ORIGINAL ARTICLE

Administration of Anti-Reg I and Anti-PAPII Antibodies Worsens Pancreatitis

Domenico Viterbo[1], Gordon E Callender[1], Theresa DiMaio[2], Cathy M Mueller[1], Tamar Smith-Norowitz[3], Michael E Zenilman[1], Martin H Bluth[1,4]

Departments of [1]Surgery, [2]Pathology, and [3]Pediatrics, SUNY Downstate Medical Center. Brooklyn, NY, USA. [4]Department of Pathology, Wayne State School of Medicine. Detroit MI, USA

ABSTRACT

Context The regeneration protein family (Reg), which includes Reg I and PAPII, is expressed in pancreas acinar cells, and increases in acute pancreatitis. We have demonstrated that Reg gene knockdown worsens severity of acute pancreatitis in the rat and hypothesize that the proteins offer a protective effect in this disease. **Objective** We investigated the ability of anti-Reg and anti-PAP antibody to neutralize pancreatic Reg protein and affect pancreatitis severity. **Intervention** Pancreatitis was induced in rats by retrograde ductal injection of 4% sodium taurocholate. **Animals** Eighty-four rats: 48 with induced pancreatitis, 30 sham operated, and 6 normal animals. **Setting** Intraductal anti-Reg I and/or anti-PAPII antibody was administered at induced pancreatitis and sham operated subgroups of 6 rats each. **Main outcome measure** Serum and pancreata were harvested 24 and/or 48 hours later and assessed for pancreatitis severity by pancreatic wet weight, serum C-reactive protein (CRP), amylase, PAPII levels, and histopathology. **Results** Animals induced with pancreatitis with administration of anti-Reg/PAP antibodies had significantly higher wet weights compared with taurocholate and histopathological analysis revealed that anti-Reg/PAP treated animals had worse tissue inflammation and necrosis compared with controls. Serum CRP, amylase, and Reg levels did not significantly differ between experimental and sham control groups. **Conclusions** Administration of anti-Reg/PAP antibody worsened taurocholate-induced organ specific pancreatitis. These data suggest that the Reg family of proteins is protective in acute pancreatitis.

INTRODUCTION

Acute pancreatitis has a spectrum of severity ranging from a mild, self-limiting course treated with conservative methods, to a more aggressive variety characterized by sepsis, pancreatic necrosis and hemorrhage. It is estimated that 25% of patients with acute pancreatitis will progress in severity and require operative management or die [1]. Pancreatic regenerating protein may play a role in the pathophysiology of acute pancreatitis. The regeneration family of proteins (Reg), which include Reg I (pancreatic stone protein) and Reg III (pancreatitis-associated protein: PAP), are a family of proteins minimally expressed in normal pancreas but strongly induced in acute pancreatitis [2, 3, 4]. We have previously demonstrated that antisense mediated gene knockdown of Reg/PAP *in vivo* worsens pancreatitis [5]. In those studies, inhibition of Reg/PAP expression significantly worsened pancreatitis in that serum amylase activity, pancreas wet weight, reflecting edema, and serum C-reactive protein levels all increased in antisense-treated animals compared with controls. Furthermore, histopathologic evaluation of pancreas revealed worsened edema, elevated leukocyte infiltration, and fat necrosis after antisense-treatment compared with controls [5]. Here we examined the ability of anti-Reg/anti-PAP antibodies to neutralize Reg/PAP proteins and their affect on pancreatitis severity.

Received April 24th, 2008 - Accepted May 13th, 2008

Key words Pancreatitis; pancreatitis-associated protein

Abbreviations NaT: 4% sodium taurocholate; NaT-N: 4% sodium taurocholate plus non specific antibody; NaT-P: 4% sodium taurocholate plus anti-PAPII; NaT-R: 4% sodium taurocholate plus anti-Reg I; NaT-RP: 4% sodium taurocholate plus anti-Reg I plus anti-PAPII; Reg: regeneration protein family; PAP: pancreatitis-associated protein; S: sham operated control rats; S-N sham operated plus non specific antibody; S-P: sham operated plus anti-PAPII; S-R: sham operated plus anti-Reg I

Correspondence Martin H Bluth
Wayne State University School of Medicine, Department of Pathology, 8203 Scott Hall, 540 E. Canfield, Detroit, Michigan 48201, USA
Phone: +1-313.577.1102; Fax: +1-313.577.0057
E-mail: mbluth@med.wayne.edu

Document URL http://www.joplink.net/prev/200901/03.html

MATERIALS AND METHODS

Experimental Design

Seventy-eight 225 g Sprague-Dawley male rats (Charles River, Wilmington, MA, USA) were utilized for this model in the experimental sodium taurocholate induced pancreatitis (n=48) and in the control (sham operated; n=30) groups. In addition, 6 normal rats were also studied.

Induction of Pancreatitis

Retrograde intra-ductal infusion of 4% sodium taurocholate (NaT) in saline was performed using a polyethylene catheter (0.011" and 0.024", internal and outer diameter, respectively; Adams, Parsipanny, NJ, USA) as previously described [5]. All rats were first anesthetized using sodium pentobarbital (Abbott Laboratories, Abbott Park, IL, USA) using a loading dose of 40 mg/kg administered intraperitoneally. A midline incision was then performed. The common bile duct was identified and cannulated in an antegrade direction with PE-10 tubing (Fisher Scientific, Pittsburgh, PA, USA) such that the proximal end of the tube was beyond the ampulla of Vater in the duodenum. The bile duct was then ligated to prevent the flow of bile, and 4% NaT in sterile saline was consistently infused into the pancreatic duct at a rate of 1 mL/kg over 10 min [5].

The experimental animals received a total volume of 1 mL/kg of 4% sodium taurocholate into the pancreatic duct and were subdivided into subgroups of 6 rats each which were simultaneously administered with non specific antibody (NS IgG: 1.6-2 mg; Sigma, St Louis, MO, USA) (NaT-N), anti-Reg I alone (NaT-R), anti-PAPII alone (NaT-P), anti-Reg I together with anti-PAPII (NaT-RP), while no antibody was administered to 6 rats (NaT).

Controls

Controls consisted of sham-operated rats which underwent open laparotomy with infusion of saline alone (S), saline with non-specific antibodies (S-N), saline with PAPII alone (S-P), and saline with anti-Reg I alone (S-R).

