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BASIC RESEARCH

Caerulin-induced pancreatitis in rats: Histological and genetic expression changes from acute phase to recuperation

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Abstract

AIM: To study the histological and pancreatitis-associated protein mRNA accumulation changes of pancreas from acute phase of caerulin-induced pancreatitis to recuperation in rats.

METHODS: Acute pancreatitis was induced by caerulein in male Wistar rats and followed up for 90 d by histological and mRNA analyses of pancreas. Pancreases were dissected at 0, 9, 24 h and 3, 5, 15, 30, 60, 90 d post-induction. Edema (E), polymorphonuclear neutrophil (PMN) infiltration, cytoplasmic vacuolization (V), zymogen granule depletion (ZD) and acinar disorganization (AD) were microscopically evaluated. Accumulation of pancreatitis-associated protein (PAP) and L13A mRNAs were quantified by real-time PCR.

RESULTS: The main histological changes appeared at 9 h post-induction for PMN infiltration and cytoplasmic V, while at 24 h and 3 d for E and ZD, respectively. All the parameters were recovered after 5 d, except for ZD which delayed more than 30 d. The main AD was observed after 15 d and values returned to normal after 30 d. Similarly to histological changes, accumulation of the PAP mRNA was increased at 9 h with the highest accumulation at 24 h and differences disappeared after 5 d.

CONCLUSION: From the acute phase to recuperation of pancreatitis, regeneration and re-differentiation of pancreas occur and PAP expression is exclusively an acute response of pancreatitis.

Key words: Acute pancreatitis; Histological changes; PAP mRNA; L13A mRNA

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with autodigestion of the gland, as a consequence of the intrapancreatic activation and releasing of digestive enzymes[1]. The pathogenic mechanisms involved in the development of acute pancreatitis are not well understood[2,3] although many studies on it are available. The acute period of pancreatitis is characterized by exocrine insufficiency as a result of important morphological alterations and changes on the expression of a number of genes in the pancreas[4,5]. If pancreatitis is not fatal, a stepwise regeneration of the morphology is followed[6].

Some events that regulate the severity of acute pancreatitis are known, involving common inflammatory and repair pathways[7,8]. Recently it was demonstrated that pancreatitis-associated protein (PAP), an acute phase protein, is itself an important determinant of disease severity[9]. The expression of this gene is low in the normal pancreas and becomes strongly augmentation after even mild pancreatic inflammation[10]. This augmentation is assumed in AP induced by caerulein, although it can be assayed in AP induced by retrograde injection of sodium taurocholate. Currently, pancreatitis is induced by supramaximal dose of caerulein and there is no report yet on histological and PAP mRNA changes in the pancreas as response of AP induction.

Therefore, the objective of this study was to evaluate the morphological and PAP mRNA changes in the acute and adaptive phases of pancreatitis induced by caerulein in rats. Morphological changes were evaluated by light microscopy and PAP mRNA levels by real-time PCR.

MATERIALS AND METHODS

Bioassay

The experiments were carried out on 33 male Wistar rats (mean weight 100.8 g and 4-wk old). The animals were fed with standard laboratory chow and water *ad libitum*

under controlled room conditions. Acute pancreatitis was induced according to the method of Dusseti *et al*[11]. The rats were injected intraperitoneally with caerulein (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 μg/kg of body weight per hour for 6 h. The rats were sacrificed at h 9 and 24, and on d 3, 5, 15, 30, 60 and 90 respectively. Three control rats without caerulein injection were sacrificed at h 0 and on d 30 and 90 and dissected. Prior to sacrifice, the rats were anesthetized with 10-15 mg of tiletamine chlorohydrate and zolazepam chlorohydrate (Zoletil 50®) per kg of body weight. Pancreas was dissected for histological or RNA analysis. Feeding protocol and animal handling were approved by the local ethics committee.

