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Gene Expression Profiles in Cells of Peripheral Blood Identify New Molecular Markers of Acute Pancreatitis

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Abstract

Introduction—Blood leukocytes play a major role in mediating local and systemic inflammation during acute pancreatitis. We hypothesize that peripheral blood mononuclear cells (PBMC) in circulation exhibit unique changes in gene expression, and could provide a "reporter" function that reflects the inflammatory response in pancreas of acute pancreatitis.

Methods—To determine specific changes in blood leukocytes during acute pancreatitis, we studied gene transcription profile of in peripheral blood mononuclear cells (PBMC) in a rat model of experimental pancreatitis (sodium taurocholate). Normal rats, saline controls and a model of septic shock were used as a controls. cRNA obtained from PBMC of each group (n = 3) were applied to Affymetrix rat genome DNA Gene Chip Arrays.

Results—From the 8,799 rat genes analyzed, 140 genes showed unique significant changes in their expression in PBMC during the acute phase of pancreatitis, but not in sepsis. Among the 140 genes, 57 were upregulated, while 69 were downregulated. Platelet-derived growth factor receptor, prostaglandin E2 receptor and phospholipase D1 are among the top upregulated genes. Others include genes involved in G protein-coupled receptor and TGF-β-mediated signaling pathways, while genes associated with apoptosis, glucocorticoid receptors and even the cholecystokinin receptor are downregulated.

Conclusions—Microarray analysis in transcriptional profiling of PBMC showed that genes that are uniquely related to molecular and pancreatic function display differential expression in acute pancreatitis. Profiling genes obtained from an easily accessible source during severe pancreatitis may identify surrogate markers for disease severity.

Keywords

Acute pancreatitis; oligonucleotide microarray; abdominal sepsis; peripheral blood mononuclear cells; PBMC

Introduction

Acute pancreatitis is a severe inflammatory disease frequently diagnosed by acute abdominal pain associated with a concomitant rise of serum amylase and lipase concentration. There are over 180,000 new cases per year in the United States (1). While the injury and systemic manifestations are typically mild, up to 20% of the patients will exhibit a severe reaction-typified by pancreatic necrosis- and among them, the morbidity can be over 80%, and mortality about 25% (2). The pathophysiology of the disease includes the activation and release of

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pancreatic enzymes within the ductal system (3-5), the autodigestion of the pancreas (6) and multiple organ dysfunction following their release into the systemic circulation. A major challenge has been to identify markers of disease severity.

It likely that the synthesis and release of pro-inflammatory cytokines and chemokines are responsible for progression of the local injury to the pancreas and retroperitoneum (7). Also, inflammatory mediators produced within the gland increase the pancreatic injury and spread to distant organs (8-10), transforming a local inflammation into a severe systemic disease. The mediators involved in this systemic inflammation are similar to those encountered during sepsis.

Because it is important to predict the severity of the disease as early as possible in order to optimize the therapy and to prevent organ dysfunction and local complications, several scores such as Ranson, Glasgow and the Acute Physiology Anad Chronic Health Evaluation (APACHE II) (11-15) scores have been used. New serum markers have emerged and their ability to provide additional information on the severity of the disease has been evaluated (15).

The current prognostic indicators available for acute pancreatitis rely heavily on clinical markers combined with an increase in serum levels of proteins such as C reactive protein & trypsinogen-activation peptide. Newer proteins such as PAP (16) have not reached clinical utility as originally hoped.

Since pancreatitis is an immunologic response to the injury, we explored the possibility that peripheral blood mononuclear cells (PBMCs) would harbor specific markers of progression of disease. We employed gene chip microarray technology to study genetic expression patterns of PBMCs obtained from rats with pancreatitis. While typically one compares these patterns to normal controls, we further compared them to PBMCs obtained from rats with intra-abdominal bacterial sepsis, to elucidate unique patterns of expression in acute pancreatitis.

