Proteomics analysis of chinese hamster ovary cells undergoing apoptosis during prolonged cultivation

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Abstract The degradation of environmental conditions, such as nutrient depletion and accumulation of toxic waste products over time, often lead to premature apoptotic cell death in mammalian cell cultures and suboptimal protein yield. Although apoptosis has been extensively researched, the changes in the whole cell proteome during prolonged cultivation, where apoptosis is a major mode of cell death, have not been examined. To our knowledge, the work presented here is the first whole cell proteome analysis of non-induced apoptosis in mammalian cells. Flow cytometry analyses of various activated caspases demonstrated the onset of apoptosis in Chinese hamster ovary cells during prolonged cultivation was primarily through the intrinsic pathway. Differential in gel electrophoresis proteomic study comparing protein samples collected during cultivation resulted in the identification of 40 differentially expressed proteins, including four cytoskeletal proteins, ten chaperone and folding proteins, seven metabolic enzymes and seven other proteins of varied functions. The induction of seven ER chaperones and foldases is a solid indication of the onset of the unfolded protein response, which is triggered by cellular and ER stresses, many of which occur during

prolonged batch cultures. In addition, the upregulation of six glycolytic enzymes and another metabolic protein emphasizes that a change in the energy metabolism likely occurred as culture conditions degraded and apoptosis advanced. By identifying the intracellular changes during cultivation, this study provides a foundation for optimizing cell line-specific cultivation processes, prolonging longevity and maximizing protein production.

Keywords Apoptosis · Chinese hamster ovary cells · Proteomics · Cell culture

Introduction

Mammalian cell cultures are the preferred expression hosts for the production of glycosylated recombinant proteins since they are able to ensure proper protein folding and post-translational modification. To date, Chinese hamster ovary (CHO) cells remain the most commonly used mammalian host for the generation of recombinant cell lines (Hacker et al. 2009). It has been estimated (Jayapal et al. 2007) that nearly 70% of all therapeutics employ CHO cell platforms, producing over \$30 billion US dollars annually. To achieve high yield during the culturing process, researchers have extensively explored strategies involving genetic modification of cell lines and alteration of culture media and conditions. These optimization methods usually seek to improve culture

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longevity, cell density, cell viability or protein productivity, leading to an increase in the total product yield. However, many cell lines undergo apoptotic cell death in response to the stressful culture environment in bioreactors (Al-Rubeai et al. 1990), negatively impacting the overall production.

Nutrient limitation, especially deprivation of glucose and amino acids, is one of the primary causes of cell death during the stationary and death phase of batch culture (Mercille and Massie 1994). Other possible causes of cell death include hyperoxia, hypoxia, and mechanical agitation in the hydrodynamic environment (Al-Rubeai and Singh 1998). Regardless of how apoptosis is triggered, it poses a major limiting factor in the production of recombinant proteins in cell cultures (Moore et al. 1995). Ongoing cell death in bioreactors makes it difficult to maintain high cell density with high viability. In addition, cell death results in the release of cellular debris and associated degradative enzymes into the medium, which can complicate purification and negatively affect the quality of the products (Wong et al. 2006b). Hence, an understanding of cell death is an essential step towards the optimization of recombinant protein-producing cell cultures.

As a mode of programmed cell death, apoptosis is a controlled and regulated mechanism for cell suicide triggered by external death stimuli or internal cell stress signals. Apoptotic cells can be characterized by distinct morphological changes including cell shrinkage, nuclear DNA fragmentation, chromatin condensation, membrane blebbing and the formation of membrane-enclosed cellular fragments called apoptotic bodies (Elmore 2007; Jin and El-Deiry 2005). Unlike necrosis, apoptosis is energy dependent. It can be initiated and executed via different pathways that trigger the cleavage of procaspases, resulting in activated caspase proteins (*cysteine-aspartic proteases*). The extrinsic and intrinsic apoptotic pathways are two major cell death-inducing signalling pathways that are well characterized (Jin and El-Deiry 2005; Twomey and McCarthy 2005).

Triggered by intracellular stress signals generated from non-receptor mediated stimuli such as growth factor withdrawal, toxin accumulation and Ca^{2+} flux, the intrinsic pathway causes apoptotic cell death via the mitochondrial outer membrane permeabilization, which result in mitochondrial dysfunction and subsequently the activation of many proapoptotic

proteins such as initiator caspase 9. The extrinsic pathway is mediated by the binding of death ligands to their corresponding cell surface death receptors, followed by the activation of many extrinsic-specific pro-apoptotic molecules, including initiator caspase 8. More recently, a third significant mechanism of apoptosis, the ER stress pathway, which involves caspase 9 and caspase 12, begins from prolonged ER stresses and persistent activation of the unfolded protein response (UPR). All three pathways depend on the activation of initiator caspases to trigger the execution of apoptosis by executioner caspases such as caspase 3, caspase 6 and caspase 7. (Reviewed in Elmore 2007; Jin and El-Deiry 2005; Krampe and Al-Rubeai 2010).

Since the initial CHO protein reference map constructed in 1999 by Champion et al. with 25 identifications, a few additional CHO 2D maps have been published, including up to 224 identifications (Lee et al. 2003; Hayduk et al. 2004). Genomic and proteomic studies involving CHO cell lines have also been performed to investigate the effects of treatments which can enhance protein production (Yee et al. 2008; Baik and Lee 2010), to compare metabolic profiles of different CHO cell lines varied in protein productivities (Pascoe et al. 2007), to study protein profiles in resting and proliferating phases (Naryzhny and Lee 2001), to understand cellular response to hyperosmotic pressure and to examine the effect of transfected cell lines (Carlage et al. 2009). Currently, there are no studies on proteomic expression of non-induced apoptotic cells that occurred in prolonged cell cultures, regardless of the cell types. With the exception of transcriptional profiling of non-induced apoptotic cell death in batch and fed-batch CHO cultures (Wong et al. 2006a), strategies taken by most apoptotic studies focused on examining changes when a specific apoptotic pathway was induced by a particular agent.

