenic response associated with acute pancreatitis

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ABSTRACT

Pancreatitis is a common and potentially lethal necro-inflammatory disease with both acute and chronic manifestations. Current evidence suggests that the accumulated damage incurred during repeated bouts of acute pancreatitis (AP) can lead to chronic disease, which is associated with an increased risk of pancreatic cancer. While parathyroid hormone-related protein (PTHrP) exerts multiple effects in normal physiology and disease states, its function in pancreatitis has not been previously addressed. Here we show that PTHrP levels are transiently elevated in a mouse model of cerulein-induced AP. Treatment with alcohol, a risk factor for both AP and chronic pancreatitis (CP), also increases PTHrP levels. These effects of cerulein and ethanol are evident in isolated primary acinar and stellate cells, as well as in the immortalized acinar and stellate cell lines AR42J and irPSCc3, respectively. Ethanol sensitizes acinar and stellate cells to the PTHrP-modulating effects of cerulein. Treatment of acinar cells with PTHrP (1–36) increases expression of the inflammatory mediators interleukin-6 (IL-6) and intracellular adhesion protein (ICAM-1), suggesting a potential autocrine loop. PTHrP also increases apoptosis in AR42J cells. Stellate cells mediate the fibrogenic response associated with pancreatitis; PTHrP (1–36) increases procollagen I and fibronectin mRNA levels in both primary and immortalized stellate cells. The effects of cerulein and ethanol on levels of IL-6 and procollagen I are suppressed by the PTH1R antagonist, PTHrP (7–34). Together these studies identify PTHrP as a potential mediator of the inflammatory and fibrogenic responses associated with alcoholic pancreatitis.

1. Introduction

It is well established that acute pancreatitis (AP) can be precipitated by exposure to risk factors such as smoking, hypertriglyceridemia, and, most commonly, ethanol. At the cellular level, ethanol induces acinar cell injury and stellate cell activation [1–3]. Damaged acinar cells are responsible for releasing the first inflammatory signals in response to pancreatic injury, leading to activation of the immune system [4–6]. Cytokines, chemokines and adhesion molecules are all produced by acinar cells in response to injury. Factors released from damaged acinar cells in turn activate the pancreatic stellate cells (PSC). These cells are key participants in pancreatitis after transforming from a quiescent into an "activated or myofibroblastic" state [7]. Activated PSC produce high levels of extracellular matrix (ECM) proteins, which most likely play a critical role in tissue repair following injury, but can also contribute to the pathologic fibrosis characteristic of chronic pancreatitis (CP) if unregulated [7]. Unfortunately, our current knowledge of the initial mediators of the ethanol-induced pathophysiology in pancreatic cells is still limited.

Parathyroid hormone-related protein (PTHrP), a peptide hormone with sequence homology to parathyroid hormone (PTH) at the N-terminus, is known to exert multiple effects in both normal and disease states, where it modulates critical cellular functions such as proliferation, apoptosis and differentiation, in part through paracrine and/or autocrine activation of the PTH/PTHrP receptor (PTH1R), a G protein-coupled receptor (GPCR) [8–10]. In the normal pancreas, PTHrP is expressed by islet cells and regulates cell proliferation, apoptosis, and insulin release [11,12]. PTHrP has also been reported to induce a proinflammatory response in a number of pathophysiological settings, including the injured kidney, atherosclerosis and rheumatoid arthritis [13–17]. Furthermore, PTHrP plays a role in tubulointerstitial apoptosis and fibrosis after folic acid-induced nephropathy [18]. These effects of PTHrP are mediated via both autocrine and paracrine pathways [13–18]. In this study, we asked whether PTHrP plays a role in the inflammatory response and fibrosis which accompany

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alcohol-induced pancreatitis. We show that PTHrP levels are transiently elevated in a mouse model of cerulein-induced AP. Treatment with ethanol also increases PTHrP levels. These effects of cerulein and ethanol are evident in primary and immortalized acinar and stellate cells. In addition, ethanol sensitizes the pancreas to the PTHrP-modulating effects of cerulein. Treatment of acinar cells with PTHrP (1–36) increases expression of the inflammatory mediators interleukin-6 (IL-6) and intracellular adhesion protein (ICAM-1), as well as apoptosis. PTHrP (1–36) also increases procollagen I and fibronectin mRNA levels in primary and immortalized stellate cells. The cerulein- and ethanol-induced upregulation of IL-6 and procollagen I are suppressed by the PTH1R antagonist PTHP (7–34). These studies identify PTHrP as a potential mediator of the inflammatory and fibrogenic responses evident in alcoholic pancreatitis.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Tissue culture supplies were purchased from Gibco (Carlsbad, CA). Antibodies for Western blot analysis, immunohistochemistry and immunofluorescence were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PTHrP (1–36) and PTHrP (7–34) were purchased from Bachem (Torrance, CA). Alexa Fluor 488 and Alexa Fluor 594 were obtained from Invitrogen (Carlsbad, CA).

2.2. Treatment with ethanol in vivo

All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol. C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) received ethanol (3.2 g/kg; administered in a 33.3% ethanol:67.7% water solution) by ip injection once a day for 7 days [19]. Control mice received water. Mice were sacrificed 2 days after the last ethanol injection. A portion of the pancreas was flash frozen and stored at −80 °C for Western blot analysis. The other portion was fixed for immunohistochemistry (IHC) and immunofluorescence analyses.

