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Proteomic analysis of apoptotic and oncotic pancreatic acinar AR42J cells treated with caerulein

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Abstract This study aims to determine the differentially expressed proteins in the pancreatic acinar cells undergoing apoptosis and oncosis stimulated with caerulein to explore different cell death process of the acinar cell. AR42J cells were treated with caerulein to induce cell model of acute pancreatitis. Cells that were undergoing apoptosis and oncosis were separated by flow cytometry. Then differentially expressed proteins in the two groups of separated cells were detected by shotgun liquid chromatography-tandem mass spectrometry. The results showed that 11 proteins were detected in both apoptosis group and oncosis group, 17 proteins were detected only in apoptosis group and 29 proteins were detected only in oncosis group. KEGG analysis showed that proteins detected only in apoptosis group were significantly enriched in 10 pathways, including ECM-receptor interaction, cell adhesion molecules, and proteins detected only in oncosis group were significantly enriched in three pathways, including endocytosis, base excision repair, and RNA degradation. These proteins we detected are helpful for us to understand the process of cell death in acute pancreatitis and may be useful for changing the death mode of pancreatic acinar cells, thus attenuating the severity of pancreatitis.

Keywords Proteomics · Acute pancreatitis · Apoptosis · Oncosis

Introduction

Though acute pancreatitis (AP) has been studied for more than a century, its pathogenesis is still unclear. It is inferred that the incidence and progress of AP are related to trypsin autodigestion [1], oxygen free radical formation [2], excessive release of inflammatory mediators [3] and apoptosis [4]. Researchers generally presume that one of the most important pathological changes in AP is the early abnormal activation of trypsinogen in pancreatic acinar cells, which may lead to injuries in autologous tissues and subsequent release of trypsin. Trypsin can damage adjacent cells, recruit, and activate inflammatory cells, and then lead to inflammatory storm followed by disorders of multiple organs [5, 6]. If the release of trypsin is inhibited, inflammatory reactions may be blocked from the origin. It is found that the release of the contents in pancreatic acinar cells and subsequent inflammatory reactions is mainly dependent on the death mode of cells—apoptosis or oncosis [7].

Apoptosis possesses typical morphological characteristics, including chromatic agglutination, nuclear fragmentation, cell shrinkage, plasma membrane bubbling, and apoptotic body formation [8, 9]. Its significance lies in the fact that it is not accompanied with inflammatory responses. On the contrary, oncosis has no special morphological characteristics and it has ever been considered as a kind of uncontrollable cell death mode induced by non-specific pathological pressure [10], but the opinion is still disputable now. Oncosis is mainly characterized by the increase in the permeability of cell membrane, and it is always

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accompanied with leakage of cellular contents into the extracellular environment and secondary inflammatory reactions.

In previous investigations, we found that pancreatic acinar cells will undergo different modes of death under the same etiological factor. It will develop into mild AP if pancreatic acinar cells die mainly by apoptosis, while it will evolve into severe AP if oncosis is the major mode of cell death [11]. These conclusions indicate that pancreatic acinar cells may employ some mechanism to regulate apoptosis and oncosis processes under external stimuli. By investigating the mechanism of apoptosis and oncosis, and striving to induce apoptosis of acinar cells and reduce the incidence of oncosis at an early stage of AP, the incidence of serious inflammations can be effectively inhibited. Cell death is a process that needs numerous functional proteins to participate in, and the study of single functional protein cannot fully elucidate the problem enough. This study was carried out to screen differentially expressed proteins during the processes of apoptosis and oncosis by high-flux proteomics techniques to identify the death mechanism of acinar cells in AP and offer new clues for the treatments of AP.

Materials and methods

Cell culture

AR42J cells, a pancreatic acinar cell line from rats, purchased from CCTCC (Wuhan, China), were cultured in Ham's F12k medium (Gibco, USA) containing 10 % fetal bovine serum in a humidified, 5 % CO_2 incubator at 37 °C.

Establishment of the cell model of AP

AR42J cells were incubated in six-well cultivation plates for 24 h, then 10 nmol/l caerulein (Sigma, USA) was added. The dose of cerulein used in the study could induce the characteristic changes of AP, such as inflammatory cytokine expression and hypersecretion in pancreatic acinar cells [12]. After further incubated for a specified period of time, the cells were collected to detect apoptosis and oncosis.

Detection of the death mode of AR42J cells by annexin V-propidium iodide staining

Collected AR42J cells at 60–70 % confluence by EDTA-free trypsin digestion. ApoAlertTM Annexin V-FITC Kit (Becton–Dickinson, USA) was used to detect apoptosis and oncosis according to the manufacturer's instructions. Briefly, washed the cells with PBS twice and resuspended 5×10^5 cells in 500 μl annexin V-binding buffer. Added 5 μl of annexin V FITC and 5 μl of propidium iodide, then incubated at room temperature for 15 min in dark. Cells were analyzed with a flow cytometer (Aria, Becton-Dickinson, USA) and a laser confocal microscopy (Zeiss 510, Germany) within 1 h of staining.

Sorting of apoptotic cells and oncotic cells by flow cytometry

Starting the stream

The 70-mm nozzle was selected. Amplitude was adjusted to keep the first droplet forming in the uppermost third of the main stream window and adjusted the amplitude further until the "Gap value" corresponded to the reference value. The satellite drop should merge with the main drop by the 3rd or 4th drop or earlier. When the stream stabilized approximately to the target values, wrote the actual "Drop1 value" given by the software to the "Drop1 box". Here the values of "Amp 1", "Drop1", and "Gap" were 9.1, 252, and 7, respectively.

Adjusting the side streams go into the collection tubes. Turned on the voltage from the side stream window and clicked "Test sort". Adjusted the amplitude until the light spot of the 4 fork streams in the side stream window was focused and bright and had clear boundaries. After the adjustment, "Gap value" should be within the range of target value ±3.

Determination of drop delay

Used 2-way sort and chose the P1 population to the left. Selected the "Initial" in the "Precision". Loaded the diluted Accudrop beads (BD, USA) and adjusted the flow rate so that 1,000–3,000 events/s were got. Adjusted the drop delay value to keep the brightness of the left light spot no lower than 95 %.

Protein extraction and electrophoresis

5×10^5 cells sorted by flow cytometer were added into 500 μl lysis buffer, and the cells were subjected to ultrasonic treatment (80 W, an interval of 10 s, three times for 5 s each time), then the protein content was quantitated by using Bradford method (Biomiga, USA) and distributed in small vials in 100 μg each tube. Protein samples (100 μg) were separated by electrophoresis on a 12 % SDS-PAGE gel and stained with silver nitrate. Whole gel lanes were cut into four pieces of equal size and subjected to in-gel trypsin digestion, and the extracted peptides from each gel piece were analyzed using mass spectrometry.