Anti-Reg/PAP Antibody for *in vivo* Administration

Monoconal anti-Reg I antibody was purified from mouse ascites fluid after immunization with a Reg I producing hybridoma cell line [6]. Polyclonal anti-PAPII antibody was similarly obtained after injection of a 31 aminoacid PAP oligopeptide protein sequence (TMGQQPNGGGWEWSNSDVLNYLNWDGDPSST) into rabbit (Cocalico Biologicals Inc, Reamstown, PA, USA). This sequence represents a hydrophilic region of PAPII and is distinct from Reg I. The gene sequence coding for this protein was directionally cloned into PGEX-5X-1 plasmid (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) using primer EcoRI (F-agcagaattcgaagactcccagaaggc agtgccctctacacg) and XhoI (R-ctcactcgag gtc tac tgc ttg aac ttg cag aca aaa ggt aat tcc aca tc) linked sequences generating a PAPII-GST fusion protein. Recombinant plasmids were transformed into BL21 competent cells (Stratagene, La Jolla, CA, USA) and purified protein was obtained by glutathione column affinity chromatography [7].

PAPII peptide used to generate anti-PAPII antibody was generated by the Alignment Program within the ExPASy software (Expert Protein Analysis System; http://www.expasy.org). Reg isoforms were compared and sequence homology obtained. Subsequent analysis demonstrated a unique amino acid sequence which had minimal overlap with other Reg isoforms (Figure 1). Anti-Reg I did not cross react with PAPII protein and anti-PAP antibody did not cross react with Reg I protein.

Concentration of Anti-Reg/PAP Antibody

Reg I

We developed a direct enzyme linked immunosorbent assay (ELISA) to determine the amount of antibody required to completely saturate the amount of endogenous Reg I found within the pancreatic ductal system. To this end 8 µg/mL of Reg I antigen were loaded on a microplate, after which varying concentrations of monoclonal Reg I antibody was added and a saturation curve was generated; the slope of OD absorbance plateaued at 40 µg/mL Reg I antibody. We were able to obtain 200 µL of pancreatic juice per rat. Pancreatic juice contains about 7 µg/µL total protein (Bradford assay, St Louis, MO, USA) and Reg I protein comprises about 15% of pancreatic juice (1 µg/µL) [8]; an average rat would contain about 200 µg of Reg I protein. We therefore postulated that injection of 100 µL of 20 mg/mL (i.e., 2 mg total) of monoclonal Reg I antibody would effectively saturate endogenous Reg I protein at a 10:1 antibody:protein ratio.

PAPII

Unlike Reg I, the concentrations of PAPII in both the pancreatic juice and serum of healthy animals is minimal [3, 9]. In contrast, and consistent with earlier studies in our laboratory [8, 10], serum concentrations increase over 200 fold in pancreatitic rats compared with healthy controls (about 220±50 µg/mL *vs.* less than 2 µg/mL, $P<0.05$) [10], but are still a fraction of Reg I levels in serum and pancreatic juice. We injected 1.6 mg (total) of polyclonal anti-PAPII antibody into the pancreatic duct based on pilot studies which demonstrated that this amount of antibody would maximize pancreatitis (histology) presumably by inhibiting endogenous PAPII protein in the pancreatic parenchyma in acute pancreatitis.

Western Analysis

Purified anti-Reg/PAP antibodies (Reg I, PAPII) were tested for Reg specificity *in vivo*. Rats were induced with NaT pancreatitis and pancreatic juice or pancreas

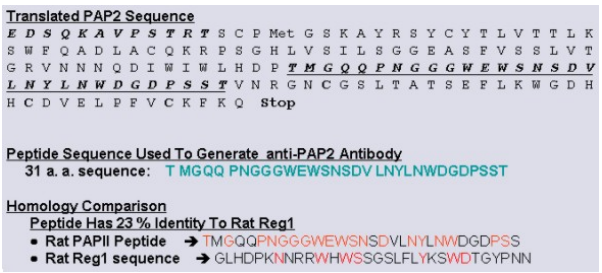

Figure 1. Peptide fragment used for anti-PAPII antibody generation: sequence comparison and homology between Reg I and PAPII.

was harvested after 24 hours. Pancreatic juice or homogenized pancreas tissue lysate was run on 15% acrylamide gel and subsequently blotted with appropriately diluted antibody (anti-Reg I antibody, 1:1,000; anti-PAP antibody, 1:400) [7].

Tissue Analysis for Edema

Pancreata were harvested at 24 and 48-hour after pancreatitis induction plus/minus anti-Reg/PAP treatment and severity was assessed by pancreatic wet weight (edema) and histopathology. To determine the degree of edema secondary to pancreatitis, the weight of the whole pancreas following harvesting was compared to the weight of the rat at the time of the pancreatectomy [5]. The pancreatic wet weight (milligrams of pancreas weight/grams of total body weight of rat; mg/g) was thus obtained from each rat belonging to each group (based on condition and time) and averaged together.

Histopathological Analysis

The head of the pancreas from each rat was fixed in 10% buffered formaldehyde solution (Fisher Scientific, Pittsburgh, PA, USA). Slides were generated for each pancreas collected using four to six micron sections, and stained with hematoxylin and eosin (H&E). The histological severity of pancreatitis was examined by pathologists who performed the evaluation in a blinded fashion by using previously described criteria [11, 12]. The degree of inflammation, hemorrhage, leukocytic infiltration, and tissue necrosis were determined for each specimen by using a scale ranging from zero (representing the least severity) to four (representing the greatest severity).

Serum Analysis for Amylase and CRP

Whole blood from each of the rats was obtained at the time of the pancreatectomy. Serum was separated and assayed for amylase (U/mL) and CRP levels (mg/dL) by the Clinical Laboratory at SUNY Downstate Medical Center.

Serum Analysis for Reg/PAP

Serum Reg levels obtained from experimental and control rats were determined by enzyme linked immunosorbent assay (ELISA) [3, 13].

Reg I

After measuring the amount of total protein within rat pancreatic juice (collected from normal rats), we estimated that 15% of the protein content was Reg I [8]. For each well, 8 µg/mL of purified rat Reg I protein was plated on a 96-well plate and left to incubate overnight. After serial washing with phosphate-buffered solution (PBS) and blocking with 3% BSA in PBS, a standard curve using our Reg I monoclonal antibody (ranging from 2.7 to 85 µg/mL) as our primary antibody was generated and a saturation curve produced as previously described [3].

