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Abstract

AIM: Pancreatic regenerating protein (reg I) stimulates pancreatic regeneration after pancreatectomy and is mitogenic to ductal and β-cells. This suggests that reg I and its receptor may play a role in recovery after pancreatic injury. We hypothesized that reg I and its receptor are induced in acute pancreatitis.

METHODS: Acute pancreatitis was induced in male Wistar rats by retrograde injection of 3% sodium taurocholate into the pancreatic duct. Pancreata and serum were collected 12, 24, and 36 hours after injection and from normal controls (4 rats/group). Reg I receptor mRNA, serum reg I protein, and tissue reg I protein levels were determined by Northern analysis, enzyme-linked immunosorbent assay (ELISA), and Western analysis, respectively. Immunohistochemistry was used to localize changes in reg I and its receptor.

RESULTS: Serum amylase levels and histology confirmed necrotizing pancreatitis in taurocholate treated rats. There was no statistically significant change in serum reg I concentrations from controls. However, Western blot demonstrated increased tissue levels of reg I at 24 and 36 h. This increase was localized primarily to the acinar cells and the ductal cells by immunohistochemistry. Northern blot demonstrated a significant increase in reg I receptor mRNA expression with pancreatitis. Immunohistochemistry localized this increase to the ductal cells, islets, and acinar cells.

CONCLUSION: Acute pancreatitis results in increased tissue reg I protein levels localized to the acinar and ductal cells, and a parallel threefold induction of reg I receptor in the ductal cells, islets, and acinar cells. These changes suggest that induction of reg I and its receptor may be important for recovery from acute pancreatitis.

http://www.wjgnet.com/1007-9327/12/4511.asp

INTRODUCTION

Since its isolation in 1988 by Terazono *et al*[1], much work has been done to elucidate the function of the regeneration protein, reg I. Reg I protein appears to function as a growth factor in the pancreas, as evidenced by its structural similarity to calcium-dependant lectins[2], as well as its mitogenic effect on beta and ductal pancreatic cell lines[3] and its ability to reverse surgically induced diabetes[4,5]. Reg I gene expression has also been found in other tissues including gastric cells[6], and rats with water immersion stress induced gastric lesions demonstrate an increase in reg I expression in gastric enterochromaffin-like cells during healing[7]. Reg I is therefore involved in the regeneration and growth of gastrointestinal tissue. Furthermore, acute pancreatitis induces reg I gene expression and protein production in the pancreas[8,9]. Recently, Kobayashi *et al* isolated the gene for reg I receptor[10], which is part of the exostoses family of genes. Transfection of the gene into the pancreatic β-cell line RINm5F cells resulted in increased mitogenesis after exposure to reg I protein.

Since reg I and its receptor are linked to cellular

mitogenesis and may affect repair of damaged pancreas, we examined the role of their expression in acute pancreatitis.

MATERIALS AND METHODS

Induction of acute necrotizing pancreatitis

Acute necrotizing pancreatitis was induced in male Wistar rats using a 30 g/L sodium taurocholate solution according to a protocol described by Aho *et al* with slight modification[11]. The animals (4 rats/group) were anesthetized with a 1:1 mixture of ketamine (100 mg/mL) and xylazine (20 g/L) dosed at 0.1 mL per 100 g of body mass injected subcutaneously. The abdominal cavity was then entered through a midline incision. The duodenum and pancreas were identified, the common biliopancreatic duct ligated at the hilum of the liver, and a duodenostomy made approximately 1 cm distal to the opening of the biliopancreatic duct into the duodenum. A polyethylene catheter with an inner diameter of 0.58 mm and an outer diameter of 0.965 mm was used to cannulate the pancreatic duct through the duodenostomy. A 30 g/L sodium taurocholate solution was slowly injected at a dose of 1 mL/kg at a constant infusion rate of 0.5 mL/min. The catheter was left in place for 30 min and then removed. The midline incision was then closed in a single layer closure. Control animals consisted of sham operated rats (open laparotomy with immediate closure) and healthy rats.