Methods

Animals

All animal experiments in this study were performed with the approval of the Animal Care and Use Committee (ACUC) of SUNY Downstate Medical Center and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats obtained from Harlan Sprague Dawley (Indianapolis, IN) weighing from 225-250 g were used for experiments. Animals were allowed *ad libitum* exposure to food and water before study, and randomly assigned to control or experimental groups.

Experimental acute pancreatitis

Acute pancreatitis was induced using retrograde infusion of 4% sodium taurocholate (NaT) (Sigma, St. Louis, MO) into the pancreatic duct as previously described (17). Briefly, under pentobarbital (Abbott Laboratories, North Chicago, IL) anesthesia (50 mg/kg given intraperitoneally), a midline incision was performed. The common bile duct was identified and cannulated in an antegrade direction with PE-10 tubing (Fisher Scientific, Pittsburgh, PA) 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 infused into the pancreatic duct at a rate of 1 ml/kg over 10 min.

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Experimental septic shock

Sepsis was induced by cecal ligation and puncture (CLP) as described by previously described (18). Briefly, under pentobarbital anesthesia, a laparotomy was performed (the size of the incision was 2.5 cm), and the cecum was ligated just below the ileocecal valve with a 3-0 silk ligature and the antimesentric cecal surface was punctured once with a 16-gauge needle proximal to the ligature. The cecum was then returned to the peritoneal cavity and fecal content in the ligated segment was allowed to extruded through the puncture to the peritoneum. The peritoneum and abdominal muscles were closed with silk sutures. After CLP, rats were returned to cages and allowed *ad libitum* access to food and water. For both experimental model of acute pancreatitis or sepsis, control rats were anesthetized and sham operated with a laparotomy. Pancreas or cecum was manipulated but neither pancreatitis induction nor CLP procedure was performed.

Peripheral blood mononuclear cell (PBMC) isolation

Twenty-four hr after pancreatitis, septic shock induction, sham operation with saline infusion or in fasted untouched, normal control animals (n =3 each group, n=12 total), approximately 8-10 mL whole blood were collected via inferior vena cava (IVC) from each rat under pentobarbital anesthesia. PBMC were isolated from rat whole blood by centrifugation through Ficoll-Paque (Pharmacia, Biotech AB, Uppsala, Sweden) (19).

Preparationi of cRNA and GeneChip hybridization

Preparation of cRNA, hybridization, and scanning of high-density oligonucleotide microarray were performed according to manufactures's protocol (Affymetryix, Inc., Santa Clara, CA). Briefly, total RNA was extracted from PBMC using TRIzol (GIBCO BRL Life Technologies, Grand Island, NY) and eluted using a RNeasy spin column (Qiagen, Inc., Valencia CA). Ten micrograms of total RNA was converted into double-stranded cDNA by reverse transcription using SuperScript Choice System (Invitrogen Corporation, Carlsbad, CA) with the T7-$(dT)_{24}$ primer [5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (dT_{24})]. The double-strand cDNA product was extracted with phenol/chloroform/isoamyl alcohol using phase lock gels (Eppendorf, Westbury, NY). Double-strand cDNA was in vitro transcribed into cRNA and nucleotides were biotinylated using the Enzo BioArray HighYield RNA Treanscrip Labeling Kit (Affymetrix). The in vitro transcription product was further purified using RNeasy mini columns (Qiagen) and fragmented as previously described (20). Fragmented in vitro transcription product was hybridized onto the rat genome U34A DNA GeneChip Array (Affymetrix) which contains approximately 7,000 full-length sequences and 1,000 EST clusters. The sequences were selected from from the UniGene database. All 12 PBMC samples were subjected to RNA extraction and transcript profiling.

Data and statistical Analysis

An absolute expression analysis was performed using Affymetrix MAS 5.0, and the data from genes were imported into GeneSpring software version 5.1 (Silicon Genetics, Redwood City, CA) for further analyses. Differentially expressed genes were selected. Differential expression was defined as a change of at least two fold versus respective controls. Non-parametric test was used, assuming non-equal means, specifically the Welch t-test and Welch ANOVA. Significance level was set at > 2-fold change between groups, $P < .05$.