We studied the whole cell proteome of anti-Rhesus D factor-producing CHO cells at various phases of the cultivation period as apoptotic cell death advanced in the culture population. Using the proteomic techniques of differential in-gel electrophoresis and mass spectrometry, we identified proteins that vary their expression levels at the onset and execution of apoptosis and during the continual degradation of culture conditions. Although apoptotic pathways are not the only cellular pathways affected during prolonged cultivation, they

are of special interest for optimization of recombinant product yield. We investigated the specific apoptotic pathways triggered in protein-producing CHO cells in batch cultures that mimic a typical industrial bioreactor setting. Flow cytometry was used to assay the occurrence of apoptosis and the advancement of apoptotic cell, and changes observed in gel electrophoresis correlated to changes in culture conditions. In contrast to previous studies, our approach did not involve the induction of apoptosis by an apoptotic agent. To the best of our knowledge, the present work is the first systematic proteomics study of the progression of non-induced apoptosis in a mammalian cell population. This work provides a biological foundation for the design of strategies to improve productivity by reducing the amount of apoptotic cell death in bioreactors. Since factors crucial in delaying the onset of apoptosis can be inferred from knowledge of the proteins involved and the specific pathway initiated in CHO cultures, strategies for inhibiting apoptosis in bioprocesses may be developed based on these results.

Materials and methods

Cell line and culture maintenance

The Chinese hamster ovary cell line CHO IgG-9 β 8 (Cangene Corp., Mississauga), producing anti-Rhesus D factor monoclonal antibody, was used in this study. Cells were cultured in SFX-CHO medium (Hyclone) supplemented with 1% Fetal Bovine Serum (#16000-077, Invitrogen, Burlington/ON, Canada) and 4 mM L-glutamine. During the batch experiment, CHO cells were seeded at 0.25×10^6 cells/mL in 500 mL spinner flasks (#1967-00250, Bellco Glass, Forest Hills/NY, USA) and grown in a 37 °C, 5% CO₂ incubator. Two replicate experiments (runs A and B), each consisting of four spinner flask cultures, were performed in a batch culture set up. Cultures in the same run were seeded from the same parent culture. Trypan blue assays were used to obtain viable and dead-cell counts throughout typical subculturing and during the experiment.

Flow cytometric detection of apoptosis

The presence and progression of apoptosis in CHO cells were assayed via flow cytometry (FCM). In both

experiments (runs A and B), cell samples were collected from four spinner flasks during the 2-weeks cultivation period, and stained with fluorescent inhibitors of caspases (FLICA) and propidium iodide (PI) before subjecting to FCM assays. The FCM analyses were performed on a Becton–Dickinson FACS Vantage SE with a 488 nm excitation and FL1-H and FL3-H detection filters for measuring FLICA and PI intensities, respectively. The presence of apoptosis was determined with the detection of activated executioner caspase 3 and 7 (ICT094, AbD Serotec, Kidlington, UK). In run B only, the detection of two major initiator caspases, namely caspase 8 (ICT910, AbD Serotec, Kidlington, UK) and caspase 9 (ICT910, AbD Serotec, Kidlington, UK) were conducted in addition to the caspase 3 tests. Analyses were conducted daily throughout culturing until the fraction of live, non-apoptotic population dropped below 30% of the cell population.

Protein extraction

Throughout cultivation, culture samples from run A were collected for protein extraction. Cells were washed with 1X phosphate buffered saline solution and resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM pH 8.0 Tris, protease inhibitor cocktail (1 μ L per 10^6 cells, P8340, Sigma, Oakville/ON, Canada)). After rotating the lysis buffer-cell mixture at 4 °C for 1 h, cell debris was pelleted by centrifugation. Protein concentrations were immediately measured after extraction using the Bio-Rad protein assay (Cat. #500-0002, Bio-Rad Laboratories Ltd., Mississauga/ON, Canada).

Differential in gel electrophoresis

Each DIGE gel compares a protein sample collected on day 4.5, 5.5 or 8.5 to that from day 2.5, which was considered as a healthy apoptosis-negative sample since it was collected very early in the culture. Run A was assayed. Four biological replicate gels were prepared for each time point comparison, resulting in a total of 12 gels for analysis. Fifty μ g of each protein sample was stained with 200 μ mol of CyDye DIGE Fluor minimal dye, either Cy3 or Cy5. Cy2 was used to label 50 μ g of internal standard, which consisted of an equal portion of all 16 protein samples analyzed in the DIGE experiment. Isoelectric focusing (IEF) was

carried out with 24 cm pH 3–10NL Immobiline DryStrip gel strips on Ettan IPGphor II followed by SDS–PAGE on 12% polyacrylamide gels using the DALTsix Electrophoresis unit (GE Healthcare). The gels were scanned with a Typhoon 9400 scanner at a PMT setting of 600 V and 200 micron resolution. The gels were analyzed using the DeCyder v6.0 software (GE Healthcare). Paired *t* tests and ANOVA with a FDR cutoff of 0.1 were used to filter and select differentially expressed protein spots for subsequent mass spectrometry identification. Manual screening of the *t* test lists was carried out to eliminate non-protein spots (such as dust), spots with fold change less than 1.1, and spots with very low abundance that were unlikely to be identified successfully by mass spectrometry.

