Identification of Differentially Expressed Proteins in Tumour Necrosis Factor-alpha-resistant and -sensitive Rat Hepatoma Cells

YASUHIRO KURAMITSU, XIULIAN ZHANG, YUFENG WANG and KAZUYUKI NAKAMURA

Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, Ube, Japan

Abstract. *Background: Our earlier studies reported that ONO-4007, a synthetic lipid A analogue with low endotoxic activity, had shown much effect on tumour necrosis factor (TNF)-alpha-sensitive rat hepatoma cells, even though it had no effect on TNF-alpha-resistant cells. Materials and Methods: To find biomarkers which relate to the sensitivity of cancer cells to TNF-alpha, proteomic differential display analysis for TNF-alpha-resistant cKDH-8/11 and -sensitive KDH-8/YK rat hepatoma cell lines was carried out using two-dimensional gel electrophoresis and liquid chromatography–tandem mass spectrometry. Results: Two-DE analysis showed 32 spots, whose expression was different between cKDH-8/11 cells and KDH-8/YK cells. Of these, 12 were up-regulated and 20 were down-regulated in cKDH-8/11 cells compared to KDH-8/YK cells. The up-regulated proteins include transitional endoplasmic reticulum ATPase, 78kDa glucose-regulated protein (GRP78), heat-shock cognate 71 kDa protein (HSC71) and protein disulfide-isomerase A6. The down-regulated proteins included alpha-enolase, aldose reductase, glutathione reductase, annexin A1, glutamate dehydrogenase 1 and dihydrolipoyl dehydrogenase. Conclusion: These findings suggest that these differentially regulated proteins could be factors responsible for the resistance of cKDH-8/11 cells to TNF-alpha-induced cell death.*

KDH-8 is a transplantable rat hepatoma cell line. From this cell line, two sublines were established. One is tumour necrosis factor (TNF)-sensitive KDH-8/YK, and the other is TNF-resistant cKDH-8/11. Although these two cell lines proliferated equally, only KDH-8/YK was TNF sensitive (1).

ONO-4007 is a lipid A analogue with low endotoxic activity. *In vivo* treatment with this agent of rats bearing KDH-8/YK cells was significantly effective, in spite of there being no effect on rats bearing cKDH-8/11 (2). Further studies suggested that the therapeutic effects of ONO-4007 depended on the TNF sensitivity of the cells, and the development of ONO-4007 as a new therapeutic agent against TNF-sensitive cancers was expected. In order to ensure that anticancer therapy with ONO-4007 is successful, it is very important to find particular proteins as biomarkers from cancer cells which show TNF resistance (3). Our previous study identified phosphatidylethanol-amine-binding protein (PEBP) as an up-regulated protein in TNF-resistant hepatoma cells compared with TNF-sensitive cells. However, it is still necessary to find other biomarker proteins (4).

Proteomic differential display is a basic method to compare the protein expression profiling among different sample groups. For this method, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques have been employed in many studies (5, 6). By means of 2-DE, proteins are separated according to their charge in isoelectrofocusing (IEF) gels and to their size in sodium dodecyl sulfate (SDS) gels (7). In this study, we used 2-DE to compare the expression patterns of intracellular proteins in TNF-sensitive KDH-8/YK cells and TNF-resistant cKDH-8/11 cells.

The purpose of present study was to identify biomarkers which may be responsible for TNF resistance and account for ONO-4007-treatment results.

Materials and Methods

Tumour cell lines and culture condition. KDH-8 is a rat hepatoma cell line induced by 3'-methyl-4-dimethylaminoazo-benzene in WKAH rat. It has been maintained *in vivo* by intraperitoneal passage every 5 days (1). KDH-8/YK is a cell line isolated from the primary culture of KDH-8 tumor cells. cKDH-8/11 is a sub-clone

Correspondence to: Yasuhiro Kuramitsu, MD, Ph.D., Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel/Fax: +81 836222212, e-mail: climates@yamaguchi-u.ac.jp

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isolated from the primary culture of KDH-8 tumor cells by limiting dilution (2). These cell lines have properties similar to those of the parent KDH-8 cells *in vivo* and have been maintained in a continuous *in vitro* culture in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Sample preparation. Cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4˚C, centrifuged at 21,500 ×*g* for 30 min at 4˚C, and the supernatants were used as samples for 2-DE.

Two-dimensional gel electrophoresis. IEF was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip. IEF was performed in the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (8).