Histological examination

Pancreatic sections were fixed in 10% formaldehyde, embedded in paraffin, cut (3-5 μm in thickness) with a semi-motorized rotary microtome (Leica RM-2145) and mounted onto slides. The sections were processed for H&E staining and examined by conventional light microscopy (Olympus microscope B × 50) with 20 ×, 40 × and 100 × objectives. Eight random images by objective were recorded by microscope video camera. A pathologist who was blinded to the treatment protocol scored the tissues according to Kyogoku *et al*[12] for zymogen granule depletion (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%), interstitial edema (0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; 3, separated individual acini), polymorphonuclear neutrophil (PMN) infiltration (0, absent; 1, less than 20 PMNs per intermediate power field (IPF); 2, 20-50 PMNs per IPF and 3, more than 50 PMNs per IPF), grade of vacuolization based on the percentage of acinar cells with cytoplasmic vacuoles per IPF (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%) and acinar disorganization based on the percentage of area without normal acinar distribution (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%).

RNA preparation

Extracted pancreas was immediately rinsed with RNase-free water (treated with 0.05% diethyl pyrocarbonate, DEPC), homogenized in 1 mL of TRIzol® reagent (GIBCO-BRL, Grand Island, NY, USA), frozen and stored at -70℃. Total RNA was extracted by phenol/chloroform extraction and isopropanol precipitation[13]. Accumulation of PAP mRNA (GenBank access No. NM_053289.1) was analyzed as indicator of pancreatic injury. L13A mRNA (GenBank access No. X68282) was also analyzed as housekeeping gene presumed to be invariant[14]. All the primers were designed with Primers3 software and synthesized by Sigma Genosys (Woodlands, TX, USA). Their sequences and PCR product sizes are listed in Table 1. Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Invitrogen, Carlsbad, CA, USA) followed by reverse-transcribed using specific primers and SYBR Green RT-PCR reagents (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Synthesized complementary DNA (cDNA) corresponding to 100 ng total RNA was used for real-time PCR.

Table 1 Oligonucleotide primer pairs used for real-time quantitative PCR, binding site and expected size

Primer	Sequence	GenBank No.	Product size (bp)
PAPfw	5′ TGAATTATGTCAACTGGGAGAGG 3′	NM-053289.1	318
PAPrv	5′ TTACTGCTTTCCAAGACATGAGG 3′		
rL13A/Fw	5′ AAGCAGGTACTGCTGGG 3′	X68282	261
rL13A/Rv	5′ CCAACACCTTGAGGCGTT 3′		

Standard synthesis

Standards for the absolute quantification for PAP and L13A mRNAs were synthesized by Titan one Tube RT-PCR system (Roche Biochemicals, Indianapolis, IN, USA) with the same PCR parameters described above, according to the manufacturer's instructions. Then, PCR products were purified with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA) and quantified by fluorometry.

Quantitative PCR analysis

Real-time quantification of PAP and L13A mRNAs was performed with an iCycler iQ detection system (BIO-RAD, Hercules, CA, USA). SYBR Green I assay was used for quantification of all specific genes. For PAP mRNA quantification of each 50 μL SYBR Green PCR, 9.0 μL cDNA (corresponding to 100 ng total RNA), 1.8 μL sense primer (10 μmol/L), 1.8 μL antisense primer (10 μmol/L), 25 μL SYBR Green PCR Master Mix (PE Applied Biosystems) and 12.4 μL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94℃ for 4 min, then 35 cycles at 94℃ for 30 s, at 61℃ for 30 s and at 70℃ for 30 s, with a single fluorescence measurement at the end of the 70℃ for 30 s. For L13A mRNA quantification of each 50 μL SYBR Green PCR, 9.0 μL cDNA (corresponding to 100 ng total RNA), 1.2 μL sense primer (10 μmol/L), 1.2 μL anti-sense primer (10 μmol/L), 25 μL SYBR Green PCR Master Mix (PE Applied Biosystems) and 13.6 μL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94℃ for 3 min, then 38 cycles at 94℃ for 45 s, at 55℃ for 45 s and at 72℃ for 1 min, with a single fluorescence measurement at the end of the 72℃ for 30 s. In both cases, a melting curve program (60-95℃ with a heating rate of 0.5℃ for 30 s and continuous fluorescence measurement) and a cooling step to 4℃ were added.