Harvesting of pancreas and serum

A total of 12 rats were operated on for induction of pancreatitis. Four rats were sacrificed at each time point (12, 24, and 36 h). Four rats per group (unoperated and sham operated) were used for controls. The pancreas was harvested and blood collected by cardiac puncture from each rat. A small portion of the head of the pancreas was fixed in formalin and sent for staining with hematoxylin and eosin (HE) and immunohistochemical staining with reg I mAb[12]. The remaining pancreas was divided in half. One portion was immediately snap-frozen in liquid nitrogen and used for RNA isolation (below), and the other for protein isolation. This was performed by homogenization in buffer containing 125 mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 5 mmol/L piperizine ethane sulfuric acid-Tris (PIPES-Tris) (pH 6.7). The homogenate was centrifuged at 4100 g for 30 min and the supernatant collected and frozen at -20℃. The concentration of total protein in the pancreas homogenates was quantified using the BioRad Protein Assay solution according to the manufacturer's protocol. The blood was centrifuged at 1800 g for 15 min. The supernatant (serum) was collected and stored at -20℃.

Serum amylase activity was determined by enzymatic reaction. Formaldehyde-fixed pancreatic specimens from each rat were stained with HE.

Preparation of reg I cDNA probe

Reg I cDNA probe was prepared by PCR using a plasmid with a reg I insert as a template[3]. The sequences of the oligonucleotide primers are 5'ACGCGTCGACTCATGACTCGCAACAAATATTTC3' and 5'GGCACTGCAGTCAGGCTTTGAACTTGCAGAC3'. The PCR was carried out using digoxigenin-labeled uridine triphosphate (DIG-labeled UTP) (Roche Diagnostics, Mannheim, Germany) under the following conditions: denaturing at 94℃ for 30 s, annealing at 57℃ for 30 s, and elongation at 72℃ for 1 min; 30 cycles were used.

Preparation of Reg I receptor cDNA probe

A Reg I receptor cDNA was prepared using double digestion of pCIneo-Reg I receptor cDNA plasmid [10] with *Hin*dⅢ and *Not* I . Electrophoresis of the digestion complex was performed on 8 g/L agarose gel, the receptor band cut from the gel, and the cDNA extracted from the gel using the QIAEX Ⅱ agarose gel extraction protocol (Qiagen, Valencia, CA). The reg I receptor cDNA fragment was labeled with digoxigenin (DIG) using the DIG High Prime DNA Labeling and Detection Starter Kit Ⅱ (Boehringer Mannheim, Germany) as per manufacturers' protocol.

Northern blot

RNA was extracted from rat pancreatic and intestinal tissue using Trizol reagent (Gibco, Life Technologies, Rockville, MD). RNA extraction was performed with 1-bromo-3-chloropropane. RNA concentrations were determined by absorbance measurements at 260 nm, and the integrity confirmed by polyacrylamide gel electrophoresis. Equal amounts (20 μg) of total RNA were then used for all Northern blots. The membrane was equilibrated in 10 × saline-sodium citrate (SSC) buffer for 5 min. The RNA samples were prepared as follows: 50 μL TE buffer (TE: 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0), 20 μL of 37 g/L formaldehyde, 30 μL of 20 × SSC, and 20 μg RNA. The samples were heated at 60℃ for 15 min and immediately chilled on ice for 3 min. Two hundred microliters of 10 × SSC were then added to each tube. The samples were applied to the nylon membrane using a Northern slot blot apparatus. Each well was washed with 500 μL SSC. The membrane with RNA was UV cross-linked and placed in 50 mL of DIG pre-hybridization solution at 48℃ for 6 h. Hybridization with the DIG-labeled reg I receptor cDNA probe was carried out at 58℃ for 12 h. After washing and blocking, the membrane was incubated in a 1:10 000 dilution of anti-DIG-alkaline phosphatase conjugate antibody solution at room temperature for 30 min. CSPD solution (Boehringer Mannheim) was used for detection. Gels were photographed using Polaroid 667 film and digitized using an Epson 636 scanner. Band density analysis was performed using the public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/) to determine the quantity of nucleic acid product. To account for differences in the amounts of starting RNA between samples, the density of each reg band was normalized to that of the β-actin band for the same sample. Samples were run in duplicate and data are expressed as mean optical densitometric (OD) measurements ± SE.