PAPII

Goat anti-rat PAPII polyclonal antibody (R&D Biosystems, Minneapolis, MN, USA) (100 µg/µL diluted in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6; Bethyl Labs Inc., Montgomery TX, USA), was plated in 96 well plates (100 µL/well) and incubated overnight at 4°C. Plates were washed three times with wash solution (50 mM Tris-buffered saline, pH 8.0, 0.05% Tween 20; Bethyl Labs, Montgomery, TX, USA) after which blocking postcoat solution (50 mM Tris-buffered saline, pH 8.0, 1.0% BSA; Bethyl Labs, Montgomery, TX, USA) was added and plates were incubated for 30 min at room temperature. Plates were washed three times with wash solution and experimental and control rat sera, appropriately diluted in dilution buffer (50 mM Tris-buffered saline, pH 8.0, 1.0% BSA, 10% Tween 20; Bethyl Labs, Montgomery, TX, USA), were added and incubated for 60 min at room temperature. Plates were washed 5 times with wash solution and biotinylated anti-rat PAPII polyclonal antibody (R&D Systems, Minneapolis, MN, USA) in sample diluent (1:200; 100 µL/well) was added and plates were incubated for 1 hour at room temperature. Plates were washed 5 times with wash buffer and streptavidin-HRP (R&D Systems, Minneapolis, MN, USA), (1:200 in sample diluent; 100 µL/well) was added and plates were incubated for 1 hour at room temperature. Plates were washed 5 times in wash solution, were developed for 15 min in the dark using 3,3,5,5, tetramethylbenzidine (TMB) substrate (100 µL/well; Bethyl Labs, Montgomery, TX, USA), according to manufacturer's recommendations, and the reaction stopped with H_2SO_4 (2 mol/L). Plates were read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Standard curves were generated using recombinant PAPII protein (used to generate anti-PAPII antibody above: Bethyl Labs, Montgomery, TX, USA) appropriately diluted in normal rat serum/PBS (1:3). Range of assay was 0.075-7.5 ng/µL with coefficient of variation (CV) no greater than 9.1% [13].

ETHICS

Institutional guidelines regarding animal experimentation were followed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals (1996)" prepared by the National Academy of Sciences.

STATISTICS

All data were expressed as mean±standard error of the mean (SEM) representing a minimum of six animals per group. Median values of the histopathology scores were also evaluated. During initial studies the statistical analysis was performed by using the unpaired Student's independent-sample t-test for between group comparisons, which consisted of control *vs*. insulted (NaT) animals, and the paired Student's t-test within groups observed over two different time

Table 1a. Pancreatic wet weights (mean±SEM; mg/g).

Groups (6 rats in each group)	**0 h**	**24 h**	**48 h**	**P values** [a]
Normal	4.07±0.06	N/A	N/A	-
S: Sham	N/A	4.54±0.21	3.93±0.16	0.056
S-N: Sham + non specific IgG	N/A	3.68±0.18	NT	-
S-P: Sham + anti-PAPII	N/A	4.72±0.16	NT	-
NaT: 4% sodium taurocholate	N/A	5.61±0.25	4.64±0.14	0.007
NaT-N: 4% NaT + NS IgG	N/A	5.71±0.20	6.04±0.21	0.286
NaT-P: 4% NaT + anti-PAPII	N/A	5.53±0.19	NT	-
NaT-R: 4% NaT + anti-Reg I	N/A	5.90±0.16	6.60±0.21	0.024
NaT-RP: 4% NaT + anti-Reg I+ anti-PAPII	N/A	6.57±0.25	NT	-

[a] paired Student's t-test
N/A: not applicable; NT: not tested

Table 1b. Results of the pairwise comparison of the pancreatic wet weight after 24 hours among groups (P values are reported in the table).

	S	**S-N**	**S-P**	**NaT**	**NaT-N**	**NaT-P**	**NaT-R**	**NaT-RP**
S: Sham	-	0.004	0.997	0.002	0.001	0.005	<0.001	<0.001
S-N: Sham + non specific IgG	0.004	-	0.005	<0.001	<0.001	<0.001	<0.001	<0.001
S-P: Sham + anti-PAPII	0.997	0.005	-	0.017	0.005	0.040	0.001	<0.001
NaT: 4% sodium taurocholate	0.002	<0.001	0.017	-	1.000	1.000	0.959	0.010
NaT-N: 4% NaT + NS IgG	0.001	<0.001	0.005	1.000	-	0.998	0.997	0.029
NaT-P: 4% NaT + anti-PAPII	0.005	<0.001	0.040	1.000	0.998	-	0.854	0.004
NaT-R: 4% NaT + anti-Reg I	<0.001	<0.001	0.001	0.959	0.997	0.854	-	0.177
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	<0.001	<0.001	<0.001	0.010	0.029	0.004	0.177	-

ANOVA with Tukey post-hoc multiple comparison.

Table 1c. Results of the pairwise comparison of the pancreatic wet weight after 48 hours among groups (P values are reported in the table).

	S	**NaT**	**NaT-N**	**NaT-R**
S	-	0.003	0.001	<0.001
NaT	0.003	-	0.999	0.846
NaT-N	0.001	0.999	-	0.890
NaT-R	<0.001	0.846	0.890	-

ANOVA with Tukey post-hoc comparison.

points. Upon compilation of all data sets representing all groups (controls and NaT insulted) analysis of variance (ANOVA) with Tukey post-hoc multiple comparison procedure was applied when three or more groups were pairwise analyzed. Initially ANOVA was employed among all control groups (normal and sham plus/minus antibody treatment). If no significance was obtained then normal animal data was compared with all insulted groups (NaT plus/minus antibody treatment). If there were differences among controls groups then all controls would be compared with all insulted groups. The Statistical Package for Social Sciences (Version 11.0; SPSS, Inc., Chicago, IL, USA) was used. Statistical significance was defined as two-tailed P value less than 0.05.