Results

Rats treated with NaT showed pancreatic edema and necrosis, as we have previously observed (17,21). Those treated with CLP were not autopsied. Adequate numbers of PBMCs were obtained from each animal studied for gene array analysis.

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We compared gene transcription profiles of PBMCs in normal, untouched control animals, to those with acute pancreatitis, to identify those genes induced in pancreatitis (Figure 1). From the 8,799 rat gene analyzed on the chip, we identified 947 genes significantly changed by 2-fold in pancreatitis.

These 947 genes were then subjected to comparison between animals with acute pancreatitis and saline controls, and identified 170 genes which changed expression (Figure 2). Similarly, 201 unique genes were identified between between rats with cecal ligation and puncture (abdominal sepsis) and saline controls, and 409 differentially expressed genes were identified between animals with acute pancreatitis and intra-abdominal sepsis.

As shown in Figure 2, of the 170 genes changed between pancreatitis and saline controls, 15 overlapped when compared to septic/pancreatitic animals, and another 18 overlapped with sepsis/normal animals. In total, 140 genes were unique to PBMCs in animals with pancreatitis. Figure 3 shows a cluster analysis of the genes from the three groups analyzed in Figure 2.

Among the 140 genes whose expression changed in pancreatitis alone, 57 were upregulated, while 69 were downregulated; 25% corresponded to ESTs. Table 1 and 2 shows the most significantly (greater than threefold) up-regulated (n=14) and down-regulated genes (n=33), respectively, and their known functions.

Discussion

Acute pancreatitis is a disease which begins with an insult followed by autodigestion of pancreatic tissue. Inflammatory cells are recruited into the organ, an inflammatory cascade is induced, and this leads to local and systemic sequelae, which can include the respiratory, immunologic and cardiac systems. 10-20% of patients will develop severe disease, and of those, up to 50% can die. While patients can die from overwhelming sepsis as a result of bacterial translocation (22,23), most patients are not infected in the first few weeks of the disease (24). Therefore, disease severity and prognostic indicators are not initially associated with bacterial infection.

The early immunologic response leads to the severe systemic reaction, and likely the subsequent morbidity. The immunologic response in acute pancreatitis is different from abdominal sepsis, since the pancreas is not invaded by bacteria at the early stages; no abscesses are seen until late, and the time lines are very different.

Predicting which patients are going to progress to severe disease has been a challenge for physicians. To date, very few serum markers have been shown to be effective predictors, and the best prognostic indicators are still the clinical parameters (2,11-15,24,25). Our lab has been interested in identification of new potential markers.

One method to identify new potential markers is to map out those genes which are uniquely expressed during the disease process. To date, no one has actually studied the role of blood derived inflammatory cells as markers for pancreatic inflammation. We postulate that the response to pancreatitis, and potentially the prognostic indicator of disease severity, rests in peripheral blood mononuclear cells. These are the cells which mediate the immunologic reaction to pancreatitis. While investigators have measured gene expression of the pancreas in experimental pancreatitis (26), and others have studies changes in the population of leukocytes in pancreatitis (27), no one has studied the genetic map of PBMC during disease.

We postulated that peripheral blood mononuclear cells can serve a “reporter function” as biomarkers of acute pancreatitis, and as such would express unique genes during pancreatitis. Further, PBMC represent an easily accessible, non-invasive source of material. We employed

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microarray technology to asses the gene transcription profile of PBMCs in a rat model of sodium taurocholate-induced acute necrotizing pancreatitis. We first compared the profile of pancreatitic animals with pancreata from normal, unoperated controls. This identified 947 genes induced during pancreatitis. In order to determine which of these genes were uniquely expressed during pancreatitis, we compared the PBMC RNA obtained from rats induced with pancreatitis with PMBC RNA obtained from rats who underwent saline infusion alone, and with rats with intra-abdominal sepsis.