Mass spectrometry protein identification

The majority of the ESI MS/MS analyses were carried out at the University of Waterloo Mass Spectrometry Facility using Waters/Micromass Q-ToF Ultima Global with nano injection, positive electrospray ionization (ESI) and Quadrupole-Time of Flight (QToF) detection. For low abundance protein spots, LC–MS/MS analyses were performed at the Atlantic Research Center of Dalhousie University using an Applied Biosystems QTRAP 2000. Separation of

peptide extracts by C18 reverse-phased nano-LC was performed before the ESI MS/MS analysis with Quadrupole Ion Trap (QIT) detection. MS results were analyzed and matched to known protein databases using PEAKS v2.5 (Bioinformatics Solutions Inc., Waterloo/ON, Canada) and MASCOT MS/MS Ions Search (Matrix Science Inc., Boston/MA, USA).

Results

Cell growth and the progression of apoptosis

In addition to cell growth and viability measured from Trypan blue assays (Fig. 1), the progress of apoptosis in cells was determined with FCM by the presence of executioner caspase 3 and 7 and cell permeability to propidium iodide (PI). Cells permeable to PI are considered nonviable and an indicator of the loss of membrane integrity. Using this bi-color assay, cells can be categorized as (1) C3–PI– (live non-apoptotic) cells that possess intact membrane and no activated executioner caspases, (2) C3+PI– (early apoptotic) cells that contain activated caspases yet still maintain their membrane integrity, (3) C3+PI+ (late apoptotic) cells which have non-intact membrane and activated caspases 3 and 7, and (4) C3–PI+ (primary and secondary necrotic) cells that

Fig. 1 Average cell growth and viability trend in run A and run B cultures

Fig. 2 Apoptosis progression in run A and run B based on FCM caspase 3/7 assay. FCM assays were performed for two additional time points in run B

display a damaged membrane but contain no activated caspase.

The overall pattern of the four cell subpopulations over time between the two runs is similar (Fig. 2). The total apoptotic percentage remained less than 30% of the total cell count until day 9.5. The peak amount of early apoptotic cell population occurred between days 5.5 and 8.5. After day 8.5, the proportion of late apoptotic cells (which are considered no longer viable) exceeded that of the early apoptotic cells. A similar percentage of apoptotic cells was previously found in similar conditions by fluorescence microscopy (Naderi et al. 2010).

FCM comparison between the activation of caspase 3, 8 and 9

Flow cytometry assays on the amount of activated initiators caspase 8 and caspase 9 were performed to monitor and compare the onset of extrinsic and intrinsic/ER pathways, respectively in run B. Figure 3

compares the results from all three caspase tests. The proportion of C–PI– cells appears to be significantly less when comparing results for the caspase 9 assay to those of caspase 3 and caspase 8. Based on *t* test statistics ($p < 0.05$) the relative number of C9+PI– cells increased more significantly over the course of the runs than either C3+PI– or C8+PI– cells. The fast accumulation of live caspase 9-positive cells began soon after day 2.5 and lasted until day 8.5 when the dead caspase 9-positive cells (C9+PI+) began accumulating. The level of cells showing activated caspase 3 and activated caspase 8 were similar.

Analysis of whole cell protein extracts using differential in gel electrophoresis

Based on the FCM assays of caspase 3 and the cell count results, four time points were selected for examining altered protein expressions in CHO cells undergoing prolonged cultivation and apoptosis in run A. Cell samples from day 2.5 cultures, which were still in the mid-exponential phase, were used as healthy, apoptosis-negative control samples. Day 4.5 was sampled as an early stationary phase time point when levels of both early and late apoptotic cells were just starting to increase. Cells collected on day 5.5 were considered as a mid-stationary phase sample, since the FCM assays showed peak amounts of early apoptotic population. Lastly, day 8.5 was selected since it marked the beginning of the death phase and contained equal quantities of late apoptotic and early apoptotic cells. To avoid the expected interference of deteriorated cellular contents, protein samples beyond day 8.5 were not considered.

Table 1 shows the number of differentially expressed proteins when comparing protein abundance on day 2.5 to those on day 4.5, 5.5 and 8.5, respectively. As expected, the number of differentially expressed proteins increased as the culture aged and the proportion of apoptotic cells grew (as indicated by FCM). The total number of unique differentially expressed protein spots, based on *t* test scores, was 62. In addition, the ANOVA test yielded 66 potential candidates. More than 70% of the protein spots were common between *t* tests and ANOVA, leading to 79 unique spots when the two tests were combined. In general, many spots showed unidirectional abundance changes through time, exhibiting up to threefold change in abundance ratios (Table 2).

Fig. 3 Comparison between cell subpopulations based on three FCM caspases assays of run B cultures. **a** C–PI–, **b** C+PI–, **c** C+PI+, **d** C–PI+ subpopulations. Caspase 8 and 9 are pathway-specific initiator caspases while caspase 3 is the common executioner caspases in different apoptotic pathways. Abbreviations: *C* caspase, *PI* propidium iodide

Identified differentially expressed proteins

Using ESI MS/MS and LC–MS/MS, a total of 38 candidate spots were identified. Out of these, 25 identifications were unique. One spot matched to two distinct identifications (see Table 2; Fig. 4). Of the identified cytoskeletal proteins, vimentin/ α -tubulin and actin were downregulated. A significant proportion of protein spots were chaperones (18 out of 40) and metabolic enzymes (10 out of 40). The identified molecular chaperones include calreticulin, ERp60, PDI, PDIA6, BiP, GRP94, GRP170, HSC70, HSP90 β and FKBP52. All of the ER chaperone proteins were upregulated, but the three cytosolic chaperones, HSC70, HSP90 β and FKBP52, were not. The glycolytic and metabolic enzymes triosephosphate isomerase, phosphoglycerate mutase, pyruvate kinase isozyme M1/M2, phosphoglycerate kinase 1, glyceraldehyde-3-phosphate dehydrogenase, enolase-1 and lactate dehydrogenase chain A/C all increased their abundance over time during the cultivation period. Lastly, the other proteins were identified as 14-3-3

protein epsilon isoform transcript variant 1, galectin-1, laminin receptor, 80K-H and galectin-3.