2.3. Treatment with cerulein in vivo

Pancreatitis was induced in C57BL/6 mice (Harlan Laboratories) by intraperitoneal (ip) injection of cerulein (50 μg/kg) at 1 h intervals [20]. Different groups of mice received 1 to 9 injections, and mice were sacrificed 1 h after the last injection. For longer term studies, mice were sacrificed 16 h after the last injection. Control mice were injected with PBS, using the same injection schedule. Pancreata were harvested and processed as described in Section 2.2.

2.4. Preparation of primary acinar and stellate cells

Primary pancreatic acinar and stellate cells were isolated as described [21,22]. Briefly, pancreata were removed from 2 to 3 mice sacrificed under anesthesia, and washed quickly with 3 ml isolation buffer (0.1% BSA-PBS with 10 μg/ml trypsin inhibitor). Pancreatic tissue was finely minced with scissors and digested with collagenase type IV (1 mg/ml) (Invitrogen) for 15 min at 37°C with vigorous shaking. Collagenase was inactivated by addition of 6 ml cold isolation buffer. Cells were washed 3 times in cold isolation buffer. The cell suspension was filtered through 70 μm mesh and cells were spun and resuspended in 10 ml of 10% FBS-DMEM containing 0.025% trypsin inhibitor. The identity of acinar cells was verified by measuring secreted amylase levels, using the Phadebas® Amylase Test kit (Lund, Sweden).

The identity of stellate cells was verified by staining with Oil Red O (Sigma) and α-smooth muscle actin (α-SMA). The absence of acinar cell contamination in stellate cells cultures was verified by measuring amylase levels in the stellate cell culture medium. For acinar cell culture, cells were plated onto 6-well dishes coated with 50 μg/ml laminin (Invitrogen). Treatment was initiated 24 h after plating. Stellate cells were plated onto 6-well dishes. Treatments were initiated when cells reached 70–80% confluence.

2.5. Cell culture and treatment

AR42J and Saos-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). AR42J cells were grown at 37 °C in a humidified 95% O_2/5% CO_2 atmosphere in F12-K medium supplemented with L-glutamine and 20% FBS. Saos-2 cells were grown under the same conditions in McCoy's 5a medium supplemented with L-glutamine and 15% FBS. The irPSCc3 rat pancreatic stellate cell line was obtained from Dr. Raul A. Urrutia (Mayo Clinic Cancer Center, MN) [23] and was grown under the same conditions in DMEM high glucose medium supplemented with 10% FBS and L-glutamine.

For all experiments requiring the preparation of RNA, AR42J, irPSCc3 and Saos-2 cells were plated in 6-well dishes. When the cells were ~70% confluent, they were serum-starved for 16 h. The cells were then treated with cerulein (10^{-9} M to 10^{-7} M), ethanol (5 mM to 50 mM), cerulein (10^{-8} M) plus ethanol (10 mM), or PTHrP (1–36) (10^{-7} M) for the time-intervals indicated in the Results section. The treatment protocols utilized in the Results section were chosen for further studies after performing the dose–response and time-course experiments. Cerulein (10^{-8} M) and ethanol (10 mM) represent the highest dose at which individual treatment had no effect. An effect was observed after individual treatment with a dose of 10^{-7} M cerulein or doses of 25 mM or higher of ethanol. In some experiments, the cells were pre-treated for 1 h with the PTH1R antagonist PTHrP (7–34) (10^{-5} M) prior to treatment with cerulein, ethanol or PTHrP (1–36).

2.6. Immunohistochemistry

Portions of the dissected pancreata were fixed immediately in 10% neutral buffered formalin for 24 h at room temperature after harvesting, and then placed in 70% ethanol. Formalin-fixed tissues were embedded in paraffin, and sections (5 μm) were cut from the paraffin blocks. The sections were deparaffinized in xylene, and rehydrated in descending ethanol series. Protein staining was performed using the DAKO EnVision Kit (Dako Corporation, Carpinteria, CA). Briefly, sections were incubated overnight at 4 °C with monoclonal antibodies (diluted in 0.05 mol/l Tris–HCl + 1% BSA) against PTHrP (N-19 and H-137), PTH1R or α-SMA (Santa Cruz Biotechnology, Inc.). After 3 washes with TBST, the sections were incubated for 30 min with secondary antibody labeled with peroxidase, then washed 3 times with TBST. Lastly, peroxidase substrate DAB was added for staining. All sections were counterstained with hematoxylin and observed by light microscopy. For negative controls, sections were incubated with rabbit IgG (Santa Cruz Biotechnology) in place of the primary antibody. To detect the presence of markers of pancreatitis, sections were stained with hematoxylin and eosin. Images were recorded using an Olympus BX51 microscope at 40× and 100× magnification.

For IHC staining of primary stellate cells and the AR42J and irPSCc3 cell lines, the cells were grown to 70% confluence in 8-well chamber slides before treatment. Primary acinar cells were treated 24 h after plating. After treatment, the cells were fixed in 95% ethanol and stained as described above.

2.7. Immunofluorescence

Pancreas sections and cells were processed as described in Section 2.6. They were then co-incubated with anti-PTHrP antibody and anti-GFAP antibody overnight. After washing 3 times with TBST, the sections were incubated in the dark for 1 h with Alexa Fluor 488 and Alexa Fluor 594. The sections were then washed 3 times with TBST in the dark. Nuclei were counterstained with DAPI.