Table 1 Primers for real-time PCR

Gene	Primer sequence	Length of products (bp)
Hspa8	F5′ TGTGGTCTCGTCATCAGCACAG 3′	97
	R5′ ACACAGGAGTAGGTGGTGCCAAG 3′	
Ahsg	F5′ TGACACGCTGGAGACAACCTG 3′	158
	R5′ TGGAATGACACTGGGCGTGTA 3′	
Psmd4	F5′ CCTGAGAATAACGTGGGCTTGA 3′	119
	R5′ ATCTTGCCTTTGGGCTGGAC 3′	
Sohlh2	F5′ GCTGACCAGGGCTTGAATGTCTA 3′	163
	R5′ CAGAGCAGTATCTGACAGCATGGAA 3′	
Col7a1	F5′ GGTCCACCGTCAGAACACA 3′	132
	R5′ CACACACAACTCTAGCACCAAATG 3′	
Cul2	F5′ TCGTGCTGTGTCCAGTGGTTTAC 3′	150
	R5′ ACGAATTTACCGTGCACTTCCAA 3′	
Blm	F5′ CACGCCATGACCTTCACTG 3′	129
	R5′ CCACTCATCGGTCATGCACA 3′	
Cd99	F5′ GGTGATGGTGACTTCAGACTGGAC 3′	91
	R5′ CTGAGGATGGCTTCTTGGGTGT 3′	
βactin	F5′ CCTCTATGCCAACACAGTGC 3′	211
	R5′ GTACTCCTGCTTGCTGATCC 3′	

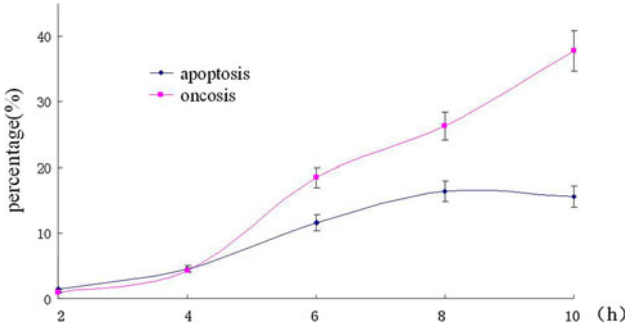


Fig. 1 Time-dependent curve of cell death of AR42J cells treated with 10 nmol/L caerulein. The ratio of apoptotic and oncotic cells at different time points (2, 4, 6, 8, and 10) after 10 nmol/L caerulein treatment was shown

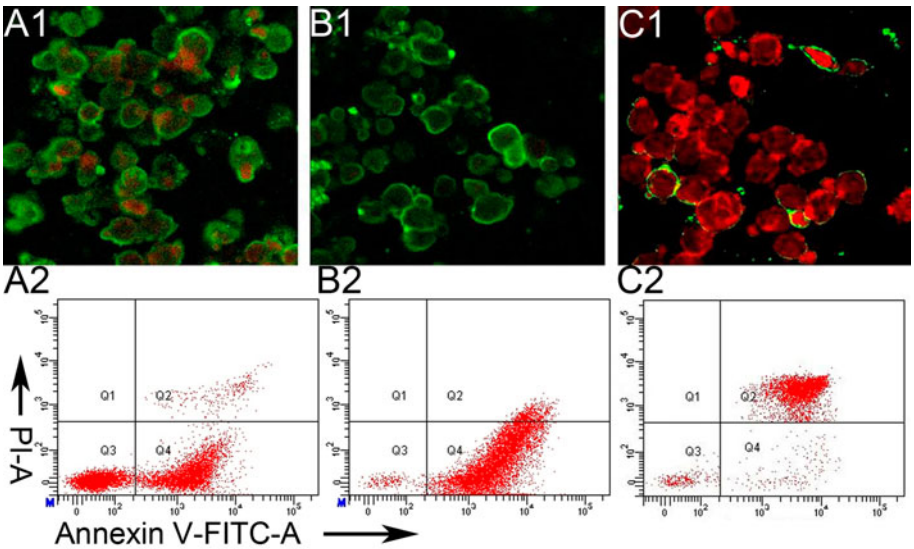
Procedure of shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS)

EttanTM MDLC system (GE Healthcare, USA) was applied for desalting and separation of tryptic peptides mixtures. In this system, samples were desalted on RP trap columns (Zorbax 300 SB-C18, Agilent Technologies, USA), and then separated on a RP column (RP-C18, 150 μm × 100 mm, Column Technology Inc. USA). Mobile phase A (0.1 % formic acid in HPLC-grade water) and the mobile phase B (0.1 % formic acid in acetonitrile) were selected. 20 μg of tryptic peptide mixtures was loaded onto the columns, and separation was done at a flow rate of 2 μl/min by using a linear gradient of 4–50 % B for 120 min. A FinniganTM LTQ-VELOSTM linear ion trap MS (Thermo Electron, USA) equipped with an electrospray interface was connected to the LC setup for eluted peptides detection. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of one full MS scan in profile mode followed by ten MS/MS scans in centroid mode with the following Dynamic Exclusion-TM (Thermo Electron, USA) settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

RNA extraction and identification of several genes by real-time PCR

Total RNA was extracted from 10^5 sorted cells or normal AR42J cells using the RNeasy Kit (Qiagen), according to the manufacturer's instructions. RNA quantity and quality were measured by NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

Real-time PCR was carried out on total RNA prepared using the RNeasy Kit (Qiagen) as described above using differently treated AR42J cells. The HiFi-MMLVcDNA reverse transcription kit (CWbio. Co. Ltd, Cat#CW0744) and RealSuper Mixture (with Rox) (CWbio. Co. Ltd, Cat#CW0767) were used according to the manufacturer's instructions and plates were read using an ABI Prism 7500 Real-Time PCR system (Applied Biosystems). The primers used in real-time PCR were list in Table 1.



Quad	%Gated	Quad	%Gated	Quad	%Gated
UL(Q1)	0.58	UL(Q1)	1.24	UL(Q1)	0.96
UR(Q2)	21.15	UR(Q2)	8.94	UR(Q2)	81.82
LL(Q3)	35.74	LL(Q3)	13.25	LL(Q3)	12.94
LR(Q4)	42.53	LR(Q4)	76.57	LR(Q4)	4.28

Fig. 2 Flow cytometric analysis of apoptosis and oncosis in AR42J cells before and after cell sorting. **A1** represents the result of staining with annexin V and PI before cell sorting, **A2** is the result of flow cytometric analysis before cell sorting. **B1** represents cells that will undergo apoptosis after cell sorting, **B2** is the result of flow cytometric analysis of apoptotic cells after cell sorting. **C1** represents cells that will undergo oncosis after cell sorting. **C2** is the result of flow cytometric analysis of oncotic cells after cell sorting

Data analysis

MS/MS spectra were automatically searched against the non-redundant international protein index Rat protein database (version 3.87) using the Bioworks Browser rev. 3.1 (Thermo Finnigan, USA). The peptides were constrained to be tryptic and up to two missed cleavages were allowed. Carbamidomethylation of cysteines were treated as a fixed modification, whereas oxidation of methionine residues was considered as variable modifications. The mass tolerance allowed for the precursor ions was 2.0 Da and fragment ions was 0.0 Da, respectively. The protein identification criteria were based on Delta CN ($\geq$0.1) and cross-correlation scores (Xcorr, one charge $\geq$1.9, two charges $\geq$2.2, three charges $\geq$3.75). The identified proteins with the unique peptide count $\geq$2 or the unique peptide count = 1 while the total count $\geq$4 were compared between the two groups.

Enrichment analysis

Enrichment analysis was performed by using cumulative hypergeometric distribution. The formula was as follows:

$$P = \sum_{i=k}^{m} \frac{\binom{m-j}{n-i}\binom{j}{i}}{\binom{m}{n}}.$$

The enrichment analysis was used in the identification of significant GO terms or pathways, in which our interested genes were significantly enriched. Here, we supposed that the mouse whole genome had n genes, and among that i genes were included in the pathway. The number of our interested genes is m, and j genes out of the m targets were involved in the pathway.

InterPro annotation, significant gene ontology (GO) term, and KEGG pathway

Protein sequences, which were selected from experiment, were compared with InterPro member databases to find meaningful matches signatures by the soft ware of InterProScan45. The output proteins were used to find whether there was significant enriched GO term or pathway by using enrichment analysis. The cutoff of significant P value from enrichment analysis is 0.05.