Discussion

Although the time of initiation of apoptosis in the (unsynchronized and non-induced) cultures varied, some consistent trends regarding the progression of apoptosis could still be observed. Up until day 4.5, the CHO-IgG-9 β 8 cultures mostly consisted of healthy viable cells that proliferated normally. During this time, the degree of apoptosis remained at a basal level (less than 5%). However, nutrient concentrations continue to decrease and waste product levels began building up (Naderi et al. 2010), both of which are potential triggers for apoptosis (Mercille and Massie 1994). The deterioration in extracellular conditions led to the initiation of apoptosis in cells after day 4.5. The early apoptotic cell populations accumulated to a maximum of 15–20% of the culture. Moreover, from day 4.5 to day 8.5, more apoptotic cells progressed

Table 1 Mass spectrometry results from PEAKS and MASCOT analyses

Protein name	Fold change/ <i>t</i> test, <i>p</i> value			ANOVA <i>p</i> value	PEAKS		MASCOT							
	Day 2.5 versus 4.5	Day 2.5 versus 5.5	Day 2.5 versus 8.5		Score (%)	Accession	Mass (kDa)	# Peptides matched	Sequence coverage (%)	Score	Accession	Mass (kDa)	PI	# Peptides matched
(a) Cytoskeletal proteins														
Vimentin	−1.29/0.34	−1.18/0.23	−1.39/0.14	3.96e−2	93.8	BAD74030	53.6	5	12	60	P02544	53.6	5.06	3
α-tubulin	−1.11/0.71	−1.20/0.45	−1.41/0.08	8.04e−2	−	−	−	−	−	74	Q3TIZ0	50.6	4.96	2
Actin (β/γ)	−1.12/0.55	−1.22/0.26	−1.46/0.04	4.77e−3	98.3	P83751	36.3	6	20.1	194	Q3UGS0	42.1	5.37	5
	−1.12/0.44	−1.22/0.22	−1.47/0.04	1.88e−4	95.9	P20359	41.6	6	16	177	O35247	42	5.37	5
	−1.10/0.56	−1.23/0.31	−1.47/0.07	2.30e−3	99.7	O76784	41.8	9	23.9	203	O35247	42	5.37	7
	−1.08/0.74	−1.24/0.44	−1.47/0.11	3.11e−2	99.9	P60712	41.6	9	22.5	334	O35247	42	5.37	8
					99.8	O76784	41.8	10	26.6	336	Q3UGS0	42.1	5.37	10
(b) ER and cytosolic chaperone proteins														
Calreticulin	1.41/0.48	1.44/0.31	3.17/0.06	4.39e−3	93.1	Q8K3H7	48.2	8	23.5	131	Q8K3H7	48.4	4.33	5
	1.43/0.31	1.64/0.21	3.11/0.05	1.54e−5	90.9	Q8K3H7	48.2	5	16.8	84	Q8K3H7	48.4	4.33	3
ERp60	1.40/0.19	1.54/0.11	2.25/0.03	1.54e−5	95	Q91Z81	56.7	8	16.8	110	Q91Z81	57.2	5.98	5
	1.42/0.19	1.52/0.26	2.37/0.07	1.73e−3	85	P11598	56.6	5	11.3	72	P27773	57	5.98	3
	1.42/0.17	1.53/0.22	2.40/0.05	1.88e−4	92.4	Q91Z81	56.7	5	9.9	85	Q91Z81	57	5.98	4
	1.41/0.34	1.54/0.10	2.33/0.02	1.40e−5	83	P11598	56.6	4	9.5	65	P27773	57	5.98	3
	1.40/0.42	1.45/0.13	2.21/0.02	2.10e−4	66.7	Q91Z81	56.7	3	6.9	−	−	−	−	−
PDI	1.45/0.01	1.57/0.09	1.93/0.03	2.36e−7	94.2	P05307	57.2	4	9	126	P05307	57.6	4.8	4
PDIA6	1.34/0.17	1.50/0.09	2.08/0.03	6.49e−6	97.3	P38660	48.1	6	19.4	222	P38660	48.5	5.04	5
	1.36/0.26	1.50/0.17	2.15/0.04	1.88e−4	89.1	P38660	48.1	4	13.7	96	P38660	48.5	5.04	3
	1.29/0.23	1.46/0.22	1.97/0.06	8.08e−4	88.3	P38660	48.1	5	16	131	P38660	48.5	5.04	4
	1.34/0.18	1.51/0.14	2.10/0.05	7.73e−5	95.8	P38660	48.1	6	21.2	88	P38660	48.5	5.04	2
BiP	1.43/0.17	1.57/0.10	2.55/0.03	6.49e−6	99.8	P07823	72.3	11	22.5	205	P06761	72.5	5.07	5
GRP94	1.42/0.17	1.49/0.22	2.65/0.02	9.48e−6	99.6	Q3UBU0	92.4	7	11.4	210	Q3UBU0	92.7	4.74	4
GRP170	1.35/0.23	1.59/0.17	1.81/0.12	5.13e−4	92.8	Q9JKR6	111.1	6/5	6.6	262	Q9JKR6	111.4	5.14	8
HSC70	−1.13/0.42	−1.15/0.30	−1.18/0.04	6.06e−2	73.3	Q53HF2	53.4*	8/2	16	234	P19120	42.2*	6.62	5
HSP90β	−1.13/0.28	−1.24/0.23	−1.31/0.07	3.05e−2	−	−	−	−	−	53	P34058	83.6	4.95	6
FKBP52	−1.09/0.57	−1.13/0.21	−1.29/0.05	6.52e−2	−	−	−	−	−	168	P27124	51.7	5.34	3
(c) Glycolytic proteins and other metabolic enzymes														
Triosephosphate isomerase	1.55/0.19	1.93/0.10	2.05/0.07	4.85e−5	98.6	Q53HE2	26.7	10/6	31.7	153	P00939	26.9	7.1	2
Phosphoglycerate mutase1	1.34/0.26	1.52/0.22	1.57/0.10	7.22e−3	86.9	P18669	28.6	8	36.4	167	P18669	28.9	6.67	8