Image analysis and spot picking. After 2-DE, the gels were washed with ultra-pure water three times to remove SDS. After fixing, the gels were stained with highly sensitive Coomassie Brilliant Blue SeePico™ (Benebiosis Co., Ltd, Seoul, Korea) (9), and expression levels of the protein spots were quantified with Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) (10). The differences in expression between KDH-8/YK and cKDH-8/11 cells were analyzed statistically by ANOVA test. The 2-DE analysis was repeated three times. The spots whose expression was significantly different between KDH-8/YK and cKDH-8/11 cells were selected for further analysis (11).

High performance liquid chromatography (LC)-MS/MS. After in-gel digestion, samples dissolved in 0.1% formic acid were centrifuged at 21,500×*g* for 5 min and the supernatant was used for HPLC-MS/MS analysis (12). An Agilent 1100 LC/MSD Trap XCT instrument (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five microliters of each sample was applied and separated on a Zorbax 300SB-C18 column, 75 μm, 150 mm; Agilent Technologies) (13). Protein identification was performed in the Agilent Spectrum Mill MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS Ions Search engine (http://www.matrixscience.com/search_form_select.html). The criteria for positive identification of proteins were as follows: filter by protein score>10.0, and filter peptide by score>8, % scored peak intensity (% SPI) >70. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (14).

Results

Figure 1A and B show the 2-DE patterns of KDH-8/YK and cKDH-8/11 cells, respectively. More than 600 protein spots were detected. Twelve spots (no. 1-12) appeared to be up-regulated, and 20 spots (no. 13-32) appeared to be down-regulated in cKDH-8/11 compared with KDH-8/YK cells.

The 32 differentially expressed protein spots were picked up and identified by the Agilent 1100 LC/MSD Trap XCT LC-MS/MS system. The results are shown in Table I.

Discussion

The results of the present study showed that 32 protein spots were differentially expressed between TNF-alpha-resistant and -sensitive rat hepatoma cell lines as identified by proteomic differential display analysis employing 2-DE and LC-MS/MS. The up-regulated proteins in TNF-resistant cKDH-8/11 cells were identified as transitional endoplasmic reticulum ATPase, heat-shock 90 kDa protein (HSP90)-alpha, 78 kDa glucose-regulated protein (GRP78), hea-shock cognate 71 kDa protein (HSC71), vimentin, protein disulfide-isomerase A6, heterogeneous nuclear ribonucleoprotein F, suppressor of G2 allele of S-phase kinase-associated protein 1 (SKP1) homolog, elongation factor 1-delta, ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3), peroxiredoxin-2 and myotrophin. The 20 down-regulated proteins in TNF-resistant cKDH-8/11 included endoplasmin and alpha-enolase.

GRP78 and HSC71 belong to the HSP70 family. GRP78 is a molecular chaperone, involved in the unfolded protein response. It facilitates the assembly of multimeric protein complexes inside the endoplasmic reticulum. HSC71 is a constitutively expressed chaperone. It binds to nascent polypeptides to facilitate correct folding. Down-regulation of GRP78 sensitizes prostate cancer cells which are resistant to adenovirus carrying Dickkopf-3-induced apoptosis (15). Castagna *et al*. reported that the basal level of HSC71 in cisplatin-resistant cervix squamous cell carcinoma cells was up-regulated compared to cisplatin-sensitive cells (16). Since TNF is very strong apoptosis inducer, the protectant role of GRP78 and HSC71 from apoptosis induced TNF could be strong in cKDH-8/11 cells.

UCHL3 is a member of the ubiquitin *C*-terminal hydrolase family. This enzyme de-ubiquitinates ubiquitin–protein conjugates in the ubiquitin-proteasome system. Although the function of UCHL3 in apoptosis is not completely understood, from the report of the analysis for UchL3-deficient mice, loss of UCHL3 leads to mitochondrial oxidative stress-related photoreceptor cell apoptosis in a caspase-independent manner (17).

Vimentin is a type III intermediate filament found in various non-epithelial cells, especially mesenchymal cells. Vimentin plays a role in the regulation of cell motility. Although its role in cell apoptosis is still controversial, Maxwell *et al*. reported that vimentin induced resistance against the CHOP chemotherapeutic regimen (cyclophosphamide, doxorubicin, vincristine, prednisone) in diffuse large B-cell lymphoma (18).