We identified 140 unique genes which were induced or inhibited in PBMCs during NaT-induced (necrotizing) pancreatic disease. Not surprisingly, some of the genes highly induced are of cytokines previously implicated in pancreatitis, such as receptors for platelet-derived factor, transforming growth factor-beta, and a variety of G-protein related signal transduction genes. The role of phospholipase D gene 1 is involved in the intracellular modulation of cellular mitogenesis and even pancreatic organ regeneration (28,29). The prostaglandin E2 receptor is induced, which is interesting since inhibition of PGE2 by cyclo-oxygenase inhibitors improves survival (30)

Interestingly, genes associated with cell death, such as caspase 1 and BH3 interacting domain 3, and cell membrane integrity were uniquely downregulated in PBMCs of rats with acute pancreatitis. Caspase 1, associated with cellular apoptosis, has been studied in acute pancreatitis (31), its activation within the pancreas is associated with severe necrosis (32), but its inhibition may be protective in sepsis (33). BH3 domain proteins, also involved in mitochondrial-mediated cell death (34) via BCL-2 mediate apoptosis (35). How these PBMC-associated apoptosis processes relate to pancreatic necrosis remains to be studied.

What is striking is that some genes associated with the disease of pancreatitis, namely glucocorticoid receptor, cholecystokinin receptor and lipase are significantly inhibited, each by 7-fold. We have shown that glucocorticoid treatment can improve survival in animal models of pancreatitis (36), and others have described a relationship between glucocorticoids and the cholecystokinin receptor in the disease process (37). Use of either compound after the induction of the disease has not been studied.

Our data show that in acute pancreatitis, PBMCs express genes which are related to pancreatic illness and not intra-abdominal sepsis. This observation, that pancreatitis-related genes are induced in cells outside the pancreas -in peripheral blood mononuclear cells- during necrotizing pancreatitis, has not been previously described. It should not be surprising that such genes are induced- these genes were originally identified from inflamed pancreatic tissue which likely already had similar PBMCs infiltrating them. But, since the PBMCs are involved in the systemic inflammatory response to pancreatitis, following their expression/activation during the evolution of pancreatitis should be illuminating. The ability of easily accessible blood leukocytes to provide a reporter function for solid organ disease will likely prove useful in pancreatitis as we have shown in other diseases (38). Mapping the expression pattern of these genes in the clinical arena might help differentiate the patients who are suffering from mild, moderate and severe pancreatitis, including necrosis and systemic complications. Future studies will establish a panel of these genes to differentiate between sepsis, pancreatitis and severe, necrotizing pancreatitis and determine if serum concentration of these new molecular markers - obtained from cells within the blood which modulate the inflammatory response to pancreatitis - correlate to the severity of pancreatitis. If they predict occurrence of multiple organ dysfunction, it is then conceivable that these markers will predict the outcome of the disease.