Table 1 continued

Protein name	Fold change/ <i>t</i> test, <i>p</i> value			ANOVA <i>p</i> value	PEAKS		MASCOT							
	Day 2.5 versus 4.5	Day 2.5 versus 5.5	Day 2.5 versus 8.5		Score (%)	Accession	Mass (kDa)	# Peptides matched	Sequence coverage (%)	Score	Accession	Mass (kDa)	PI	# Peptides matched
Pyruvate kinase isozyme M1/M2	1.25/0.40	1.41/0.26	1.51/0.07	2.93e-2	96.2	P11980	57.7	6	12.6	147	P52480	58.3	7.42	5
Phosphoglycerate kinase	1.43/0.12	1.78/0.09	1.71/0.09	4.94e-4	89.4	P50310	44.5	5	17	141	P50310	44.8	8.02	4
Glyceraldehyde-3- phosphate	1.19/0.37	1.32/0.26	1.26/0.25	3.05e-2	65.5	P00355	35.7	5	21.1	225	P17244	36	8.49	5
dehydrogenase	1.19/0.42	1.36/0.21	1.27/0.16	1.21e-2	98.3	P00355	35.7	7	28.6	218	P17244	36	8.49	5
Enolase-1	1.14/0.53	1.24/0.29	1.09/0.61	6.29e-2	90.9	P04764	50.6	4	11	213	Q9PVK2	47.5	6.62	4
L-lactate dehydrogenase chain A/C	1.22/0.36	1.31/0.23	1.14/0.38	2.07e-2	77.1	P07864	28.9	2	7.9	149	P00340	36.7	8.18	2
	1.19/0.49	1.29/0.23	1.20/0.31	6.52e-2	35.7	Q5R1W9	36.5	1/3	5.7	89	Q06BU8	36.8	7.01	2
(d) Other proteins														
14-3-3 epsilon	-1.07/0.46	-1.07/0.39	-1.15/0.07	3.80e-3	72.2	Q1HPT4	29.6	2/1	9.5	213	Q4VJB6	26.7	4.76	3
Galectin-1	1.03/0.72	1.14/0.13	-1.08/0.09	1.00e-2	42.1	P48538	14.6	2	18.7	42	P48538	15.1	5.52	2
Laminin receptor 1	-1.11/0.62	-1.16/0.30	-1.19/0.09	2.09e-1	78.5	P08865	31.8	3	11.2	165	P26452	32.9	4.8	3
80 K-H	1.20/0.58	-1.04/0.81	1.82/0.14	6.29e-2	49.5	Q3U518	58.6	2	5.4	101	P14314	45.3	4.27	2
Galectin-3	-1.16/0.24	-1.20/0.15	-1.65/0.05	3.59e-5	33	P08699	27	1 (+3)	5.4	-	-	-	-	-

A false discovery rate cutoff of 0.1 was used to establish statistical significance of fold changes, and FDR corrected *p* values are shown below

*based on the estimated MW of ~70 kDa from the gel, this is likely the larger 71 kDa isoform of Hsc70

Table 2 Number of differentially expressed proteins obtained from *t* tests and ANOVA

Statistical test	Number of differentially expressed proteins after FDR cutoff and manual screening	Fold change (up/down/mixed ^a)
<i>t</i> test: day 2.5 versus 4.5	2	2/0/0
<i>t</i> test: day 2.5 versus 5.5	8	8/0/0
<i>t</i> test: day 2.5 versus 8.5	59	33/26/0
ANOVA	66	41/23/2

^a When comparing the changes across all 4 time points in ANOVA test, a few spots did not show uniform direction of expression changes

from the early stage into the late phase where the plasma membrane became permeable, rendering the cells nonviable. From day 8.5 onward, the proportion of late apoptotic and caspase-negative dead cells continuously increased and eventually accounted for a large portion of the culture.

Although some cells likely underwent apoptosis via the extrinsic pathway, the dominant trigger for apoptosis seems to be related to intracellular death stimuli as shown by the steep increase of activated caspase 9 early in the culturing process. As early as 5.5 days after seeding, CHO cultures showed an average of 70% cells with active caspase 9 (and intact plasma membrane) whereas less than 15% of the culture exhibited active caspase 8 and caspase 3. Although death receptor-mediated cell death in some cell lines might engage a crosstalk with the mitochondrial pathway, it is more likely that the activation of caspase 9 in this work corresponds to the onset of mitochondrial and/or ER pathway. Prolonged cultivation of cell cultures is known to result in nutrient limitation and accumulation of toxic waste products, both of which are linked to intrinsic cell death. Moreover, expression of recombinant protein in cell cultures can overload the ER folding system and lead to ER apoptotic cell death.