Western blot on tissue homogenate to determine reg I levels

The tissue was homogenized in buffer containing 125

mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L EDTA, 5 mmol/L PIPES-Tris (pH 6.7), and 1 tablet of protease inhibitor cocktail per 50 mL of buffer. Total protein concentrations were determined using Bio-Rad protein assay. Electrophoresis was performed using equal amounts of total protein (10 μg/well) in a 100 g/L sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The electrophoresis was carried out at 80 V for the first 15 min and then at 130 V for 45 min in SDS electrophoresis buffer. The protein was transferred to nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) at 100 V for 1 h. The membrane was blocked overnight in 50 mL/L milk. A dilution of 1:5000 was used for the mouse anti-reg I mAb and incubated for 1 h with the membrane. After washing with T-TBS, the membrane was incubated for 45 min with a 1:2000 dilution of horse radish peroxidase-linked anti-mouse IgG. Detection was done using the ECL Western Blotting Detection System (Amersham Pharmacia, Piscataway, NJ). Human Reg 1 (hReg1) (10 μg) was used as a positive antibody control.

### ELISA on serum samples to determine reg I concentration

Each well of the microtiter plate was coated with a mixture of 50 μL serum and 50 μL of coating buffer (20 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$). Serial dilutions of human reg I were used as standards. The wells were coated overnight, followed by blocking with a 3% BSA solution for 4 h. The mouse reg I mAb was diluted 1:1000 and incubated for 1 h[12,13]. A dilution of 1:1000 was used for the secondary antibody and incubated for 45 min. Detection was performed by adding 100 μL of detection buffer (4.84 mL of 0.05 citric acid, 5.14 mL of 0.1 Na_2HPO_4, 30 μL of 300 mL/L peroxide, and 1 tablet of o-phenylenediamine) and measuring absorbance at 450 nm.

Development of anti-receptor antibodies

Two non-overlapping peptide sequences were identified that were contained in the extracellular domain of the Reg I receptor[10] with predicted antigenicity and directly flanking specific amino acids thought to directly bind to Reg I (phage display experiment, unpublished data). PAELEKQLYSLPHWRTDC and RLLPEKDDAGLPPPKATRGC were synthesized by solid phase synthesis (Biomolecules Midwest Inc., St. Louis, MO), purified by reverse phase high performance liquid chromatography (HPLC), and characterized by Mass Spectrometry. Peptides were coupled to KLH and used for immunization of rabbits. Rabbits were boosted at monthly intervals and bled 10-14 d after each boost. Antibody titer was determined in a microtiter plate ELISA using the conjugated peptide as the immobilized antigen. The immunoglobulin fraction was isolated using ImmunoPure (A/G) IgG purification system (Pierce, Rockford, IL) and the final antibody concentration was determined with the BCA Assay (Pierce).