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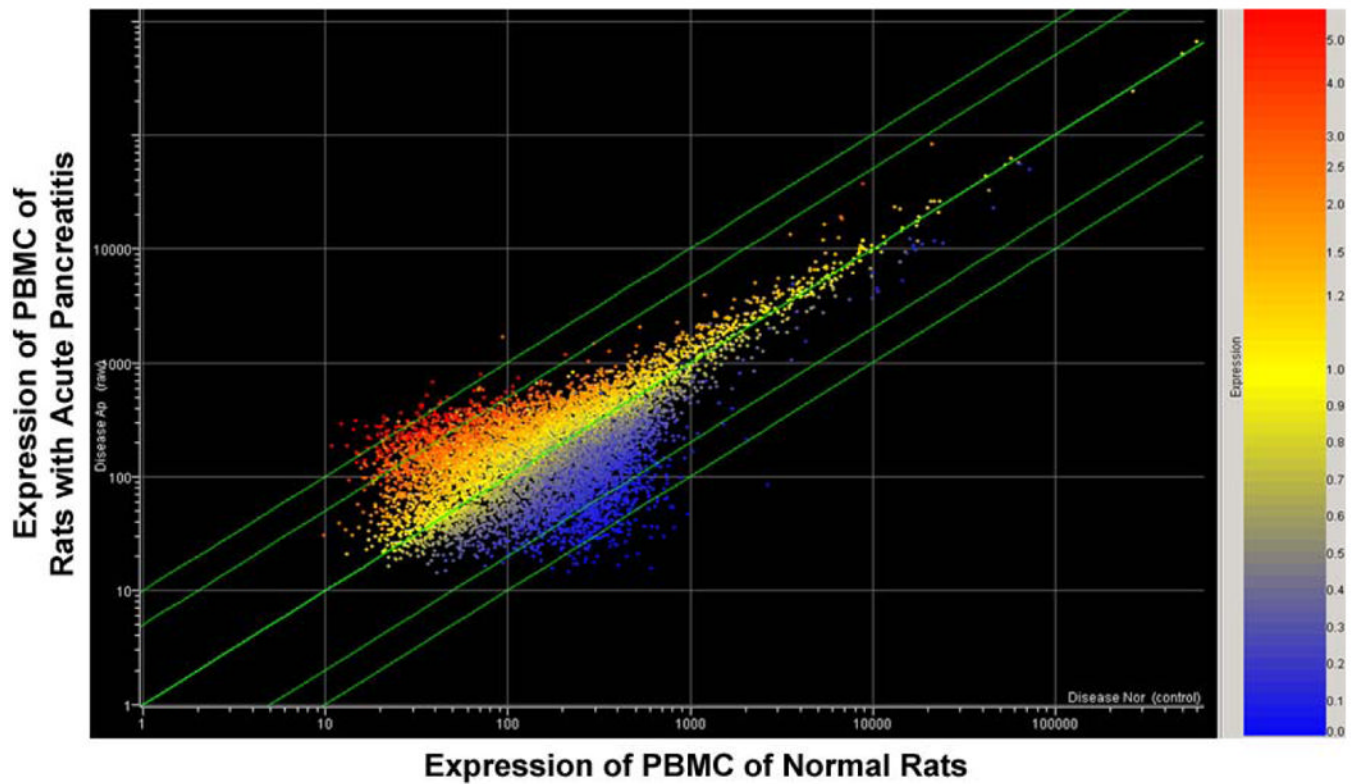

Figure 1.

Scatter plot of the differentially express genes in peripheral blood mononuclear cells isolated from rats with acute pancreatitis compared to those from normal, control, unoperated animals. The axes are arbitrary fold changes. The red points above the line are up-regulated genes, the blue ones below are down-regulated genes. 947 genes were identified and subjected to further analysis (see Figure 2).

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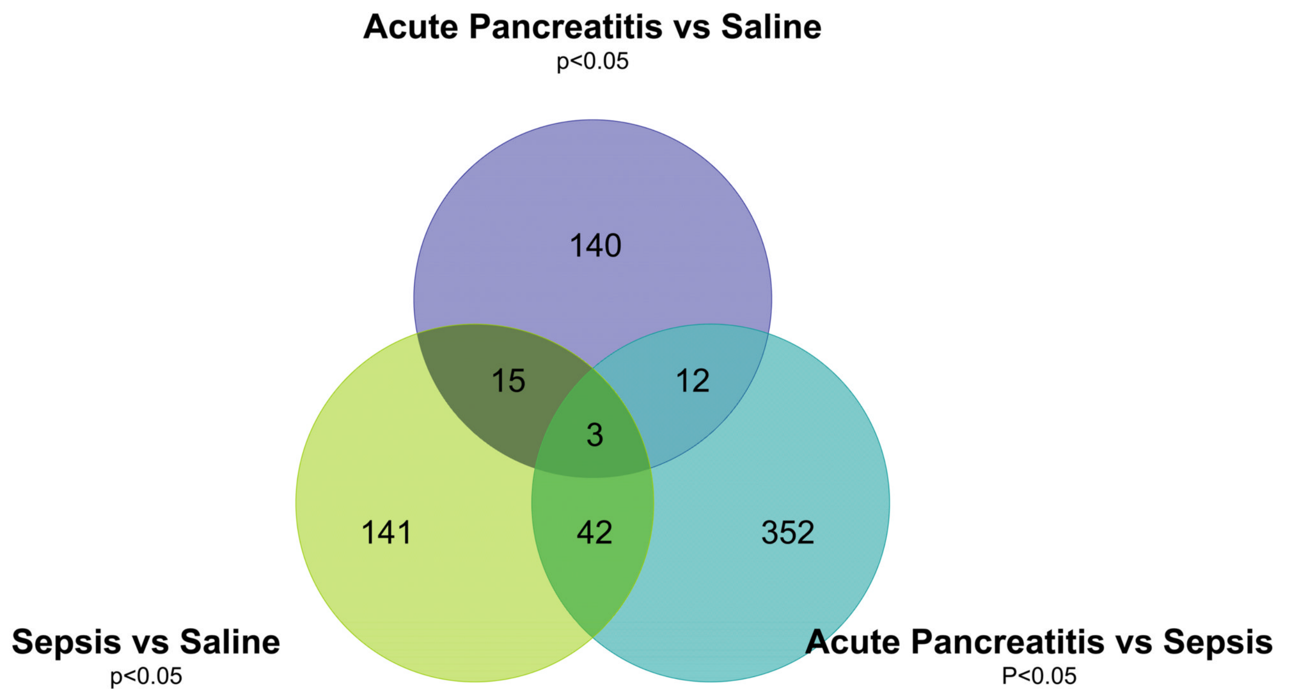