RESULTS

Generation of PAPII Antibodies

The Reg protein family which contains Reg I and PAPII, possesses considerable homology to one another [3]. In light of this, the sequences of Reg I and PAPII were compared and a unique hydrophilic region for PAPII was identified: T Met G Q Q P N G G G W E W S N S D V L N Y L N W D G D P S S T. As shown in Figure 1 this oligopeptide contains about 20% homology to Reg I. This distinction allows for analysis of antibody-mediated neutralization of individual Reg proteins with minimal overlap; cross reactivity was not observed (data not shown).

Specificity of Anti-Reg/PAP Antibody

Prior to injection of antibody, the specificity to the Reg I and PAPII proteins were confirmed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Protein isolated from pancreatic juice or pancreas obtained from animals induced with 4% NaT demonstrated a band at approximately 17 kDa representing Reg protein [3] when blotted with monoclonal Reg I antibody against human and rat Reg I, (Figure 2; a1 and a2, respectively). Similarly, a 17 kDa band was observed with anti PAPII antibody blotted against pancreata obtained from rats induced with NaT pancreatitis (Figure 2, c1) when compared with pancreata obtained from normal rats (Figure 2, c2). In contrast, non-specific control IgG did not demonstrate any bands representative of Reg I or PAPII (Figure 2, b1 and b2).

Wet Weight

Animals induced with NaT pancreatitis had higher pancreatic wet weights at 24 hours when compared

Figure 2. Validation of specificity of monoclonal anti-human Reg I antibody to human and rat Reg I protein using SDS-PAGE and Western blot. Lanes **a1** and **a2** demonstrate reactivity of monoclonal antibody against human and rat Reg I protein respectively, revealing a band at approximately 17 kDa; Reg I protein was obtained from pancreatic juice from human or rat with pancreatitis (ammonium sulfate precipitation). Lane **b** contained normal mouse IgG against human Reg I and rat Reg I (**b1** and **b2** respectively), which did not reveal any bands. Lane **c**: anti-PAPII antibody blotted against pancreas obtained from NaT induced pancreatitis (**c1**) or normal pancreas (**c2**).

Table 2a. Serum amylase levels (mean±SEM; U/L).

Groups (6 rats in each group)	**0 h**	**24 h**	**48 h**	**P values** [a]
Normal	1,551±31	N/A	N/A	-
S: Sham	N/A	2,489±617	1,543±75	0.159
S-N: Sham + non specific IgG	N/A	2,923±453	NT	-
S-P: Sham + anti-PAPII	N/A	3,187±306	NT	-
NaT: 4% sodium taurocholate	N/A	4,250±471	3,119±1,381	0.456
NaT-N: 4% NaT + NS IgG	N/A	4,155±356	2,610±281	0.007
NaT-P: 4% NaT + anti-PAPII	N/A	5,606±650	NT	-
NaT-R: 4% NaT + anti-Reg I	N/A	4,789±563	4,879±1,281	0.950
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	N/A	4,502±756	NT	-

[a] paired Student's t-test
N/A: not applicable; NT: not tested

Table 2b. Results of the pairwise comparison of the serum amylase levels after 24 hours among groups (P values are reported in the table).

	S	**S-N**	**S-P**	**NaT**	**NaT-N**	**NaT-P**	**NaT-R**	**NaT-RP**
S: Sham	-	0.999	0.977	0.047	0.245	0.001	0.027	0.081
S-N: Sham + non specific IgG	0.999	-	1.000	0.541	0.636	0.005	0.134	0.310
S-P: Sham + anti-PAPII	0.977	1.000	-	0.792	0.863	0.017	0.292	0.553
NaT: 4% sodium taurocholate	0.047	0.541	0.792	-	1.000	0.512	0.996	1.000
NaT-N: 4% NaT + NS IgG	0.245	0.636	0.863	1.000	-	0.421	0.987	1.000
NaT-P: 4% NaT + anti-PAPII	0.001	0.005	0.017	0.512	0.421	-	0.943	0.757
NaT-R: 4% NaT + anti-Reg I	0.027	0.134	0.292	0.996	0.987	0.943	-	1.000
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	0.081	0.310	0.553	1.000	1.000	0.757	1.000	-

ANOVA with Tukey post-hoc comparison.

Table 2c. Results of the pairwise comparison of the serum amylase levels after 48 hours among groups (P values are reported in the table).

	S	**NaT**	**NaT-N**	**NaT-R**
S	-	0.065	0.087	0.012
NaT	0.065	-	0.999	0.846
NaT-N	0.087	0.999	-	0.773
NaT-R	0.012	0.846	0.773	-

ANOVA with Tukey post-hoc comparison.

with sham controls (5.61±0.25 mg/g *vs*. 4.54±0.21 mg/g, P=0.002) (Table 1). Pancreatitic animals treated with anti-Reg I or anti-PAPII antibodies did not significantly differ from NaT treated animals alone (P=0.959 and P=1.000). In contrast, pancreatitis animals treated with both anti-Reg I and anti-PAPII at 24 hours demonstrated significantly increased pancreatic wet weight (NaT-RP *vs*. NaT: 6.57±0.25 mg/g *vs*. 5.61±0.25; P=0.010). Furthermore, at 48-hour post pancreatitis induction, the NaT treated animals demonstrated improvement of edema compared with 24-hour time point (NaT 48 h *vs*. NaT 24 h: 4.64±0.14 mg/g *vs*. 5.61±0.25 mg/g, P=0.007) whereas anti-Reg I treated animals continued to demonstrate worsening evidence of edema (NaT-R 48 h *vs*. NaT-R 24 h: 6.60±0.21 mg/g *vs*. 5.90±0.16 mg/g, P=0.024). These data suggest that the degree of edema was worse when an animal received anti-Reg I and anti-PAPII antibody treatment and may continued to worsen over time.