Analysis and normalization of real-time PCR data

Optical data obtained by real-time PCR were analyzed by using the default and variable parameters available in the software provided with the iCycler iQ optical system (BIO-RAD, Hercules, CA, USA). The PCR threshold cycle number (CT) for each cDNA standard and cDNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10-20 SDs above the average background fluorescence. The number of amplicon cDNA copies was expressed relative to the amount of total cDNA present (ng). Although it might be difficult to determine the absolute amount of a

Table 2 Histological changes in the pancreas of rats with caerulein-induced acute pancreatitis (mean ± SE)

Period	Zymogen granule depletion	Edema	PMN infiltration	Vacuolization	Acinar disorganization
0 h	$0.25 \pm 0.23^{a,b}$	$1.25 \pm 0.31^{a,b}$	0.00 ± 0.14^{a}	0.25 ± 0.26^{a}	0.5 ± 0.36^{a}
9 h	$1.00 \pm 0.23^{b,c}$	$2.25 \pm 0.31^{b,c}$	0.75 ± 0.14^{b}	2.75 ± 0.26^{c}	$0.75 \pm 0.36^{a,b}$
24 h	1.40 ± 0.20^{c}	3.00 ± 0.28^{c}	0.60 ± 0.12^{b}	2.00 ± 0.24^{b}	$1.00 \pm 0.32^{a,b}$
3 d	3.00 ± 0.18^{e}	$2.17 \pm 0.25^{b,c}$	0.67 ± 0.11^{b}	2.00 ± 0.22^{b}	$1.33 \pm 0.29^{a,b}$
5 d	$2.00 \pm 0.23^{c,d}$	$1.25 \pm 0.31^{a,b}$	0.00 ± 0.14^{a}	$1.00 \pm 0.26^{a,b}$	$0.75 \pm 0.36^{a,b}$
15 d	$2.57 \pm 0.17^{d,e}$	0.86 ± 0.23^{a}	0.00 ± 0.10^{a}	0.00 ± 0.20^{a}	2.14 ± 0.28^{b}
30 d	1.60 ± 0.20^{c}	0.60 ± 0.83^{a}	0.00 ± 0.12^{a}	0.00 ± 0.24^{a}	$0.80 \pm 0.33^{a,b}$
60 d	0.00 ± 0.19^{a}	0.83 ± 0.25^{a}	0.00 ± 0.11^{a}	0.17 ± 0.22^{a}	0.66 ± 0.29^{a}
90 d	$0.25 \pm 0.13^{a,b}$	0.42 ± 0.18^{a}	0.00 ± 0.07^{a}	0.00 ± 0.15^{a}	$0.92 \pm 0.21^{a,b}$

Different letters within a same column differ significantly ($P < 0.05$).

cDNA present in different samples, quantification of test mRNA transcripts was normalized to a reference gene, the L13A gene.

Statistic analysis

Histological data were expressed as mean ± SE of the scores assigned by the pathologist and evaluated by ANOVA. Also, relation between PAP and L13A after absolute quantification was expressed as mean ± SE and compared using ANOVA test. $P < 0.05$ was considered statistically significant. NCSS 2001 statistical program was used.

RESULTS

Histological examination

At the beginning (0 h), the architecture of pancreas was normal (Figure 1) with acinar cells exhibiting the typical epithelial polarity. Basal portion of the cells contained their nuclei and apical portion of the secretory vesicles and zymogen granules. Acinar cells in AP displayed moderate vacuolization and light inflammatory infiltration of neutrophils, with the highest score after 9 h post-induction. After 5 d, levels returned to the normal scores maintained to the end of the study. Edema was increased 9 h post-induction with the highest score at 24 h and returned to the normal level on d 5. Zymogen granule depletion was most pronounced on d 3 with later recovery. However, an additional depletion occurred on d 15, returned to the normal levels 30 d post-induction (Table 2). Although some degree of acinar disorganization was observed in the pancreas 9 h after induction, a significant increase was observed on d 15, and returned to the normal level 30 d post-induction (Table 2).