Figure 2.

Venn diagram of differential expression of the 947 genes (identified in Figure 1) in peripheral blood mononuclear cells. Expression was compared from rats who underwent laparotomy and ductal infusion with saline, to those undergoing infusion with sodium taurocholate to induce with acute pancreatitis, to those with intra-abdominal sepsis induced by cecal ligation and puncture. 140 genes induced specifically during sodium taurocholate acute pancreatitis were identified, the most induced and inhibited are depicted in Tables 1 and 2 respectively.

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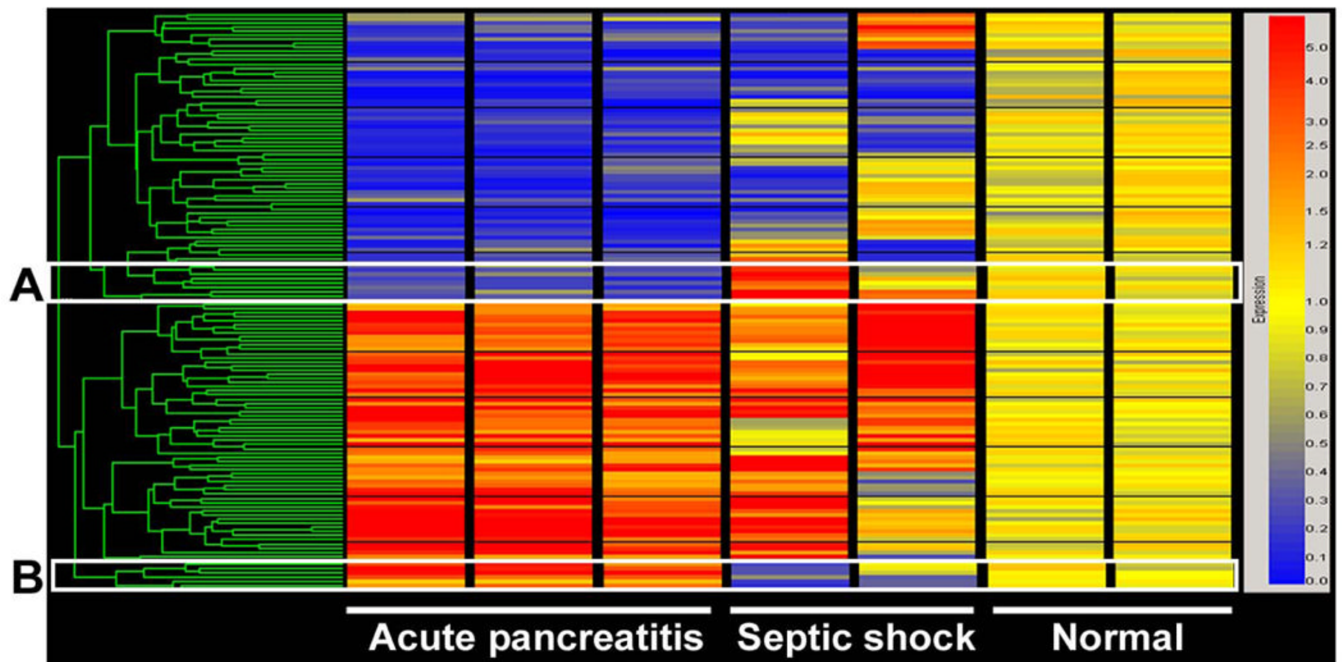