Reg I receptor immunohistochemistry

Paraffin embedded sections were treated with Ficin (1:100 dilution, Sigma, St. Louis, MO) for 30 min at room temperature, washed, and were then treated with Avidin/Biotin blocking reagent (Vector Labs, Burlingame, CA) for
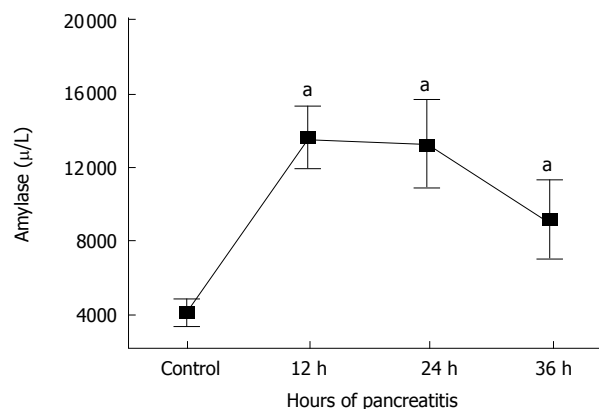
Figure 1 Serum amylase activity in pancreatitis rats (12, 24, 36 h) *vs* sham operated and normal controls. [a]$P < 0.05$.
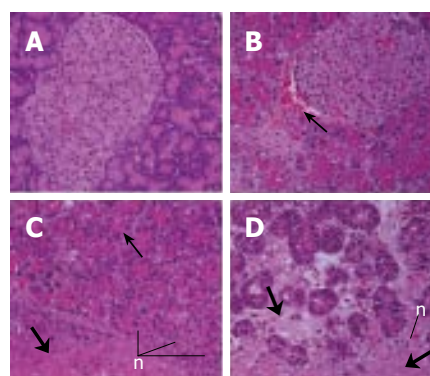
Figure 2 HE stain of normal rat pancreas (**A**) and after taurocholate injection at 12, 24, and 36 h (**B**, **C**, and **D**). Induction of pancreatitis resulted in hemorrhage (thin arrows), infiltration of neutrophils ("n"), and necrosis (thick arrows).

20 min. Slides were subsequently treated with a Protein Block Reagent (Dako, Carpenteria, CA) and incubated with primary antibodies overnight at 4℃. Goat anti-rabbit biotinylated IgG secondary antibody was applied for 1 h at room temperature (1:1500, NEN Life Science, Boston, MA). Endogenous peroxidase activity was quenched by treatment with 10 mL/L hydrogen peroxide/PBS and sections were then incubated with streptavidin-horseradish peroxidase (SA-HRP) (1:1000 dilution, Dako) for 30 min. For receptor experiments, tyramide amplification was applied for 3 min (NEN Life Science, Boston, MA). Slides were extensively rinsed and again incubated with SA-HRP (Dako, 1:1000) for 30 min at room temperature. Slides were developed in DAB (Sigma, St. Louis, MO) and counterstained with hematoxylin (Richard Allan, Kalamazoo, MI).

Statistical analysis

Data were expressed as means ± SE. Student's *t* test was used for analysis. $P < 0.05$ was taken as significant.
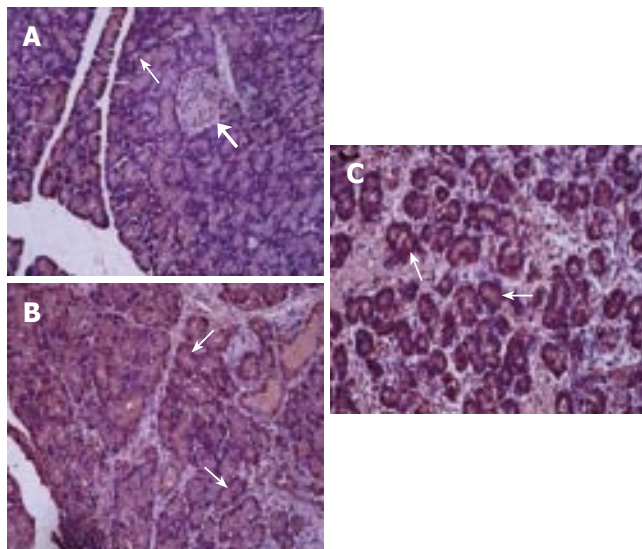
RESULTS