In agreement with the FCM results obtained in this work, a CHO cDNA array study comparing mRNA expression over 5 days (Wong et al. 2006a) showed an increase in both caspase 8 and caspase 9 mRNA levels within cells after the exponential phase. However, evidence from their study seems to indicate that the Fas-mediated extrinsic pathway was the

dominant mechanism for apoptotic cell deaths in these IFN- γ producing CHO batch cultures. It is possible that this difference is cell line specific, or is influenced by the expression of recombinant proteins. For instance, the CHO IFN- γ cell line in the study by Wong et al. produces a recombinant human interferon gamma, which has been documented to activate Fas death receptor signaling (Curtin and Cotter 2003; Ossina et al. 1997; Spanaus et al. 1998). Therefore, the observed transcriptional changes in the CHO IFN- γ batch culture might not be universally applicable to other CHO cell lines.

The onset and progression of apoptosis within the CHO culture was clearly indicated by the flow cytometry results, but not all changes brought about by apoptosis were reflected explicitly by the proteomic analysis. This can be attributed to the facts that apoptosis is controlled both at transcriptional and post-translational levels (Twomey and McCarthy 2005) and that some of the changes necessary to inflict apoptotic cell death do not result in differential protein abundance. Although it is possible that minute abundance changes are below our threshold of detection, many of the apoptotic proteins involved are expected to maintain their expression level and function via mechanisms such as localization, association with other proteins and post-translational modification. Since apoptosis within an individual cell requires a quick response and usually takes no longer than a few hours, post-translational mechanisms are most likely involved (as opposed to slower translational effects). In theory, it is possible to detect these changes in a 2D gel, since, for example, the cleavage of pro-caspases into their active forms leads to changes in molecular weight and PI. Nonetheless, caspase proteins were not among those identified as differentially expressed. This is not surprising, given the discovery-driven (non-specific) nature of proteome analysis.

Of the 79 differentially expressed protein spots from the DIGE experiments, identification of 38 protein spots were obtained from mass spectrometry analyses. We characterized these into four categories based on their functions as discussed below.

The cytoskeletal proteins identified, namely actin, vimentin and α -tubulin are among the known set of substrates for caspase-mediated proteolysis during apoptosis. The disruption and disintegration of the actin network is crucial for the reorganization of the network to facilitate the orderly dismantling of dying

Fig. 4 MS identified differentially expressed protein spots from the comparison of healthy (day 2.5) to aging/apoptotic (day 8.5) CHO protein samples. *Dotted line* indicates proteins that upregulated over time while *solid line* indicates a reduction in expression. Estimates of molecular weight and PI were obtained from the protein identifications. Abbreviations: *ATPB* ATP synthase beta subunit, *BiP* binding immunoglobulin protein, *ERp60* endoplasmic reticulum protein 60 kDa,

FKBP52 FK506-binding protein 52, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *GRP* glucose-regulated protein, *HSC70* heat shock cognate protein 70 kDa, *HSP* heat shock protein, *LAMR1* laminin receptor 1, *LDH* L-lactate dehydrogenase chain, *PDI* protein disulfide isomerase, *PGM* phosphoglycerate mutase, *PGK* phosphoglycerate kinase 1, *PK* pyruvate kinase isozyme M1/M2, *Prx6* peroxiredoxin-6, *TPI* triosephosphate isomerase

cells (Ndozangue-Touriguine et al. 2008) and to trigger cell shrinkage during apoptosis. Interestingly, several β - and γ -actin isoforms were differentially expressed in this study. The cleavage of vimentin prohibits the intermediate filaments from maintaining cell integrity and generates a pro-apoptotic N-terminal fragment able to amplify the cell death signal (Byun et al. 2001). Meanwhile as a regulatory region for microtubule polymerization, the C-terminus of tubulins released post-cleavage appears to affect the

dismantling of microtubules during the early apoptotic phase and the subsequent reassembling in later apoptotic stages (Moss and Lane 2006). Vimentin and α -tubulin, however, were identified from a single spot. Since their corresponding molecular weights and PI (53 kDa/pH5.06 and 50 kDa/pH4.96, respectively) are similar, we attribute this to overlapping between multiple protein spots.

Many cellular or ER stresses, including oxidative stress, amino acid depletion, protein over-expression

and inhibition of glycosylation reactions, can lead to the accumulation of misfolded proteins within the ER lumen and the activation of the unfolded protein response (UPR) (Kim et al. 2006). Hence, it is not surprising that ten ER chaperone proteins were identified from the comparative proteomic analyses. HSC70, HSP90 and FKBP52 are the only identified cytosolic chaperones and these were downregulated. Meanwhile, the remaining seven chaperone proteins, calreticulin, ERp60, BiP, GRP94, GRP170, PDI and PDIA6, which function mostly in the ER, exhibited large fold increases as cells aged and died during prolonged cultivation. All seven ER-localized chaperones and folding proteins identified are known UPR inducible proteins, suggesting the presence of ER stresses and the activation of UPR during prolonged CHO culturing.

As components of the calnexin/calreticulin ER chaperone system, calreticulin and ER protein 60 (ERp60) interact and promote the proper folding of glycoproteins (Michalak et al. 2009). Moreover, their roles in apoptosis have also been characterized. The localization and exposure of calreticulin to cell surface during apoptosis serves as a signal for the phagocytotic engulfment of cells (Michalak et al. 2009). ERp60, which folds the mitochondrial calcium-dependent neutral cysteine protease μ -calpain, is responsible for the cleavage and release of membrane bound apoptosis inducing factor (AIF) into the intermembrane space of mitochondria in preparation for subsequent release into the cytosol during apoptosis. The involvement of ERp60 perhaps was indicated by the consistent upregulation of five isoforms in this study.