Figure 3.

Cluster view of genes differentially expressed in PBMC isolated from rats with acute pancreatitis. (A.) Genes downregulated in acute pancreatitis, but upregulated in abdominal sepsis. (B.) Genes upregulated in acute pancreatitis, but downregulated in abdominal sepsis. The ‘normal’ column is RNA from saline-infused control animals.

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Table 1

Upregulated genes, which are differentially expressed in PBMC in acute pancreatitis, but not in septic shock or normal conditions (3×over controls, n=14).

Fold Change	P	Genbank	Name	Description	Biological Process
U11.9	0.02	AF017251	Pld1	Phospholipase D gene 1	intracellular signaling cascade, phospholipase D
U10.7	0.03	AI232379	Pdgfra	Platelet-derived growth factor receptor alpha	protein amino acid phosphorylation, transmembrane receptor protein tyrosine kinase
U6.1	0.03	Y07903	Adam3	a disintegrin and metalloprotease domain 3 (cyritestin)	Pep_M12B_propep;metalloendopeptidase
U5.5	0.03	U94708	Ptger2	prostaglandin E receptor EP2 subtype	GPCRs Class A Rhodopsin-like; Small ligand GPCRs rhodopsin-like receptor
U5.5	0.03	U08976	Ech1	enoyl coenzyme A hydratase 1	Enzyme
U5.0	0.01	Z22867	Pde3b	phosphodiesterase 3B	signal transduction
U4.6	0.01	Z21935	Mapk4	Rat protein kinase rMNK2.	protein amino acid phosphorylation ATP binding; protein serine/threonine kinase
U4.3	0.03	L09653	Tgfbr2	transforming growth factor, beta receptor 2	TGF Beta Signaling Pathway
U4.3	0.03	L09653	Tgfbr2	transforming growth factor, beta receptor 2	pkinase;protein kinase, proteolysis and peptidolysis, subtilase
U3.8	0.03	L07736	Cpt1a	carnitine palmitoyltransferase 1	Fatty Acid Degradation; Mitochondrial fatty acid Betaoxidation, fatty acid metabolism, mitochondrion; membrane fraction Carn_acyltransf;acyltransferase
U3.5	0.04	U52948	C9	complement component 9	Complement Activation Classical
U3.4	0.02	L26986	Adcy8	adenylyl cyclase 8	G Protein Signaling;intracellular signaling cascade
U3.4	0.02	J04486	Igfbp2	Insulin-like growth factor binding protein 2	insulin-like growth factor binding
U3.4	0.02	L26986	Adcy8	adenylyl cyclase 8	guanylate cyclase

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Table 2

Down-regulated genes, which are differentially expressed in PBMC in acute pancreatitis, but not in septic shock or normal conditions (3× under control, n=33).