Results

Apoptotic or oncotic cells were sorted by flow cytometry

As shown in Fig. 1, when the AR42J cells were treated with 10 nmol/l caerulein for different time (2, 4, 6, 8, and

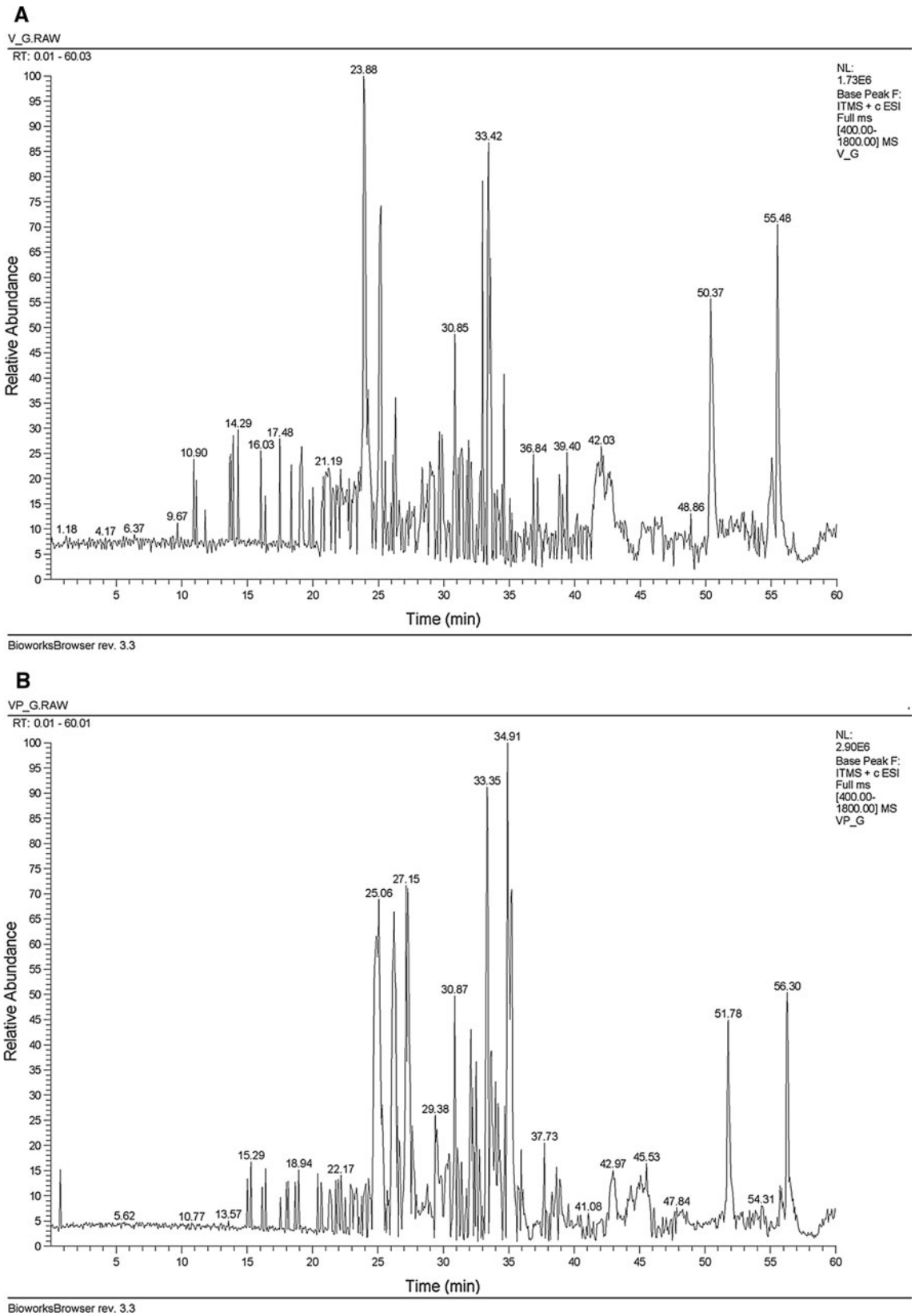


Fig. 3 The maps of total ion current. **a** V group, **b** VP group

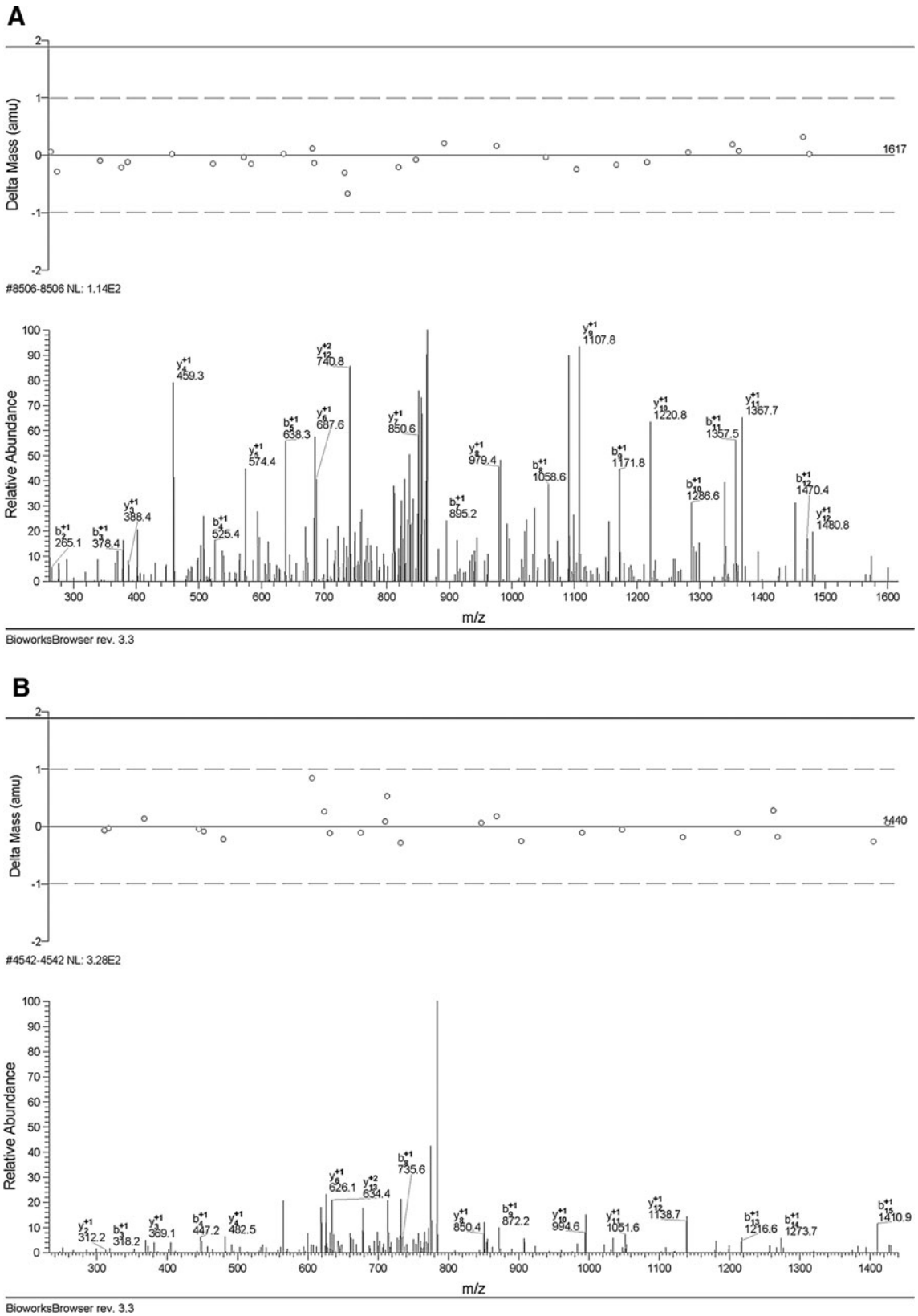


Fig. 4 Representative MS/MS spectrograms. **a** V group, **b** VP group

Table 2 Proteins detected in both V group and VP group

Number	Cover percent (%)	MW	PI	Accession number	Identified name
1	4.46	58224.34	8.47	IPI00222228.5	4732456N10Rik: hypothetical protein LOC239673
2	8.13	26134.64	4.75	IPI00130391.1	Tcrb-V20;Prss3;Prss1 protease, serine, 1
3	8.13	26273.82	5.48	IPI00128108.1	Try4: trypsin 4
4	4.18	70870.94	5.37	IPI00323357.3	Hspa8: Heat shock cognate 71 kDa protein
5	2.03	37325.59	6.04	IPI00128249.1	Ahsg: Alpha-2-HS-glycoprotein
6	4.52	40703.68	4.67	IPI00128564.1	Psmd4: Isoform Rpn10A of 26S proteasome non-ATPase regulatory subunit 4
7	3.64	51325.6	9.27	IPI00136801.2	Sohlh2: Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2
8	0.27	295233.17	5.98	IPI00134652.3	Col7a1: Collagen alpha-1(VII) chain
9	1.74	86876.99	6.54	IPI00387216.1	Cul2: Isoform 1 of Cullin-2
10	1.48	158365.93	6.9	IPI00329943.6	Blm: Bloom syndrome protein homolog
11	14.86	16782.58	5.14	IPI00187438.1	Cd99: CD99 antigen

MW molecular weight, *PI* isoelectric point

Table 3 Proteins detected only in V group

Number	Cover percent (%)	MW	PI	Accession number	Identified name
1	22.39	7500.91	8.47	IPI00808074.1	2900056M20Rik hypothetical protein LOC72997
2	2.39	63026.64	8.7	IPI00120383.1	Zfp119: Zinc finger protein 119
3	2.56	58194	9.61	IPI00226318.3	E330016A19Rik: Putative uncharacterized protein
4	3.93	54906.33	5.85	IPI00338270.5	Erlec1: Endoplasmic reticulum lectin 1
5	2.52	46472.22	8.6	IPI00310661.2	Relt: Tumor necrosis factor receptor superfamily member 19L
6	4.13	38676.23	7.55	IPI00468203.3	Anxa2: Annexin A2
7	3.34	57058.63	4.77	IPI00122815.3	P4hb: Putative uncharacterized protein
8	1.38	109101.42	6.49	IPI00265305.5	Mcf2: Mcf.2 transforming sequence
9	1.52	50082.05	5.52	IPI00404914.4	Slc39a8: Isoform 1 of Zinc transporter ZIP8
10	0.69	313630.05	4.73	IPI00122439.1	Fbn2: LOC100047082 Fibrillin-2
11	0.65	119594.93	6.54	IPI00227969.1	Itga6: Isoform Alpha-6X1A of Integrin alpha-6
12	1.69	147378.47	6.83	IPI00124242.7	Nlrp1a: NLR family, pyrin domain-containing 1A
13	2.21	59652.1	7.95	IPI00331174.5	Cct7: T-complex protein 1 subunit eta
14	2.55	50254.92	8.97	IPI00652288.1	I830012O16Rik: Putative uncharacterized protein
15	0.47	166592.64	6.26	IPI00226397.3	Gemin5: Gem-associated protein 5 isoform 2
16	4.82	34032.15	8.62	IPI00127569.4	Olfr399: Olfactory receptor 399
17	1.85	106462.06	6.68	IPI00604878.3	Pitpnm3: Isoform 1 of Membrane-associated phosphatidylinositol transfer protein 3

MW molecular weight, *PI* isoelectric point

10 h), the number of oncotic cells increased rapidly with longer treatment time, while the number of apoptotic cells increased after treated with caerulein and reached a peak at 8 h, then decreased. Therefore, 8 h was chosen as the time of caerulein treatment in the following research.