Amylase

Animals induced with NaT pancreatitis had higher serum amylase levels at 24 hours when compared with sham controls (NaT *vs*. S: 4,250±471 U/L *vs*. 2,489±617 U/L, P=0.047) (Table 2). However, similar to pancreatic wet weight results, pancreatitic animals treated with anti-Reg I or anti-PAPII antibodies did not differ from NaT treated animals alone (P=0.996 and P=0.512, respectively). Although, pancreatitis animals treated with both anti-Reg I and anti-PAPII antibodies (4,502±756 U/L, P=1.000) did not significantly differ from NaT treated animals, they also trended toward increased amylase levels. Furthermore, at 48-hour post pancreatitis induction, the NaT pancreatitis animals treated with non-specific IgG demonstrated reduction of serum amylase (NaT-N 48 h *vs*. NaT-N 24 h: 2,610±281 U/L *vs*. 4,155±356 U/L, P=0.007) whereas anti-Reg I treated animals continued to maintain elevated amylase levels (NaT-R 48 h *vs*. NaT-R 24 h: 4,879±1,281 U/L, *vs*. 4,789±563U/L, P=0.950). These data suggest that serum amylase remained elevated over time when an animal received anti-Reg I antibody treatment in the setting of taurocholate-induced pancreatitis.

Serum C Reactive Protein and PAP Levels

CRP

Animals induced with NaT pancreatitis had non-significantly higher serum CRP levels at 24 hours when compared with sham controls (NaT *vs*. S: 1.73±0.68 mg/dL *vs*. 1.43±0.46 mg/dL, P=0.999). Furthermore, pancreatitic animals treated with anti-Reg I or anti-PAPII antibodies either individually or in combination did not differ from NaT treated animals alone (Table 3).

PAP

Animals induced with NaT pancreatitis had significantly higher serum PAP levels at 24 hours when compared with sham controls (298±33 µg/mL *vs*. 20±6 µg/mL, P<0.001). However, pancreatitic animals treated with anti-Reg I or anti-PAPII antibodies either individually or in combination did not differ from NaT treated animals alone (Table 3).

Histopathology

Animals induced with chemical pancreatitis demonstrated worsening pancreatitis by histopathology

as evidenced by increased leukocytic infiltration, hemorrhage and necrosis (Table 4). When NaT animals were injected with anti-Reg I and anti-PAPII antibodies, pancreatitis severity worsened as evidenced by increased leukocytic infiltration and tissue necrosis when compared with non specific antibody treatment (Table 4, Figure 3). This suggests that anti-Reg treatment directly worsened local pancreatitis severity as demonstrated by histopathology.

DISCUSSION

In our present study we have shown *in vivo* evidence that Reg proteins provide protection from severity of pancreatitis in that antibody neutralization of Reg proteins correlated with worsening pancreatitis on the local pancreas tissue. Reg proteins are well-known components in pancreatic juice. In 1979, DeCaro *et al*. described a novel protein found in patients with chronic pancreatitis; initially it was named human pancreatic stone protein (PSP) [14]. Subsequently, after further analysis, it was later renamed to pancreatic thread protein (PTP) by Gross *et al*. [15] and finally the name was changed to pancreatic Reg I, for it was shown to be expressed in regenerating islet cells following a near total pancreatectomy [16, 17]. We have shown that Reg I is mitogenic to both pancreatic beta- and ductal cells and co-precipitates with trypsin although the mechanism of action is yet to be elucidated [18]. Reg III, an isoform of the Reg protein family, is also known as pancreatitis associated protein (PAP) is comprised of three additional isoforms of PAPI, PAPII and PAPIII. The Reg proteins are coded for by genes present on chromosome 9 in the rat, span about 100 kb and possess over 60% homology with one another [3]. The exact function of Reg is largely unknown. Knockout studies of Reg I by Unno *et al*. did not demonstrate any significant physiological changes in a mouse animal model [19]. This could be due to redundancies in the Reg gene family which are able to compensate for one another in the event of individual Reg gene damage. To this end, Bodeker *et al*. have shown that PAPI interacts with PAPII, PAPIII and lithostatin (Reg I alpha) as well as itself to form homo/heterodimers, [20] which suggests that individual Reg proteins may provide overlapping function for other members of the Reg protein family. The large span of the entire Reg gene family renders classical gene knockout strategies, including Cre-Lox, an ineffective means to study Reg function *in vivo*. We have previously employed gene knockdown strategies to study Reg III. In those studies antisence mediated gene knockdown using a consensus sequence coding for a bioactive protein component common to all three PAP isoforms (GGWEWSN) [21], was able to decrease gene expression of PAPI, PAPII, and PAPIII and worsened pancreatitis severity in an animal model of acute pancreatitis [5]. Our studies are consistent with recent studies by Gironella *et al*. who have shown that PAP has anti-inflammatory effects in PAP knockout mice induced with pancreatitis [22]. However, in

Table 3a. Serum CRP and PAP levels evaluated after 24 hours (means±SEM).

Groups (6 rats in each group)	**CRP levels (mg/dL)**	**PAP levels (µg/mL)**
Normal	NT	<5
S: Sham	1.43±0.46	20±6
S-N: Sham + non specific IgG	1.42±0.41	25±9
S-P: Sham + anti-PAPII	1.44±0.66	29±8
NaT: 4% sodium taurocholate	1.73±0.68	298±33
NaT-N: 4% NaT + NS IgG	1.65±0.60	300±26
NaT-P: 4% NaT + anti-PAPII	1.77±0.61	330±20
NaT-R: 4% NaT + anti-Reg I	1.70±0.60	316±33
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	1.79±0.65	345±28

NT: not tested

Table 3b. Results of the pairwise comparison of the serum CRP levels after 24 hours among groups (P values are reported in the table).

Groups	**S**	**S-N**	**S-P**	**NaT**	**NaT-N**	**NaT-P**	**NaT-R**	**NaT-RP**
S: Sham	-	1.000	1.000	0.999	1.000	0.997	0.998	0.997
S-N: Sham + non specific IgG	1.000	-	0.999	1.000	1.000	1.000	1.000	1.000
S-P: Sham + anti-PAPII	1.000	0.999	-	1.000	1.000	1.000	1.000	1.000
NaT: 4% sodium taurocholate	0.999	1.000	1.000	-	1.000	1.000	1.000	1.000
NaT-N: 4% NaT + NS IgG	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000
NaT-P: 4% NaT + anti-PAPII	0.997	1.000	1.000	1.000	1.000	-	1.000	1.000
NaT-R: 4% NaT + anti-Reg I	0.998	1.000	1.000	1.000	1.000	1.000	-	1.000
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	0.997	1.000	1.000	1.000	1.000	1.000	1.000	-

ANOVA with Tukey post-hoc comparison.

Table 3c. Results of the pairwise comparison of the serum PAP levels after 24 hours among groups (P values are reported in the table).