Fold Change	P	Genbank	Name	Description	Biological Process
D10.7	0.04	J00758	Ngfg	rat pancreatic preprokallikrein	serine-type endopeptidase
D9.3	0.00	AB017044	Hnf3g	rat hepatocyte nuclear factor 3 gamma	regulation of transcription, DNA-dependent
D9.8	0.04	U14647	Casp1	caspase 1	proteolysis and peptidolysis
D9.6	0.05	AA851223	Eno3	enolase 3, beta, muscle	Glycolysis, gluconeogenesis phosphopyruvate hydratase complex
D9.3	0.00	AB017044	Hnf3g	rat hepatocyte nuclear factor 3 gamma	nucleus
D8.8	0.03	AA925846	Bid3	BH3 interacting domain 3	apoptosis;inferred from mutant phenotype protein binding
D8.5	0.05	AA874784	Lipa	lipase A, lysosomal acid	sterol esterase
D7.6	0.02	Y00156	UDPGT	UDP-glucuronosyltrans ferase 2B3 precursor, microsomal	UDPGT;transferase, transferring hexosyl groups
D7.5	0.01	L15079	Pgy3	P-glycoprotein 3/multidrug resistance 2	ATP-binding cassette (ABC) transporter Membrane transport
D7.2	0.04	AI137538	Grl	glucocorticoid receptor	regulation of transcription, DNA-dependent, nuclear receptors
D7.2	0.04	L32591	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	Cell cycle
D7.2	0.04	M99418	Cckbr	Cholecystokinin B receptor	rhodopsin-like receptor
D7.2	0.04	L32591	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	Ribosomal_L7Ae;structural constituent of ribosome
D7.1	0.00	AB003478	B3galt4	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase	Galactosyl_T;galactosyltransferase
D6.6	0.05	U28938	Ptpro	protein tyrosine phosphatase, receptor type, O	transmembrane receptor protein tyrosine phosphatase signaling pathway
D6.2	0.01	S49400	Ptpn5	protein tyrosine phosphatase, non-receptor type 5	protein tyrosine phosphatase, protein amino acid dephosphorylation
D5.9	0.03	L09120	Capn	calpain 2	calpain; calcium ion binding, proteolysis and peptidolysis
D5.6	0.02	S74898	Ptgfr	prostaglandin F2 alpha receptor	membrane
D5.3	0.01	D16349	Oprm1	Opioid receptor, mu 1	immune response, GPCRs Class A Rhodopsin-like; Peptide GPCRs, plasma membrane
D5.3	0.01	Z22867	Pde3b	phosphodiesterase 3B	3′,5′-cyclic-nucleotide phosphodiesterase
D4.7	0.03	AA997614	Cyp51	Cytochrom P450 Lanosterol 14 alpha-demethylase	Monooxygenase, electron transport
D4.7	0.04	D29766	Crkas	v-crk-associated tyrosine kinase substrate	cell adhesion
D4.5	0.04	AF012347	Madh9	MAD homolog 9 (Drosophila)	TGF Beta Signaling Pathway, MH1 regulation of transcription, DNA-dependent
D4.5	0.05	AA945583	Hsd17b10	hydroxysteroid (17-beta) dehydrogenase 10	adh_short;oxidoreductase

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Fold Change	P	Genbank	Name	Description	Biological Process
D4.4	0.00	M85299	Slc9a1	Solute carrier family 9, antiporter, Na+/H+	G Protein Signaling, sodium transport integral plasma membrane protein, Na_H_Exchanger; solute:hydrogen antiporter
D4.4	0.00	U33287	Casq2	calsequestrin 2	calcium ion storage
D3.6	0.01	L26110	Tgfbr1	transforming growth factor, beta receptor 1	TGF Beta Signaling Pathway
D3.5	0.04	X06564	Ncam	neural cell adhesion molecule	cell adhesion; post-translational membrane targeting
D3.3	0.04	X72757	Cox6a1	rat cox Via gene (liver).	electron transport
D3.3	0.01	U22296	Csnk1g1	casein kinase 1 gamma 1	pkinase;protein kinase
D3.3	0.04	X72757	Cox6a1	R.norvegicus cox Via gene (liver).	cytochrome c oxidase
D3.2	0.02	M64780	Agrn	Agrin	serine protease inhibitor plasma membrane organization and biogenesis
D3.1	0.05	U24150	Tsc2	Tuberous sclerosis 2, (renal carcinoma)	GTPase activator, cell growth and/or maintenance