Specific amplification

Melting curve analysis demonstrated that each of the primer pairs (Table 1) amplified a single predominant product with a distinct melting temperature (Tm) as shown for L13A and PAP cDNA in Figure 2. The Tm of products could be seen clearly as a peak in a first derivative plot. The rapid fall of Tm at 87.5℃ and 82.0℃ for L13A and PAP cDNA respectively indicated the presence of a specific product melting at this temperature.

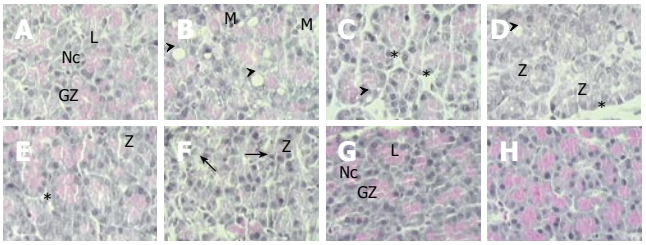

Figure 1 Light-microscopic sections of pancreas (X 133) from 0 h (**A**) and rats with caerulein-induced acute pancreatitis at 9 h (**B**), 24 h (**C**), 3 d (**D**), 5 d (**E**), 15 d (**F**), 30 d (**G**) and 60 d (**H**). Normal pancreatic acinus (0 h) shows zymogen granules (GZ) concentrated in the apical pole of the cell close to the acinar lumen (L). The basal region of the cell contains nucleus (N). In AP, PMN infiltration (M), vacuoles (arrowhead), edema (star), zymogen granule depletion (z) and acinar disorganization (arrow) were observed on d 15. On d 30, pancreatic acinus appearance was almost indistinguishable from that at 0 h.

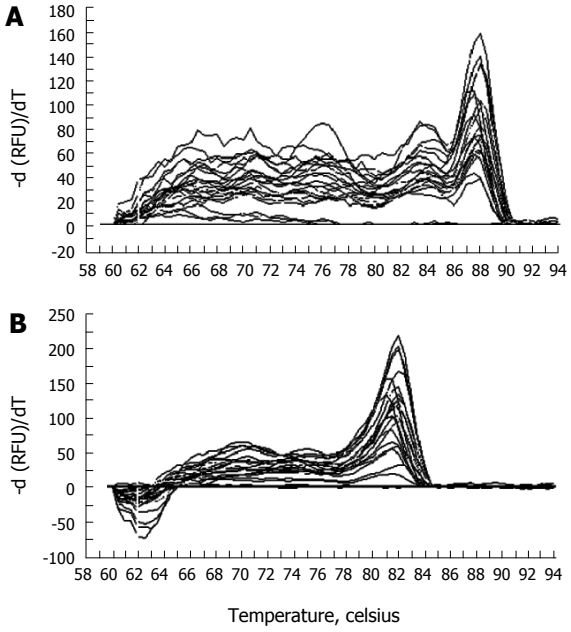

Figure 2 DNA melting curve analysis of amplification products for PAP mRNA (**A**) and L13A (**B**).

Absolute detection and quantification

The absolute quantification was estimated from real-time PCR analyses using a standard curve of tenfold serial dilutions ranging from 1.78×10^{9} copies to 1.78×10^{5} copies of L13A mRNA and 1.46×10^{9} copies to 1.46×10^{5} copies of PAP mRNA, in 50 μL of volume reaction. As shown in Figure 3, the mRNA of PAP was detected in basal levels without caerulein (0 h). However, after induction of AP, the PAP mRNA expression was slightly augmented after 9 h, and 3.8-fold the basal level at 24 h. After 5 d, differences disappeared and the accumulation of PAP mRNA was decreased gradually to the basal level.