Groups	**S**	**S-N**	**S-P**	**NaT**	**NaT-N**	**NaT-P**	**NaT-R**	**NaT-RP**
S: Sham	-	1.000	1.000	<0.001	<0.001	<0.001	<0.001	<0.001
S-N: Sham + non specific IgG	1.000	-	1.000	<0.001	<0.001	<0.001	<0.001	<0.001
S-P: Sham + anti-PAPII	1.000	1.000	-	<0.001	<0.001	<0.001	<0.001	<0.001
NaT: 4% sodium taurocholate	<0.001	<0.001	<0.001	-	1.000	0.964	0.999	0.754
NaT-N: 4% NaT + NS IgG	<0.001	<0.001	<0.001	1.000	-	0.975	1.000	0.794
NaT-P: 4% NaT + anti-PAPII	<0.001	<0.001	<0.001	0.964	0.975	-	1.000	1.000
NaT-R: 4% NaT + anti-Reg I	<0.001	<0.001	<0.001	0.999	1.000	1.000	-	0.980
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	<0.001	<0.001	<0.001	0.754	0.794	1.000	0.980	-

ANOVA with Tukey post-hoc comparison.

contrast their studies used cerulean as the inducing agent which induce pancreatitis by mechanisms distinct from NaT induction [23] and pancreatic necrosis was less severe with gene knockout compared with wild type mice. Furthermore, their studies investigated knockout effects of Reg IIIb/Reg II which is phylogenetically most consistent with the PAPI isoform of rat and PAPI is only 58% homologous to PAPII. Our studies investigated the role of PAPII with anti-PAPII antibodies raised to a oligopeptide sequence in the loop region of PAPII which has minimal overlap with any of the other PAP isoforms and its effect either alone or in conjunction with anti-Reg I antibody on pancreatitis severity. It is likely that the relationship of Reg and PAP isoforms to one another directly affect the disease course. Since gene knockdown technologies do not always translate into actual protein function [24] our current studies therefore investigated a protein knockdown approach by neutralizing Reg proteins with anti-Reg I and anti-PAPII specific antibodies.

The monoclonal anti-human Reg I antibody used was specific to Reg I found in the pancreas of rats. Despite having a homology of approximately 68% between rat and human Reg I [25], our anti-human antibody recognized the same epitope on rat Reg I. In addition, based on the ELISA data observed, we have shown we were able to saturate endogenous Reg I within the ductal juice of the rat pancreas. Based on previous studies [8] that have shown that approximately 15% of total protein content within rat pancreatic juice contains Reg, we were able to calculate that a total of 2 mg of antibody was needed to competitively bind endogenous Reg within the pancreatic juice. Reg III/PAPII is found in trace amounts in serum in healthy rats and increases in acute pancreatitis [3]. To this end since PAPII in serum and tissue appears to comprise a fraction of Reg I concentration. We injected 1.6 mg (total) of polyclonal anti-PAPII antibody into the pancreatic duct based on pilot studies which demonstrated that this amount of antibody would maximize pancreatitis (histology) presumably by inhibiting endogenous PAPII protein in the pancreatic parenchyma in acute pancreatitis (data not shown). It is possible that anti-Reg antibody administration could precipitate pancreatitis by forming antibody-antigen aggregates, protein plugs in the pancreatic ducts, or precipitates. In such a case one would likely expect to find dilated ducts, distinct areas of periductal leukocytic infiltrate

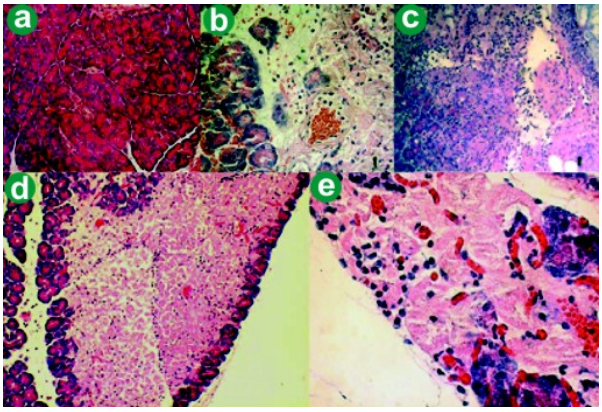

Figure 3. Photomicrographs of pancreatitis severity. Representative images of experiments described in Table 4. **a.** Normal rat pancreas. **b.** Pancreas from rats induced with pancreatitis (NaT) alone demonstrating leukocytic infiltration. **c.** After anti-Reg I or PAPII antibody treatment showing increased leukocytic infiltration. **d.** and **e.** After administration of both anti Reg I and PAPII demonstrating increased necrosis (**d.**), and leukocytic infiltration and hemorrhage (**e.**). (Magnification. a, c, d: 100x; b, e: 200x).

or evidence of complex deposition in these ducts all of which were not evident in our studies. This suggests that the worsening pancreatitis severity observed in pancreas tissue with anti-Reg or anti-PAP administration were likely due to antibody neutralization, although ultrastructural changes cannot be excluded [26]. Since PAP has been found to co-localize with intracellular zymogens in acinar cells [26], studies investigating the effect of anti-zymogen antibody neutralization in conjunction with anti-Reg and/or anti-PAP antibody administration with determination of antibody presence in duct and tissue are warranted to further elucidate the relationship of these proteins in the context of acute pancreatitis. It is also possible that the antibodies may be degraded by activated proteases when retroperfused into the duct, thereby underscoring the observed effects. Addition of protease inhibitor during antibody administration may have potentiated the observed responses.

The wet weights were used to determine the degree of edema following induction of pancreatitis. As was expected, NaT administration demonstrated increased wet weight compared with shams at 24 or 48 hours. It is interesting to note that only groups receiving combined anti Reg I and PAPII antibody treatment had worsening edema compared with NaT alone, whereas

Table 4. Histopathology scoring of pancreatitis severity (median values). Animals were sacrificed after 24 hours and pancreata were analyzed for pancreatitis severity. Severity was scored, by a pathologist blinded to specimens, (key: - no pancreatitis; + minimal; ++ mild; +++ moderate; ++++ severe) [8, 9].