After treated with 10 nmol/l caerulein for 8 h and staining with annexin V and PI, the AR42 J cells were sorted with a flow cytometer (Fig. 2). It can be found that in the apoptosis group, most of the cells were distributed in Q4 region, indicating that the cells were mainly apoptotic cells (V group, purity 76.57 %); while in oncosis group, most of the cells were distributed in Q2 region, indicating that the cells were mainly oncotic cells (VP group, purity 81.82 %).

Forty-six highly differentially expressed proteins were found after LC/MS/MS analysis

The two groups of peptides obtained from one-dimensional SDS-PAGE electrophoresis and in-gel digestion were

Table 4 Proteins detected only in VP group

Number	Cover percent (%)	MW	PI	Accession number	Identified name
1	21.71	14045.42	11.05	IPI:IPI00153400.2	H2afj: Histone H2A.J
2	2.61	48396.16	8.16	IPI:IPI00111352.5	Dcp2: mRNA-decapping enzyme 2
3	2.73	71360.89	8.87	IPI:IPI00311247.6	Sgip1: Isoform 3 of SH3-containing GRB2-like protein 3-interacting protein 1
4	0.68	119400.63	6.17	IPI:IPI00126340.1	Pmfbp1: Polyamine-modulated factor 1-binding protein 1
5	2.68	65392.98	9.26	IPI:IPI00227149.4	Ythdf3: Isoform 3 of YTH domain family protein 3
6	2.84	57740.31	8.99	IPI:IPI00352751.7	Gm4944: Hypothetical protein isoform 1
7	7.79	16533.7	6.2	IPI:IPI00119545.2	Pfdn2: Prefoldin subunit 2
8	6.98	24893.83	5.62	IPI:IPI00420261.5	Hmgb1: Gm15387 High mobility group protein B1
9	1.54	116075.98	8.3	IPI:IPI00749774.3	Tdrd5: Tudor domain-containing protein 5
10	3.29	54212.45	4.73	IPI:IPI00117011.3	Hcls1: Hematopoietic lineage cell-specific protein
11	1.23	128905.32	6.19	IPI:IPI00169846.3	Nckap1 l: Putative uncharacterized protein
12	3.77	42955.58	6.49	IPI:IPI00165815.1	Lrrc2: Leucine-rich repeat-containing protein 2
13	1.71	60993.48	9.69	IPI:IPI00343256.7	Ccdc112: Coiled-coil domain-containing 112
14	4.31	46916.24	7.15	IPI:IPI00124286.2	Ap3m2: AP-3 complex subunit mu-2
15	1.62	109985.04	6.38	IPI:IPI00165717.1	Med24: Isoform 1 of Mediator of RNA polymerase II transcription subunit 24
16	1.17	117329.96	8.54	IPI:IPI00623382.2	Morc2a: MORC family CW-type zinc finger protein 2A
17	3.76	58334.05	9.86	IPI:IPI00225153.5	Shisa7: Isoform 1 of Protein shisa-7
18	3.98	39401.41	8.45	IPI:IPI00136513.1	Inhbc: Inhibin beta C chain
19	6.27	34002.03	5.9	IPI:IPI00850732.1	Gm2981: Similar to Apoptosis inhibitor 5
20	2.87	72418.64	5.62	IPI:IPI00884541.1	Lrrc32: leucine rich repeat containing 32
21	3.91	58567.49	8.53	IPI:IPI00222724.1	Zcchc4: Isoform 1 of Zinc finger CCHC domain-containing protein 4
22	3.83	47111.67	6.02	IPI:IPI00122340.1	Tcp10a: Tcp-10
23	8.12	25952.39	6.45	IPI:IPI00119169.1	Med8: Mediator of RNA polymerase II transcription subunit 8 isoform 2
24	0.76	204575.8	5.57	IPI:IPI00761751.2	Kif13b: Kinesin family member 13B
25	5.29	26334.62	8.56	IPI:IPI00133562.1	Chchd3: Coiled-coil-helix-coiled-coil-helix domain- containing protein 3, mitochondrial
26	5.45	28575.32	7.67	IPI:IPI00224872.2	Tyw3: tRNA wybutosine-synthesizing protein 3 homolog
27	4.72	21266.49	11	IPI:IPI00223713.5	Hist1h1c: Histone H1.2
28	9.71	23260.26	5.68	IPI:IPI00857854.1	4930511M11Rik 23 kDa protein
29	1.11	108937.67	5.84	IPI:IPI00407352.4	Psd3: Isoform 4 of PH and SEC7 domain-containing protein 3