The remaining five identified upregulated ER chaperones belong to the BiP/GRP94 ER chaperone system, which folds the hydrophobic regions of proteins. Thus, far, these proteins have only been associated with UPR and cellular pro-survival mechanism. The increase of their protein expression likely was part of the UPR. Previously, the overexpression of BiP has been shown to protect cells against ER stress-induced apoptosis in human endothelial cells while the reverse leads to UPR activation and apoptotic cell death (Xu et al. 2009). Deficiency in pro-apoptotic ER chaperone proteins, calreticulin and ERp60, have also been separately shown to render cells less sensitive to the induction of apoptosis (Xu et al. 2009).

Among the ten chaperone-related proteins identified, the cytosolic proteins, HSC70, HSP90 β and FKBP52, are the only ones that decreased in abundance as the culture aged and apoptosis progressed. Both HSC70 and FKBP52 have been reported to function as HSP90 co-chaperones. In particular, HSP70 isoforms promote cell proliferation and survival by loading into HSP90 and neutralizing the effects of several pro-apoptotic proteins such as AIF, APAF-1 and BAX in both the intrinsic and extrinsic pathways (Powers et al. 2009). It is possible that the decrease of HSC70, HSP90 β and FKBP52 in CHO cells was a means to remove pro-survival signals and chaperones in order for the cells to undergo apoptosis.

Interestingly, of the ten enzymes in the glycolytic chain, six showed an increase in protein abundance in our prolonged CHO cultures. These were triosephosphate isomerase (TPI), phosphoglycerate mutase (PGM), pyruvate kinase isozyme, phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase-1. L-lactate dehydrogenase (LDH), which converts the end product of glycolysis into lactate and generates ATP, was also upregulated. This is consistent with the observed enhancement of lactate formation during this phase (Naderi et al. 2010). There is an obvious metabolic shift to primarily anaerobic energy-yielding metabolism as the mitochondrial membrane is compromised. It may be that the increase in the abundance of these metabolic enzymes was needed to ensure that sufficient energy was available for cell proliferation and maintenance (and possibly apoptosis) as glucose levels drop over time. Additionally, several studies have reported non-metabolic functions of some glycolytic enzymes. For example, the nuclear localization of several glycolytic proteins, including enolase-1, GAPDH and LDH, has been noted and linked to possible involvement in transcription and DNA replication (Kim and Dang 2005). More importantly, expression of GAPDH has been shown to induce apoptosis and the induction of GAPDH in apoptotic cells has been previously reported (Kim and Dang 2005).

Five differentially expressed proteins with diverse roles and expression changes were also identified in this study. They are 14-3-3 epsilon, laminin receptor 1 (LAMR1), galectin-1, galectin-3 and 80K-H. Under normal conditions, 14-3-3 proteins prevent the

initiation of apoptosis by binding and inactivating proteins in the apoptosis pathway, such as Bad and Bax. 14-3-3 epsilon is cleaved by caspase during apoptosis (Nomura et al. 2003), which explains the observed reduction of full length 14-3-3 proteins in prolonged CHO culture. Meanwhile, cleavage of LAMR1, which forms the structural component of ribosomes, has been reported in apoptotic cells and proposed to have a pro-apoptotic effect (Gerner et al. 2002). Galectins are beta-galactoside-binding proteins that regulate various cellular activities including differentiation, apoptosis, cell growth, and tumor progression (Carlage et al. 2009). A slight down regulation of galectin-1 was observed in this study when significant percentage of the cultures was apoptotic. Although the exact mechanisms are unknown, galectin-3 is the only member of the galectin family that exhibits an anti-apoptotic role against various stimuli (Yang and Liu 2003), in addition to other functions. The slightly reduced abundance observed during prolonged CHO cultivation might be a means to remove pro-survival signals in dying cells and eliminate any unnecessary interference during the execution of apoptosis. A recent study has shown that 80K-H, also known as glucosidase II beta subunit, can directly enhance the IP3- and ATP-induced release of Ca^{2+} by interacting with IP3 receptors (Kawai et al. 2009).

With the exception of a transcriptional profile of non-induced apoptotic cell death in batch and fed-batch CHO cultures by Wong and colleagues (Wong et al. 2006a), most apoptotic studies have focused on examining changes when a specific apoptotic pathway was induced by a particular agent. While Wong's objectives were similar to ours, comparisons using microarrays were limited to the probes built on the microarray chips, and the majority of the apoptotic genes were probed with mouse sequences. Most of the reported differentially expressed genes in Wong's study were not identified in our DIGE study. (This is often the case when comparing microarray and proteome data sets).

Induced apoptosis has been studied in various cell lines using a number of approaches (Baik and Lee 2010; Dong et al. 2006; Schmidt et al. 2007; Thiede et al. 2006). Comparative 2D gel electrophoresis of sodium selenite-induced apoptosis in human leukemia NB4 cells has been performed and confirmed by RT-PCR and Western blot (Dong et al. 2006). Out of

the 26 downregulated and four upregulated proteins that exhibited a minimum 1.5 fold change in that study, only α -tubulin, β -actin and LAMR1 were also identified in our CHO IgG-9 β 8 study. However, instead of decreasing in abundance, α -tubulin and β -actin were both upregulated in NB4 cells. The remaining proteins included regulators of signal transduction, members of the mitogen activated protein kinase family, proteins that regulate *c-fos*, *c-myc* and *c-jun* mRNA expression, metabolism related proteins and proteins associated with DNA damage. Although the authors suggested the involvement of the JNK pathway in selenite-induced apoptosis, the specific apoptotic pathway triggered was not examined.