Groups	Edema	Hemorrhage	Leukocyte infiltration	Tissue necrosis	Fat Necrosis
S: Sham	-	-	-	-	-
S-N: Sham + non specific IgG	+	-	-	-	-
S-P: Sham + anti-PAPII	-	-	+	-	-
S-R: Sham + anti-Reg I	-	-	-	-	-
NaT: 4% sodium taurocholate	-	+	++	++	+
NaT-N: 4% NaT + NS IgG	-	++	++	++	+
NaT-P: 4% NaT + anti-PAPII	-	+	+++	++++	++++
NaT-R: 4% NaT + anti-Reg I	-	++	++	+++	++
NaT-RP: 4% NaT + anti-Reg I + anti-PAPII	-	+	++++	++++	++++

individual antibody treatment did not differ from NaT induced animals. It is likely that with regard to wet weight, which is an index of pancreatic edema, there may be redundant protective mechanisms provided by both Reg isoforms. Only when both isoforms were neutralized was there evidence of increased edema. Furthermore, Reg I antibody treatment maintained the increased state of edema compared with NaT treatment alone. It could be that although anti-Reg I alone was unable to worsen pancreatitis compared with NaT treatment, it was able to maintain the severity induced by NaT.

Interestingly, although there was increased evidence of pancreatitis with NaT treatment, compared with sham controls, as evidenced by increased serum amylase and PAP levels, there were no differences with administration of either individual or dual anti Reg/PAP treatment compared with NaT treatment alone. Furthermore, there were no observed differences in CRP levels. This could be due to the fact that these represent systemic parameters of inflammation [27] and are not sensitive enough to be affected by antibody treatment. Additionally, the antibodies utilized in the ELISA were different from the experimental anti-PAPII that was administered to rats. Thus, the epitopes recognized by these antibodies differ, and the ELISA utilized may not be able to distinguish free PAPII, bound PAPII or PAPII-anti-PAPII complexes. Therefore, although the levels of PAPII in sera did not differ, it could be that the levels of active protein differed. However, the degree of pancreatic inflammation and necrosis with anti Reg treatment did increase with anti-Reg/PAP treatment. Taken together with the lack of differences in blood based markers of disease (amylase, CRP, PAP) this suggests that only the "local" pancreatic parenchyma is affected by Reg/PAP protein neutralization. We have demonstrated similar worsening of pancreatitis in the pancreatic organ with antisense gene knockdown of PAP [5]. In those studies knockdown of all three PAP isoforms correlated with increased inflammatory infiltrates and pancreatic edema. Taken together our data suggest that Reg proteins have a protective effect on pancreatitis. It is possible that the family of Reg proteins is critical in protecting the pancreas during inflammation. Another member of the Reg family may actually be more important in diminishing the inflammatory response following the induction of pancreatitis than Reg I. Other researchers [2] have also demonstrated that in acute pancreatitis, the expression of Reg I within the pancreas increased 100 fold, while the expression of Reg III/PAP increased on the order of 500-1,000 fold. In addition, serum levels of Reg III/PAP are markedly higher in the setting of acute pancreatitis in rats when compared to Reg I [18, 28]. Reg III/PAP is expressed in the same gene family, which is localized to 2p12 in humans [29, 30]. It is therefore likely that Reg I and Reg III/PAP act in concert to protect and stabilize the pancreas.

It has been suggested that Reg proteins have mitogenic and may have anti-apoptotic characteristics [31] and that Reg and its receptor are upregulated in acute pancreatitis [32]. Our collective data suggest that by interfering with the normal physiological functioning of Reg proteins, the balance between pro- and anti-apoptotic mediators in pancreatitis is shifted towards inducing cell death and subsequent necrosis. More in-depth analysis needs to be conducted to examine this interplay that Reg proteins may have in preventing cell death. Finally, since Reg proteins have been reported to have immunomodulatory effects, they may exert a direct role on the pathogenesis or progression of pancreatitis.

Acknowledgements The authors would like to thank Dr. Sameer Patel, Dr. Gabriel Levi, Gary Papierman, Marshall Weisberg, Hazel Drew, and Victor Ocasio for their efforts. In addition, the authors would also like to acknowledge Hans Von Gizycki for using SPSS to perform various statistical analyses for this project and Michael Kruger, MS for his statistical review of the manuscript

Research grant support This project was funded by the National Institute of Digestive and Kidney Disease (R01DK54511, MEZ)

Conflict of interest The authors have no potential conflicts of interest

References

1. Mayerle J, Hlouschek V, Lerch MM. Current management of acute pancreatitis. Nat Clin Pract Gastroenterol Hepatol 2005; 2:473-83. [PMID 16224479]

2. Graf R, Schiesser M, Lüssi A, Went P, Scheele GA, Bimmler D. Coordinate regulation of secretory stress proteins (PSP/reg, PAP I, PAP II, and PAP III) in the rat exocrine pancreas during experimental acute pancreatitis. J Surg Res 2002; 105:136-44. [PMID 12121700]

3. Zenilman ME, Tuchman D, Zheng Q, Levine J, Delany H. Comparison of reg I and III levels during acute pancreatitis in the rat. Ann Surg 2000; 232:646-52. [PMID 11066135]

4. Satomura Y, Sawabu N, Mouri I, Yamakawa O, Watanabe H, Motoo Y, et al. Measurement of serum PSP/reg - protein concentration in various diseases with a newly developed enzyme-linked immunosorbent assay. J Gastroenterol 1995; 30:643-50. [PMID 8574338]

5. Zhang H, Kandil E, Lin YY, Levi G, Zenilman ME. Targeted inhibition of gene expression of pancreatitis-associated proteins exacerbates the severity of acute pancreatitis in rats. Scand J Gastroenterol 2004; 39:870-81. [PMID 15513386]

6. Tezel E, Nagasaka T, Tezel G, Kaneko T, Takasawa S, Okamoto H, Nakao A. REG I as a marker for human pancreatic acinoductular cells. Hepatogastroenterology 2004; 51:91-6. [PMID 15011837]

7. Viterbo D, Bluth MH, Lin YY, Mueller CM, Wadgaonkar R, Zenilman ME. Pancreatitis-associated protein 2 modulates inflammatory responses in macrophages. J Immunol 2008; 181:1948-58. [PMID 18641332]

8. Carrère J, Guy-Crotte O, Figarella C. Human pancreatic reg protein immunoenzymatic assay and molecular form in serum. Clin Chim Acta 1998; 273:185-94. [PMID 9657348]

9. Chen CY, Lin XZ, Wu HC, Shiesh SC. The value of biliary amylase and Hepatocarcinoma-Intestine-Pancreas/Pancreatitis-associated Protein I (HIP/PAP-I) in diagnosing biliary malignancies. Clin Biochem 2005; 38:520-5. [PMID 15885230]

10. Lin YY, Bluth MH, Stanek A, et al. In vivo and in vitro administration of siRNA mediated gene knockdown of pancreatitis-associated protein (PAP) in experimental pancreatitis. Gastroenterology 2006; 130:A708.