MW molecular weight, *PI* isoelectric point

detected through mass spectrometry system, then the maps of total ion current were obtained (Fig. 3) and examples of the MS/MS spectra are shown in Fig. 4. After tandem mass spectrometry analysis and search of the protein database, 57 proteins were identified. The relative molecular mass range is (7.5–313) $\times$ 10^3 Da, and the range of the pH is 4.73–11.05. 11 out of the 57 proteins were detected in both apoptosis group and oncosis group, 29 proteins existed only in the VP group, and 17 proteins existed only in the V group (Tables 2, 3, 4). Among the 11 proteins which were detected in both apoptosis group and oncosis group, additional real-time PCR was performed to detect 8 differentially expressed genes in normal AR42J cells, apoptotic or oncotic cells, and results shown low expression of these 8 genes in normal AR42J cells ($P < 0.05$, see Fig. 5).

Results of InterPro annotation, significant GO term and KEGG pathway

Abnormal changes in various kinds of proteins and loss of balance among different cytological processes may finally lead to the death of AR42J cells in caerulein-induced AP model. However, how do these proteins and the cellular processes regulate different death modes? To elucidate this question, analyses of GO term and KEGG pathway these proteins enriched in were carried out. The GO terms

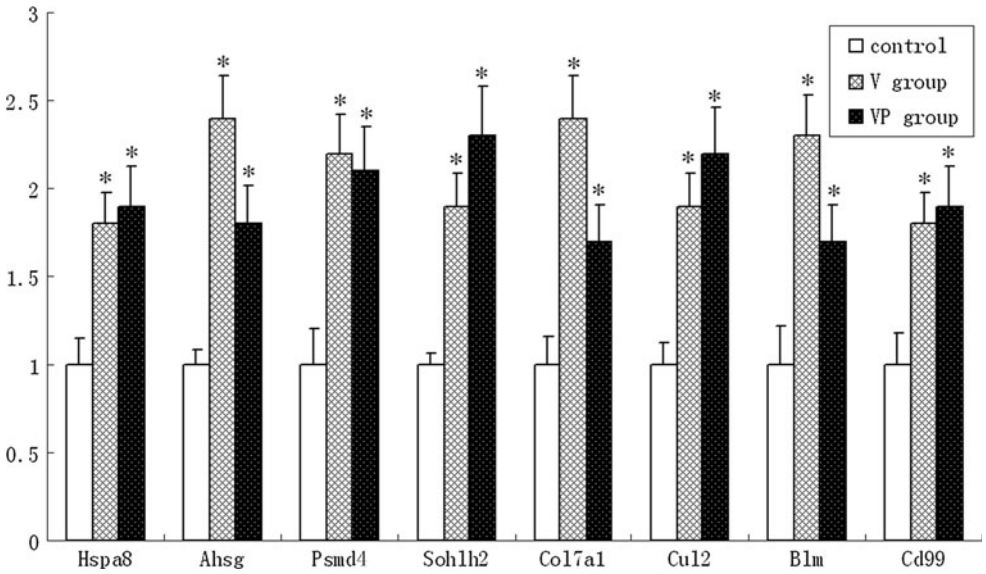


Fig. 5 Real-time PCR verification of the 8 proteins which were detected in both apoptosis group and oncosis group (compared to control group, $*P < 0.05$)

proteins in V group and VP group enriched in were listed in Tables 5, 6, and 7. The pathways these proteins enriched in were shown in Fig. 6. In total, based on 11 co-expressed proteins, analysis of GO enrichment identified 5 significantly enriched biological process terms, 5 significantly enriched cellular component terms, and 14 significantly enriched molecular function terms, while KEGG pathway enrichment analysis revealed 3 statistically enriched pathways. Seventeen proteins detected only in the V group showed enrichment within 36 biological process GO terms, 13 cellular component GO Terms, 14 molecular function GO terms and 10 KEGG pathways. Twenty-nine proteins detected only in VP group were significantly enriched in 35 biological process GO terms, 12 cellular component GO terms, 15 molecular function GO terms, and 3 KEGG pathways.

Visualization of biological processes the differentially proteins participated in

Abnormal changes in various kinds of proteins and imbalance in different cytological processes may finally lead to the death of AR42J cells in caerulein-induced AP model. However, how do these proteins and the cellular processes regulate different death modes? Based on the 57 proteins identified in either apoptosis or oncosis group, and combined GO enrichment analysis with protein–protein interaction data, we plotted a diagram for the relationship between proteins, apoptosis, and oncosis, and some functional proteins showing close relationship with cell death were discovered, such as Psmd4, CD99, Hspa8, Blm, Ahsg, Col7a1, and Cul2 were proteins existed in both V and VP group, Anxa2, P4hb, Mcf2, Itga6, and Fbn2 existed only in V group, and Hmgb1, Hist1h1c, Hcls1, Med8, and Kif13b existed only in VP group (Fig. 7).

Discussion

Proteasome degradation pathway was activated in both cell death modes

Among the proteins detected in both V group and VP group, many functional proteins, such as Psmd4, Hspa8, and Cul2, mainly participate in the degradation of proteins by proteasome. In the GO enrichment analysis, the molecular function term "ubiquitin protein ligase binding" (GO: 0031625) and the cellular component term "Cullin-RING ubiquitin ligase complex" were found enriched, and in the KEGG pathway enrichment analysis, "proteasome" was enriched in. So, it was concluded that the proteasome degradation pathway was activated in both the acinar cell death modes in the caerulein-induced AP model.

Proteasome is located in nuclear and cytoplasm in eukaryotes [13] and its major function is to degrade unwanted or injured proteins in cells by disrupting peptide bonds using protease. Proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, gene expression regulation, and stress responses (such as infections, heat shock, and oxidative injuries). As a kind of molecular chaperone, heat shock protein Hspa8a (Hsp70) can bind to the hydrophobic domain on the surface of incorrectly folded proteins and guide ubiquitin ligase (E3) to label incorrectly folded proteins with ubiquitin [14], and thus proteasomes can degrade them [15]. Proteasomes may also play multiple important roles in the process of

Table 5 The GO "biological process" terms enriched in the set of statistically significantly detected proteins