The presence of acute necrotizing pancreatitis in taurocholate-treated rats was confirmed by serum amylase activity and histology (Figures 1 and 2). As demonstrated in Figure 1 serum amylase levels at 12, 24, and 36 h were elevated ($P < 0.05$) when compared to both sham operated and normal control rats. Figure 1 demonstrates a rapid increase
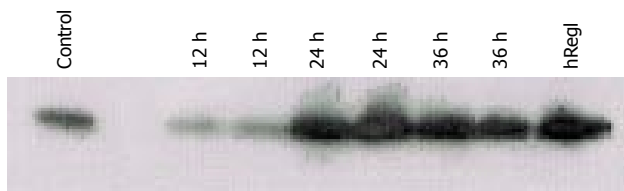
Figure 3 Slot blot analysis with anti-reg I antibody of total protein (10 μg/well) from normal rat pancreas and rat pancreas after induction of acute pancreatitis. Reg I protein levels in tissue increased after 24 h of pancreatitis. Human Reg I (hReg1) is shown as a loading control as described in MATERIALS AND METHODS.
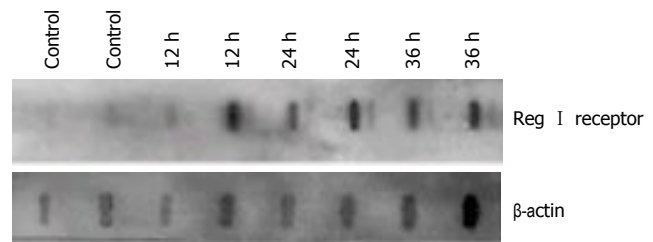

Figure 5 Slot blot of reg I receptor during pancreatitis showed an increase in reg I receptor mRNA expression after induction of pancreatitis. The density of each reg band was normalized to that of β-actin band for the same sample as described in MATERIALS AND METHODS.

Figure 4 Immunohistochemical staining of normal rat pancreas with anti-reg antibody (**A**) and of rat pancreas after 24 and 36 h of pancreatitis (**B**, **C**). There is a marked increase in reg I levels in acinar cells of the pancreas after pancreatitis. Stained acinar cells are marked with thin arrows, compared with lack of staining in Islet cells (thick arrow).

Figure 6 Immunohistochemical staining of pancreata from normal (**A**) and experimental pancreatitis (**B-D**) rats. There is an induction of reg I receptor expression in the acinar cells (a) and islets (i), and maintenance of reg I receptor expression in ductal cells (d) with pancreatitis.
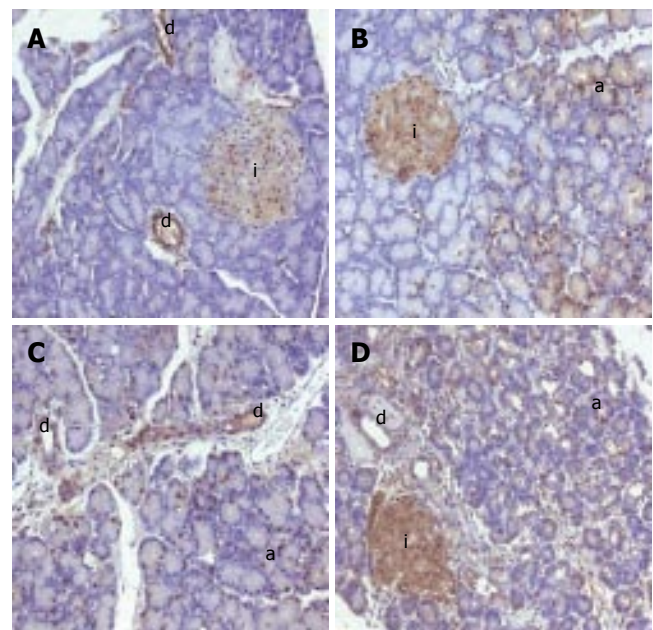
in amylase levels at 12 h with a gradual decline towards normal levels at 24 and 36 h. Figure 2 demonstrates histopathological worsening of pancreatitis in the pancreatic parenchyma after sodium taurocholate treatment as evidenced by hemorrhage (Figures 2 B, C) and necrosis (Figure 2D) when compared with control groups (Figure 2A).

Western blot analysis of pancreatic tissue showed a single band at 15-17 ku using a monoclonal antibody and demonstrated an increase in reg I protein in the 24 and 36 h pancreatitis groups compared to controls, although at 12 h, there was an initial decrease in reg I protein (Figure 3). Histologic examination showed that this appeared localized in the pancreatic acinar cells (Figure 4). Serum reg I protein levels were not significantly different in pancreatitis (0.43 ± 0.13 mg/L) when compared with sham-operated and normal controls (0.38 ± 0.15 mg/L) (data not shown).