11. Alhonen L, Parkkinen JJ, Keinanen T, Sinervirta R, Herzig KH, Jänne J. Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. Proc Natl Acad Sci U S A 2000; 97:8290-5. [PMID 10880565]

12. Niederau C, Liddle RA, Ferrell LD, Grendell JH. Beneficial effects of cholecystokinin-receptor blockade and inhibition of proteolytic enzyme activity in experimental acute hemorrhagic pancreatitis in mice. Evidence for cholecystokinin as a major factor in the development of acute pancreatitis. J Clin Invest 1986; 78:1056-63. [PMID 2428840]

13. Bluth MH, Smith-Norowitz TA, Drew H, Viterbo D, Lin YY, Pierre J, et al. A sensitive assay for the determination of pancreatitis-associated protein in experimental acute pancreatitis. Am J Clin Pathol 2006; 126:635.

14. Decaro A, Lohse J, Sarles H. Characterization of a protein isolated from pancreatic calculi of men suffering from chronic calcifying pancreatitis. Biochem Biophys Res Commun 1979; 87:1176-82. [PMID 111670]

15. Gross J, Carlson RI, Brauer AW, Margolies MN, Warshaw AL, Wands JR. Isolation, characterization, and distribution of an unusual pancreatic human secretory protein. J Clin Invest 1985; 76:2115-26. [PMID 3908481]

16. Yonemura Y, Takashima T, Miwa K, Miyazaki I, Yamamoto H, Okamoto H. Amelioration of diabetes mellitus in partially depancreatized rats by poly(ADP-ribose) synthetase inhibitors. Evidence of islet B-cell regeneration. Diabetes 1984; 33:401-4. [PMID 6323238]

17. Terazono K, Yamamoto H, Takasawa S, Shiga K, Yonemura Y, Tochino Y, Okamoto H. A novel gene activated in regenerating islets. J Biol Chem 1988; 263:2111-4. [PMID 2963000]

18. Levine JL, Patel KJ, Zheng Q, Shuldiner AR, Zenilman ME. A recombinant rat regenerating protein is mitogenic to pancreatic derived cells. J Surg Res 2000; 89:60-5. [PMID 10720454]

19. Unno M, Nata K, Noguchi N, Narushima Y, Akiyama T, Ikeda T, et al. Production and characterization of Reg knockout mice: reduced proliferation of pancreatic beta-cells in Reg knockout mice. Diabetes 2002; 51:S478-83. [PMID 12475793]

20. Bödeker H, Keim V, Fiedler F, Dagorn JC, Iovanna JL. PAP I interacts with itself, PAP II, PAP III, and lithostathine/regIalpha. Mol Cell Biol Res Commun 1999; 2:150-4. [PMID 10662590]

21. Zenilman ME, Zheng Q, Wu H, et al. Pancreatic reg and a conserved bioactive fragment are mitogenic through the MAPK p38 pathway. Surgical Forum 2000; LI:33-34.

22. Gironella M, Folch-Puy E, LeGoffic A, Garcia S, Christa L, Smith A, et al. Experimental acute pancreatitis in PAP/HIP knock-out mice. Gut 2007; 56:1091-7. [PMID 17409121]

23. Chan YC, Leung PS. Acute pancreatitis: animal models and recent advances in basic research. Pancreas 2007; 34:1-14. [PMID 17198179]

24. Voisin T, Lorinet AM, Laburthe M. Partial knockdown of G alpha i2 protein is sufficient to abolish the coupling of PYY receptors to biological response in renal proximal tubule cells. Biochem Biophys Res Commun 1996; 225:16-21. [PMID 8769088]

25. Okamoto H. The Reg gene family and Reg proteins: with special attention to the regeneration of pancreatic beta-cells. J Hepatobiliary Pancreat Surg 1999; 6:254-62. [PMID 10526060]

26. Morisset J, Iovanna J, Grondin G. Localization of rat pancreatitis-associated protein during bile salt-induced pancreatitis. Gastroenterology 1997; 112:543-50. [PMID 9024308]

27. Vermeire S, Van Assche G, Rutgeerts P. The role of C-reactive protein as an inflammatory marker in gastrointestinal diseases. Nat Clin Pract Gastroenterol Hepatol 2005; 2:580-6. [PMID 16327837]

28. Bödeker H, Fiedler F, Keim V, Dagorn JC, Iovanna JL. Pancreatitis-associated protein is upregulated in mouse pancreas during acute pancreatitis. Digestion 1998; 59:186-91. [PMID 9643677]

29. Miyashita H, Nakagawara K, Mori M, Narushima Y, Noguchi N, Moriizumi S, et al. Human REG family genes are tandemly ordered in a 95-kilobase region of chromosome 2p12. FEBS Lett 1995; 377:429-33. [PMID 8549770]

30. Perfetti R, Hawkins AL, Griffin CA, Egan JM, Zenilman ME, Shuldiner AR. Assignment of the human pancreatic regenerating (REG) gene to chromosome 2p12. Genomics 1994; 20:305-7. [PMID 8020983]

31. Dieckgraefe BK, Crimmins DL, Landt V, Houchen C, Anant S, Porche-Sorbet R, Ladenson JH. Expression of the regenerating gene family in inflammatory bowel disease mucosa: Reg Ialpha upregulation, processing, and antiapoptotic activity. J Investig Med 2002; 50:421-34. [PMID 12425429]

32. Bluth MH, Patel SA, Dieckgraefe BK, Okamoto H, Zenilman ME. Pancreatic regenerating protein (reg I) and reg I receptor mRNA are upregulated in rat pancreas after induction of acute pancreatitis. World J Gastroenterol 2006; 12:4511-6. [PMID 16874863]