No.	GO ID	GO biological process terms
Detected only in V group		
1	GO:0030097	Hemopoiesis
2	GO:0065004	Protein-DNA complex assembly
3	GO:0034728	Nucleosome organization
4	GO:0031497	Chromatin assembly
5	GO:0045931	Positive regulation of mitotic cell cycle
6	GO:0006334	Nucleosome assembly
7	GO:0051247	Positive regulation of protein metabolic process
8	GO:0043471	Regulation of cellular carbohydrate catabolic process
9	GO:0044247	Cellular polysaccharide catabolic process
10	GO:0009251	Glucan catabolic process
11	GO:0005980	Glycogen catabolic process
12	GO:0043470	Regulation of carbohydrate catabolic process
13	GO:0042098	T cell proliferation
14	GO:0032270	Positive regulation of cellular protein metabolic process
15	GO:0008284	Positive regulation of cell proliferation
16	GO:0070663	Regulation of leukocyte proliferation
17	GO:0070875	Positive regulation of glycogen metabolic process
18	GO:0050709	Negative regulation of protein secretion
19	GO:0070873	Regulation of glycogen metabolic process
20	GO:0050710	Negative regulation of cytokine secretion
21	GO:0032881	Regulation of polysaccharide metabolic process
22	GO:0050670	Regulation of lymphocyte proliferation
23	GO:0032944	Regulation of mononuclear cell proliferation
24	GO:0042127	Regulation of cell proliferation
25	GO:0016584	Nucleosome positioning
26	GO:0070168	Negative regulation of biomineral formation
27	GO:0046007	Negative regulation of activated T cell proliferation
28	GO:0031401	Positive regulation of protein modification process
29	GO:0042129	Regulation of T cell proliferation
30	GO:0005981	Regulation of glycogen catabolic process
31	GO:0045819	Positive regulation of glycogen catabolic process
32	GO:0042327	Positive regulation of phosphorylation
33	GO:0010562	Positive regulation of phosphorus metabolic process
34	GO:0045937	Positive regulation of phosphate metabolic process
35	GO:0001934	Positive regulation of protein amino acid phosphorylation
36	GO:0071103	DNA conformation change
Detected only in VP group		
1	GO:0050873	Brown fat cell differentiation
2	GO:0030282	Bone mineralization
3	GO:0046635	Positive regulation of alpha–beta T cell activation
4	GO:0000018	Regulation of DNA recombination
5	GO:0006829	Zinc ion transport
6	GO:0030199	Collagen fibril organization
7	GO:0032200	Telomere organization
8	GO:0000723	Telomere maintenance
9	GO:0051053	Negative regulation of DNA metabolic process
10	GO:0050818	Regulation of coagulation

Table 5 continued

No.	GO ID	GO biological process terms
11	GO:0046633	Alpha–beta T cell proliferation
12	GO:0070167	Regulation of biomineral formation
13	GO:0030500	Regulation of bone mineralization
14	GO:0006458	'De novo' protein folding
15	GO:0030035	Microspike assembly
16	GO:0051084	'De novo' posttranslational protein folding
17	GO:0046847	Filopodium assembly
18	GO:0046640	Regulation of alpha–beta T cell proliferation
19	GO:0051085	Chaperone mediated protein folding requiring cofactor
20	GO:0030193	Regulation of blood coagulation
21	GO:0030279	Negative regulation of ossification
22	GO:0045910	Negative regulation of DNA recombination
23	GO:0046641	Positive regulation of alpha–beta T cell proliferation
24	GO:0006457	Protein folding
25	GO:0050819	Negative regulation of coagulation
26	GO:0022407	Regulation of cell–cell adhesion
27	GO:0022409	Positive regulation of cell–cell adhesion
28	GO:0033627	Cell adhesion mediated by integrin
29	GO:0030195	Negative regulation of blood coagulation
30	GO:0051241	Negative regulation of multicellular organismal process
31	GO:0018208	Peptidyl-proline modification
32	GO:0042730	Fibrinolysis
33	GO:0018401	Peptidyl-proline hydroxylation to 4-hydroxy-L-proline
34	GO:0019471	4-Hydroxyproline metabolic process
35	GO:0019511	Peptidyl-proline hydroxylation
Detected in both V group and VP group		
1	GO:0000019	Regulation of mitotic recombination
2	GO:0045950	Negative regulation of mitotic recombination
3	GO:0030502	Negative regulation of bone mineralization
4	GO:0070168	Negative regulation of biomineral tissue development
5	GO:0006312	Mitotic recombination

apoptosis. The prediction for the involvement of proteasomes in apoptosis is based on the phenomenon that ubiquitinated proteins, ubiquitin activating enzyme E1, ubiquitin cross-linking enzyme E2, and ubiquitin ligase E3 increase in their quantities before apoptosis [16]. Moreover, the proteasomes that are originally located in nucleus can move to the external membrane of apoptotic vesicles during the process of apoptosis [17]. The inhibitory effects of proteasomes can affect the apoptosis induction in different kinds of cells [18].

Obviously, proteasome degradation pathway is activated during the apoptotic and oncotic processes of acinar cells in the present study. What kind of roles does it play in the pathogenesis of AP? Is it only a kind of cellular protective mechanism in cells under stress? Or is it involved in the development of both cell death modes—apoptosis and oncosis simultaneously? These problems still need further investigations.

Profiling of V group proteins

Analysis of the relation between identified proteins and biological processes showed that the proteins in the V group were mainly associated to the regulation of cellular metabolic processes, such as positive regulation of protein metabolic process, positive regulation of glycogen metabolic process. This phenomenon can be explained by the fact that the apoptosis is a process of initiative energy consumption. Moreover, the protein and cytokine secretion presented negative regulation, this may be the mechanism that organism confines the inflammatory reaction during apoptosis.

Table 6 The GO "cellular component" terms enriched in the set of statistically significantly detected proteins

No.	GO ID	GO cellular component terms
Detected only in V group		
1	GO:0030131	Clathrin adaptor complex
2	GO:0030119	AP-type membrane coat adaptor complex
3	GO:0000785	Chromatin
4	GO:0044422	Organelle part
5	GO:0044446	Intracellular organelle part
6	GO:0032993	Protein-DNA complex
7	GO:0016272	Prefoldin complex
8	GO:0044427	Chromosomal part
9	GO:0000786	Nucleosome
10	GO:0043234	Protein complex
11	GO:0005694	Chromosome
12	GO:0032991	Macromolecular complex
13	GO:0016592	Mediator complex
Detected only in VP group		
1	GO:0005769	Early endosome
2	GO:0008305	Integrin complex
3	GO:0042641	Actomyosin
4	GO:0032432	Actin filament bundle
5	GO:0001725	Stress fiber
6	GO:0005832	Chaperonin-containing T-complex
7	GO:0043205	Fibril
8	GO:0031012	Extracellular matrix
9	GO:0005578	Proteinaceous extracellular matrix
10	GO:0001527	Microfibril
11	GO:0005604	Basement membrane
12	GO:0044420	Extracellular matrix part
Detected in both V group and VP group		
1	GO:0005657	Replication fork
2	GO:0043073	Germ cell nucleus
3	GO:0001673	Male germ cell nucleus
4	GO:0045120	Pronucleus
5	GO:0031461	Cullin-RING ubiquitin ligase complex

From the GO terms of molecular function and cellular component proteins in V group enriched in, we can see that they are mainly referred to the functional regulation of nucleic acid and organelles, preparing for the process of apoptosis.

NOD-like receptor (NLR) signaling pathway was enriched in V group by enrichment analysis (Fig. 6). Nlrp1a is a member of the NLR family. It is a critical factor in the activation of caspase-1 in response to proinflammatory stimuli [19, 20]. The activation of caspase-1 is necessary for the secretion of pro-IL-1β and pro-IL-18 in their mature biologically active forms [21–23]. The role of Nlrp1a in the pathophysiology of AP has not been reported, but considering the fact that the proinflammatory cytokines hasten the process of AP, we presumed that inhibition of the expression of Nlrp1a may attenuate the inflammatory reaction during caerulein-induced pancreatitis. In addition, the N-terminal domain of the NLRs contains the caspase recruitment domain, which is involved in both apoptosis and inflammation [24].