DISCUSSION

The acute phase of pancreatitis is characterized by a pattern of changes in the morphology of exocrine pancreas, expression of secretory proteins and mRNA levels of different genes[4,6,15,16]. The observed changes in our study resemble a mild form of AP, characterized by edematous fluid in the extra-cellular space, causing separation of lobules

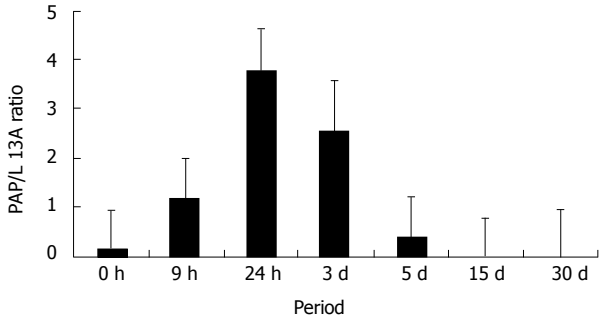

Figure 3 Relative quantification of PAP mRNA in caerulein-induced pancreatitis. Absolute quantification of PAP mRNA as copies was normalized to copies of L13A as determined from standard curves. All data are mean ± SE.

and acini[6]. Usually there is mild invasion by neutrophils, the acinar cells lose their zymogen granules, enlarging the acinar lumen. However, few studies[17-19] have analyzed the morphological changes after the acute phase of pancreatic response.

After acute response, re-differentiation of acinar cells and transition begin with regular acinar cell replete with dense zymogen granules beside cells with a smaller quantity of zymogen granules, on the same microscopic preparation. Intermediate cells change the typical architecture of the pancreas due to the loss of zymogen granules in a particular section. Additionally, the rough endoplasmic reticulum might be reduced and/or rearranged[6]. In this study, zymogen granule depletion and increase of acinar disorganization were presented after the acute phase of pancreatitis (determined by maximal PAP synthesis and its mRNA accumulation according to Iovanna *et al*[4] and our results, respectively). These changes are explained in the context of reconstitution of the normal morphology and function of the pancreas.

De Lisle and Logsdon[20] have shown the differentiation and re-differentiation of mouse pancreatic acinar cells. They immunolabeled acinar cells with monoclonal antibodies specific for acinar or duct cells and showed that more than 97% of the labeled area is acinar positive on d 3, which decreases to approximately 16% on d 9, and then returns to over 97% by day 21 of culture[20]. Lechene de la Porte *et al*[21] induced pancreatitis by the injection of trypsin in multiple sites of the rat pancreas and reported that pancreatic repair involves proliferation of cells from intact acini and tubular complexes. This process of differentiation and re-differentiation of pancreatic acinar cells explains our finding of the highest score for acinar disorganization on d 3 and 15 as well as complete recovering of zymogen granules 30 d post-induction.

In pancreatitis not only morphological changes were observed, but also molecular modifications occurred. Iovanna *et al*[4] showed that PAP is significantly increased 48 h after induction of pancreatitis by retrograde injection of sodium taurocholate in rats, compared to normal pancreas. In spite of methodological differences in the quantified PAP, our results agree with those of Iovanna *et al*[4]. However, PAP mRNA accumulation is quite different between both studies. Differences could be attributable to the pancreatitis induction method, the severity of the problem, as well as the quantification method itself. In our study, after absolute quantification, values were normalized in respect to a housekeeping gene (L13A). Therefore, quantification by real-time PCR may better reflect the actual physiological changes.

Histology of pancreas showed a mild edematous pancreatitis induced by caerulein and the acute response was followed by recuperation process after 15 d of induction, according to acinar disorganization and zymogen granule depletion scores. Although PAP is related to the severity of pancreatitis, its function *in vivo* is not well established. Our findings support that PAP is a protein of acute response, because changes in levels are not observed in reconstitution of the pancreatic normal morphology. In addition, the PAP mRNA changes in AP induced by caerulein, measured by a most sensible technique of quantification as real-time PCR, are similar to those from AP induced by sodium taurocholate.

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