In another study, Fas-induced apoptosis in human Jurkat T cells was analyzed using stable isotope labeling with amino acid (SILAC) leucine instead of CyDyes (Thiede et al. 2006). Using this method, the authors identified 28 out of 38 apoptosis-modified protein spots on 2D gels that exhibited a minimum 1.5 fold change between normal and Fas-induced apoptotic cultures. Similarly to the apoptotic NB4 proteome, most (31) of the altered protein spots were downregulated while the remaining seven spots exhibited increased expression. Of all 28 identified proteins, only LAMR1, which decreased in expression, was also identified as differentially expressed in our CHO IgG-9B8 cells.

Quantitative proteomic analyses of cisplatin-induced apoptotic Jurkat T cells with SILAC and SDS-PAGE has also been conducted (Schmidt et al. 2007). Cisplatin is thought to inhibit DNA replication and transcription, leading to cell death by apoptosis. In this proteomic study, 26 proteins with abundance changes greater than 1.5 fold were identified, 19 of which were already known to be involved with apoptosis. Upregulation in 14 and downregulation in 12 proteins were observed. Nine RNA-binding proteins and seven cytoskeletal proteins, which include actin, α -tubulin and β -tubulin, were identified in this study. However, rather than downregulation of actin, α -tubulin and β -tubulin as in "aging" CHO cultures (which included a mix of healthy, apoptotic and dead cells) cisplatin-induced apoptotic Jurkat cells showed an increase in these cytoskeletal proteins.

Some studies have reported subsets of proteins affected by apoptosis that are similar to the findings of the current work. For instance, a proteomic study

examining ionizing radiation-induced apoptosis of human prostate cells revealed abundance changes in mostly ER stress response/chaperone and cytoskeletal proteins (Prasad et al. 1999). Upregulation of ER proteins, including GRP94, calreticulin, calnexin, GRP78, GRP75, HSP70 and PDI, as well as down-regulation of cytoskeletal proteins, were reported in this study. Similarly, in a quantitative proteomics study examining staurosporine-induced apoptosis in human neuroblastoma derived cells, many molecular chaperones and ER proteins such as GRP94, BiP, ERp60 and HSP70 proteins, and cytoskeletal proteins like vimentin and α -tubulin were detected (Short et al. 2007). It was concluded that the treatment of staurosporine provokes an ER stress and induces the UPR.

Many researchers have applied proteomic approaches to the study of apoptosis. However, correlation between the results of different studies is usually low. This deviation might be due to differences in cell line, apoptotic inducer, proteomic techniques and experimental design (Thiede et al. 2001; Thiede and Rudel 2004). For instance, the heterogeneous nature of non-induced CHO cultures likely influenced our DIGE results, as the populations assayed were composed of cells at different stages. Furthermore, in comparison with our prolonged observations, the majority of the induced apoptosis studies in the literature were performed in shorter time frames, usually a few hours after treatment. In these induced studies, one would expect to observe responses more restricted to apoptosis, since abrupt changes were brought uniformly to most if not all cells. On the other hand, in our prolonged cultures, cells were subjected to slowly deteriorating culture conditions and the eventual onset of apoptosis. Many of the upregulated proteins identified were ER chaperone and metabolic enzymes, which likely increased in abundance to cope with the initial cellular stresses and changes in metabolism as key nutrients were depleted from the environment. Lastly, technical variations, such as pH range and cell lysis buffer, can significantly affect the number of observed changes made in these studies.

Conclusions

This study demonstrated that the major changes in aging CHO cultures undergoing apoptosis involve the

regulation of stress response and energy metabolism related proteins. Proteins that directly modulate apoptosis were not readily apparent and could not be identified as differentially expressed from the proteomic analyses. Furthermore, the intracellular response prior to the initiation of apoptosis included a complex network of changes that might be as important as the regulation of apoptotic proteins. Although no direct apoptosis-regulating proteins were identified, the DIGE results have established the potential importance of UPR and energy metabolism in prolonged cultures and apoptotic cell death. Along with the knowledge of the type of apoptotic pathway triggered, apoptotic proteins associated with ER stress, mitochondrial dysfunction and energy metabolism should be considered in the optimization of CHO IgG-9 β 8 cultivation.

This quantitative proteomic study associates cellular changes at the protein level with culture-level aging and progression of apoptosis during prolonged cultivation in MAb-producing CHO cultures. To our knowledge, the work presented here is the first proteome-wide investigation of non-induced apoptosis in mammalian cells. Since no treatment was made to artificially induce cell death, the trigger for apoptosis in these cells was the process of prolonged cultivation as experienced in typical industrial bioreactors. Despite the fact that several proteomic analyses of apoptosis in various cell lines have been carried out, strategies taken by these studies all involved inducing the cultures with particular apoptotic agents. From the DIGE and MS analyses of this work, many proteins expected to be modified by the degradation of culture condition and the onset of apoptosis in protein-producing CHO batch cultures were identified. Apart from the common apoptosis-cleaved substrates and the stress-induced responses, the upregulation of six glycolytic enzymes responsible for the final six steps of glycolysis, and one post-glycolysis metabolic protein were surprising and unseen in previous studies. Moreover, the differential expression of several other pro- and anti-apoptotic proteins was detected in this study. Additionally, due to the small number of proteomics CHO maps available, the CHO IgG-9 β 8 DIGE gels and the corresponding protein identifications obtained in this study will be valuable additions to the current 2D gel database. This work illustrates the potential for proteomics approaches in revealing the intracellular

changes occurring in prolonged CHO cultures. Since most recombinant protein production in CHO platforms is not growth associated, the prolongation of viability in dense populations, rather than continuous growth, is important. This study provides a foundation for optimizing cell line-specific cultivation processes, prolonging longevity and maximizing protein production.

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