Itga6 was found enriched in multiple pathways, such as extracellular matrix (ECM)–receptor interaction, cell adhesion molecules, pathways in cancer (Fig. 6). Itga6 named integrin alpha 6 and could form functional dimers with integrin beta 1 or beta 4. Integrins mediate cell adhesion and migration, activate signal transduction pathways, and orchestrate the organization of the cytoskeleton [25]. The ECM consists of a complex mixture of structural and functional macromolecules and plays an important role in tissue and organ morphogenesis and in the maintenance of cell and tissue structure and function. Specific interactions between cells and the ECM are mediated by transmembrane molecules. Integrins are transmembrane receptors composed of an alpha- and a beta-subunit that transmit signals from ECM components to the cell interior. Integrin-mediated intracellular signaling plays pivotal roles in cellular functions such as proliferation, migration, invasion, and survival. Accumulated investigations of an association between Itga6 and carcinoma show that high expression levels of the Itga6 facilitate carcinoma cell invasion, metastatic capacity, apoptosis evasion, and negative patient outcome [26, 27]. In this study, high expression levels of the Itga6 may be associated with cellular defense mechanism against caerulein-induced cell death.

Some other proteins are also involved in the cellular defense mechanism. Endoplasmic reticulum (ER) plays key roles in the process of protein synthesis and distribution in many cells, and disulfide bond, which is of great importance for its folding, must be formed before the synthesized protein is transported to a specific location. Erlec1 exists in ER lumen; it can selectively bind to structurally abnormal luminal proteins and participate in the metabolism of ER associated non-glycosylated proteins and glycoproteins. Investigations by Yanaqisawak et al. [28] showed that Erlec1 could affect the stress response pathway of cells, including hypoxia inducing factor pathway and ER response pathway; it could also interact with the key ER-response protein-Bip and affect the cell proliferation under stress responsive status. P4hb is localized primarily to ER as well where it acts as a protein disulfide isomerase/oxidoreductase. P4hb is involved in the process of ubiquitination and is a mediator of ER protein homeostasis. Chen et al. [29] suggested in their study that the reduction of P4hb in the development of arginine-induced pancreatitis could be associated with the induction of apoptosis processes. So the expression of P4hb in the V group may associate with the cellular defense mechanism

Table 7 The GO "molecular function" terms enriched in the set of statistically significantly detected proteins

No.	GO ID	GO molecular function terms
Detected only in V group		
1	GO:0016251	General RNA polymerase II transcription factor activity
2	GO:0016455	RNA polymerase II transcription mediator activity
3	GO:0003676	Nucleic acid binding
4	GO:0016741	Transferase activity, transferring one-carbon groups
5	GO:0008168	Methyltransferase activity
6	GO:0019209	Kinase activator activity.
7	GO:0005086	ARF guanyl-nucleotide exchange factor activity
8	GO:0030295	Protein kinase activator activity
9	GO:0000217	DNA secondary structure binding
10	GO:0000400	Four-way junction DNA binding
11	GO:0000402	Crossed form four-way junction DNA binding
12	GO:0010858	Calcium-dependent protein kinase regulator activity
13	GO:0000401	Open form four-way junction DNA binding
14	GO:0003681	Bent DNA binding
Detected only in VP group		
1	GO:0008289	Lipid binding
2	GO:0005201	Extracellular matrix structural constituent
3	GO:0016706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors
4	GO:0005509	Calcium ion binding
5	GO:0005544	Calcium-dependent phospholipid binding
6	GO:0005178	Integrin binding
7	GO:0016862	Intramolecular oxidoreductase activity, interconverting
8	GO:0016864	Intramolecular oxidoreductase activity, interconverting keto- and enol-groups, transposing S–S bonds
9	GO:0019798	Procollagen-proline dioxygenase activity
10	GO:0031543	Peptidyl-proline dioxygenase activity
11	GO:0003756	Protein disulfide isomerase activity
12	GO:0055102	Lipase inhibitor activity
13	GO:0004859	Phospholipase inhibitor activity
14	GO:0004656	Procollagen-proline 4-dioxygenase activity
15	GO:0031545	Peptidyl-proline 4-dioxygenase activity
Detected in both V group and VP group		
1	GO:0051082	Unfolded protein binding
2	GO:0031625	Ubiquitin protein ligase binding
3	GO:0030414	Peptidase inhibitor activity
4	GO:0030234	Enzyme regulator activity
5	GO:0004857	Enzyme inhibitor activity
6	GO:0004866	Endopeptidase inhibitor activity
7	GO:0008094	DNA-dependent ATPase activity
8	GO:0003678	DNA helicase activity
9	GO:0004869	Cysteine-type endopeptidase inhibitor activity
10	GO:0004003	ATP-dependent DNA helicase activity
11	GO:0043140	ATP-dependent 3′–5′ DNA helicase activity
12	GO:0016887	ATPase activity
13	GO:0042623	ATPase activity, coupled
14	GO:0043138	3′–5′ DNA helicase activity

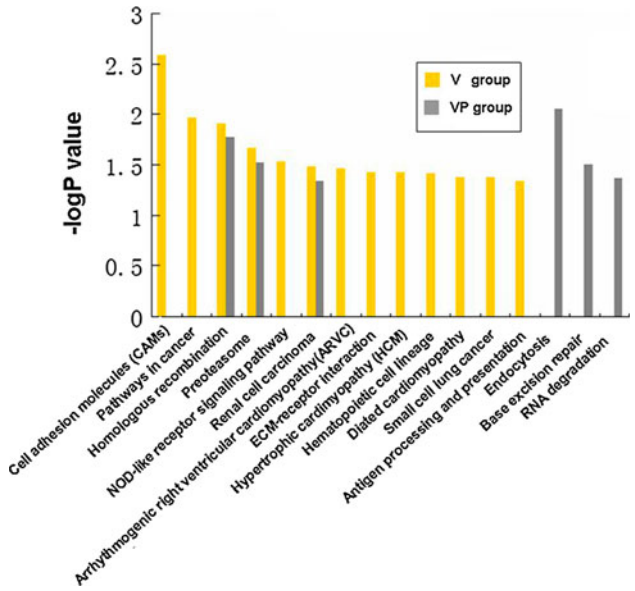


Fig. 6 The comparison chart of pathways, which proteins in the V group and VP group significantly enriched. The *X* axis is the detail significant enriched pathway name from the KEGG database. The *Y* axis is negative log value of the enrich *P* value. The *yellow bar* presents the V group, the *gray bar* is the VP group. (Color figure online)

against caerulein-induced cell death as well. CCT7 containing TCP1 complex is a kind of molecular chaperone, and it can assist the folding of proteins after connecting with ATP and inducing configuration changes. It is involved in the folding of actin and tubulin, and researchers gradually realize that the establishment of spatial configuration of important proteins during various kinds of cellular processes also requires the participation of molecular chaperones [30]. Yu et al. [31] found that the expression of chaperonin-containing TCP-1 beta subunit in caerulein-treated AR42J cells was up-regulated and then presumed that this up-regulation was involved in the cellular protection mechanism, which may provide protections for acinar cells during the pathogenesis of AP. ANNEXin A2, which possesses redox sensitive cysteine(s), is a Ca^{2+}-dependent phospholipid-binding protein. Annexin A2 depleted cells are more sensitive to oxidative stress-induced death [32]. So expression of annexin A2 referred to cellular defense mechanism as well.

Relt is involved in the apoptosis pathway. Relt is a member of TNF receptor family, which is an important modulator of some biological processes, such as immune responses, hematopoiesis, and tumor suppression [33, 34]. Cusick et al. [35] observed that a morphology characteristic of apoptotic cells could be induced by transient over expression of Relt family members in HEK 293 cells. Distinct from the cellular defense mechanism, the expression of Relt may solely participate in the process of caerulein-induced apoptosis.

Profiling of VP group proteins

There are apparent differences between the GO terms that proteins in VP group and V group participate in. From the cellular component GO terms (Table 6), we can see that proteins in VP group were mainly referred to the construction of cytoskeleton and ECM, which indicated that cytoskeleton of cells undergoing oncosis disintegrated earlier. This finding is in accordance with the fact that the disassembly of acinar cell cytoskeleton happened in the early steps of AP [36, 37]. This process can interfere with intracellular vesicular transport and is believed to be responsible for the inhibition of digestive enzyme secretion [36–38].