Northern/slot blot analysis demonstrated a single band corresponding to the Reg I receptor mRNA and showed an increase in reg I receptor RNA expression in pancreatic tissue from the experimental pancreatitis groups compared to controls (Figure 5). Mean absorbance (A) ± SE of reg I receptor signals on Northern/slot blot for control, 12, 24, and 36 h of pancreatitis were 24 ± 6, 71 ± 15, 66 ± 26, and 75 ± 20 (all were significantly increased compared to control, $P < 0.05$). Immunohistochemical staining with anti-reg I receptor antibody showed that although staining persisted in the ductal population, there was significant induction of receptor protein in both the islets and acinar cells (Figure 6).

DISCUSSION

Reg I is mitogenic to ductal and β-cells likely through the induction of the MAP kinase p38 pathway[14]. This has been shown to be true in both cell culture and animal models of pancreatectomized rats. The data presented support the hypothesis that reg I is involved in regeneration after injury to the pancreas in the form of a sodium taurocholate-induced acute necrotizing pancreatitis. The pancreatitic rats demonstrated an increase in reg I protein levels in pancreatic tissue after 24 h of pancreatitis. In the acutely inflamed pancreas it appeared that reg I protein was overproduced in the acinar cell population. This is consistent with the findings of others in both acute and chronic forms of pancreatitis[15]. Although many genes are

differentially regulated in inflammatory states, microarray data by us and others have demonstrated increased Reg expression in experimental pancreatitis[16,17]. Interestingly, serum reg I protein levels did not increase in parallel with acinar reg I RNA expression levels[13]. This may be due to differences in cellular RNA turnover prior to stabilization of protein levels in serum, possibly through unidentified reg I binding proteins. Similar discord between protein and gene expression has been found in other systems[18].

Baseline expression of reg I receptor is present in pancreatic ductal and beta cell lines, but not in acinar cell lines[10,19]. These cells respond to reg I protein by proliferating, as measured by thymidine incorporation. Cells transfected with the receptor respond to reg I in a similar fashion[10]. Acinar cells, which typically do not grow in response to reg I, do not express the receptor. It is therefore likely that the expression of the receptor is linked to the activity of cellular mitogenesis, and may be important in the proliferative response of pancreatic regeneration.

We found that pancreatitis significantly induced the expression of reg I receptor RNA in pancreatic tissue compared with baseline level. Immunohistochemical analysis with a polyclonal antibody to the reg I receptor localized the induced protein to the acinar cells and islets, and was only mildly present in ductal cells. This pattern of induction was in sharp contrast to the increase in reg I which was initially induced primarily in the acinar cells. While ductal cells typically proliferate after pancreatitis[20], the additional induction of the protein in islets and acinar cells likely means that they are becoming responsive to the reg I protein which is being produced and secreted by the acinar cells.

Our present data suggest that increased reg I protein and receptor are important in the response of the pancreas to injury. Specifically, reg I may exert its mitogenic effect on all the cell populations after injury. It likely works in a paracrine fashion, since it is produced and secreted from acinar cells. The induction of the receptor after injury in the acinar cells and islets and its maintenance of expression in the ductal cells may allow the protein to enhance its regenerative effect.

Another new protein induced in pancreatitis is reg Ⅲ, or pancreatitis associated protein (PAP). Seventy percent homologous to reg I, the pancreatic RNA and protein levels are increased even more dramatically in pancreatitis, and PAP protein is also increased in serum [21,22]. We showed that the bovine form of reg Ⅲ is mitogenic to pancreatic β- and ductal cells as well[3]. It is possible that reg Ⅲ protein interacts with the reg I receptor which may provide an important pathway for regeneration after injury.

Future experiments need to determine whether blocking the receptor will block the regenerative effect of reg I, and whether other members of the reg family, in particular reg Ⅲ, can stimulate the receptor as well.

ACKNOWLEDGMENTS

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