Some interesting proteins were detected in VP group. Hmgb1 (high mobility group protein B1) participates in multiple biological processes. Hmgb1 is an abundant chromatin-binding protein normally residing in cell nucleus and plays a crucial role in transcription. Scaffidi et al. [39] had validated that Hmgb1 could be released to the extracellular space by oncotic or injured cells and evoke inflammation. But Hmgb1 can bind to chromatin tightly in cells undergoing apoptosis, so it cannot be released by apoptotic cells. When released to the extracellular space by oncotic cells or inflammatory cells, Hmgb1 would act as a cytokine mediator of inflammation. In recent years, many investigations have shown that Hmgb1, an important late-acting proinflammatory cytokine, plays an important role in the development of systemic inflammatory response syndrome and multiple organ dysfunction syndrome in severe AP [40, 41]. Moreover, it has been confirmed that Hmgb1 can interact with P53 and participate in the apoptosis process. Gong et al. [42] found that high expression of Hmgb1 was always accompanied with the inhibition on the expression of oncogene protein cyclin D/E and tumor suppressor protein P53, thus regulating cell proliferation and inhibiting apoptosis.

In addition, some other important functional proteins, such as Gm2981 (similar to Apoptosis inhibitor 5), Med24, Med8, Dcp2, Inhbc, H2afj, and Hist1h1c, were also detected. These proteins participated in gene expression regulation, cell proliferation and differentiation, RNA degradation, and some other biological processes. Normal of these biological processes is an essential condition for the survival of cells.

Endocytosis pathway was enriched in (Fig. 6). Hcls1 and Sgip1 participate in this process. Hcls1 is a 75-kDa intracellular protein that contains a region of 37 amino acid tandem repeats, a coil-coiled region, an Arp2/3 complex binding domain and a proline-rich domain [43, 44]. On the basis of these structural characteristic, Hcls1 may promote both receptor-mediated and actin-dependent endocytosis. Sgip1 is also involved in endocytosis. Knockdown of Sgip1

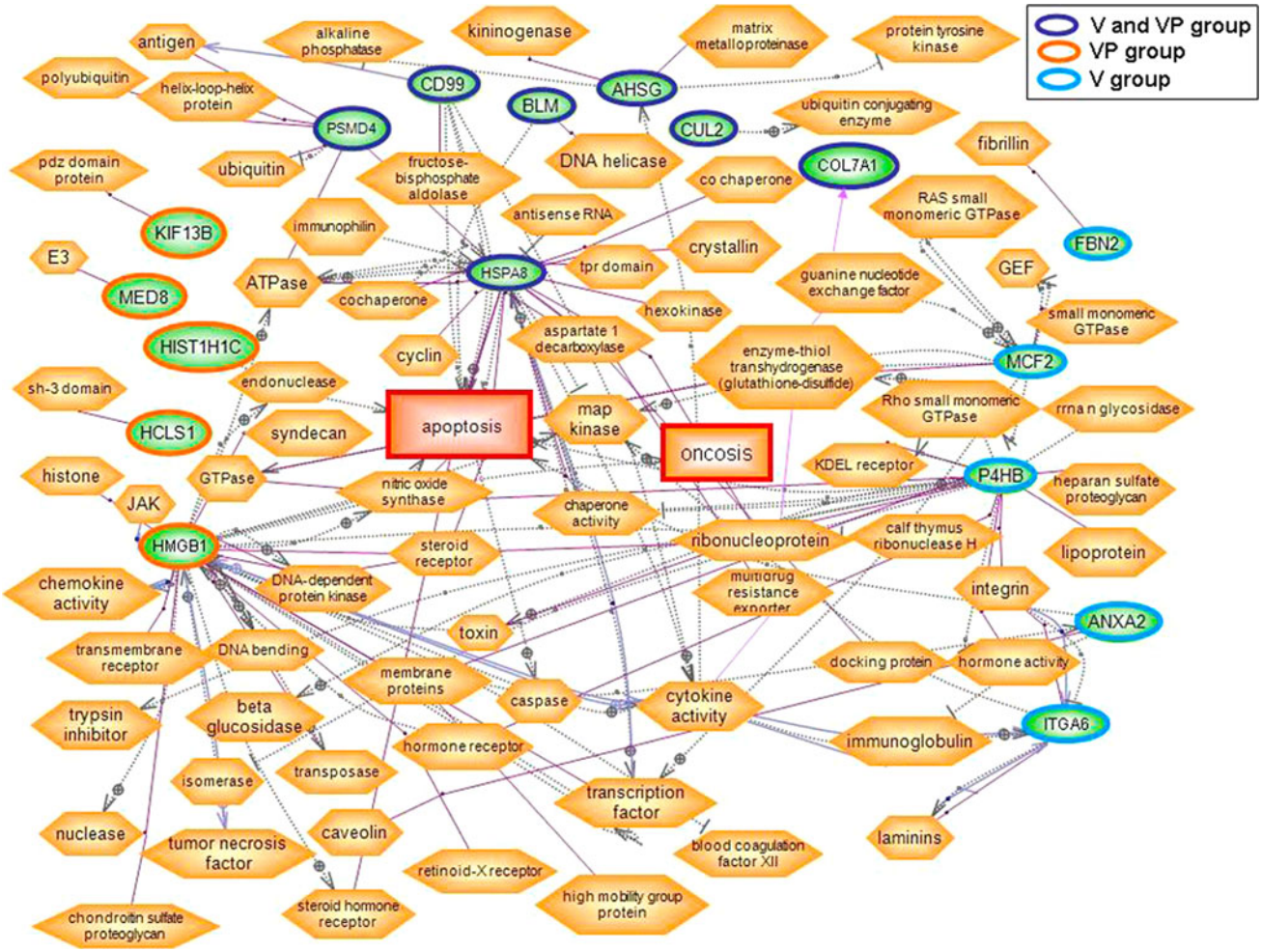


Fig. 7 The biological processes that V group and VP group proteins have participated in. All the *hexagons* without *colored circles* are the biological processes. Meanwhile, all the *hexagons* with *colored circles* are the proteins, we noted. The symbol "-⊕->" means positive regulation. "- - -|" means negative regulation. "- - - >" means unknown regulation. A link is placed between nodes if the relationship is shown in at least one paper report. (Color figure online)

expression reduces clathrin-mediated endocytosis [45]. Endocytosis is an essential process to maintain cellular homeostasis in eukaryotic cells. It regulates activities of multiple processes, such as signal transduction, nutrient uptake, neuronal synaptic transmission, clearance of apoptotic cells, intercellular interaction, and antigen presentation [46]. This process may refer to the cellular defense mechanism in cells undergoing oncosis.

Mitochondrion is the power source of cells and abnormalities in their structures and functions play crucial roles in the process of cell death. Chchd3 that was detected in the VP group is a kind of mitochondrial inner membrane protein. Darshi et al. [47] reported that Chchd3 was a scaffold protein for maintaining the structures of cristae in the mitochondrial inner membrane and protein import, which has crucial functions in maintaining the structures and functions of mitochondria. Thus, the expression of Chchd3 may be a kind of cellular defense mechanism.

Conclusion

In our study, we attempted to carry out investigations on protein expression profiles of apoptotic and oncotic pancreatic acinar cells under pathological conditions of AP. We found many important functional proteins were involved in apoptotic and oncotic processes among the identified proteins. These proteins are helpful for us to understand the process of cell death in AP. Furthermore, they may be useful for changing the death mode of pancreatic acinar cells, thus attenuating the severity of pancreatitis. But further investigations are required for comprehensive understanding on the roles of these proteins in AP.

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Conflict of interest The authors declare that they have no competing interests.

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