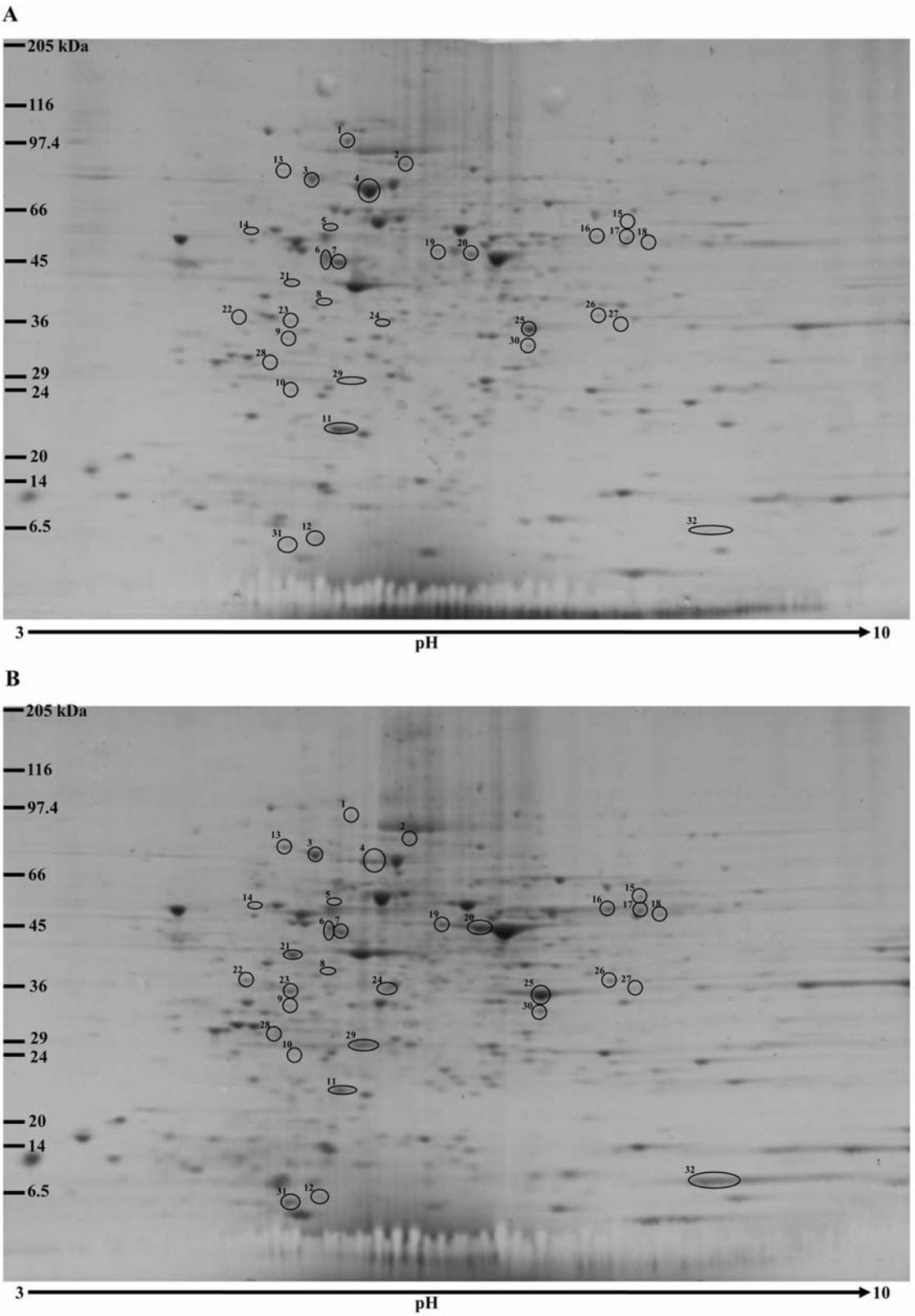

Figure 1. *2-DE gel pattern of A: cKDH-8/11 (TNF-alpha-resistant cell line), and B: KDH-8/YK (TNF-alpha-sensitive cell line) cells. Proteins (80 μg) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%. After fixing, the gels were stained with highly sensitive Coomassie Brilliant Blue SeePico™ .*

Table I. *Identification of proteins up- and down-regulated in cKDH-8/11 cells compared to KDH-8/YK cells.*

Spot no.	Protein name	Accession no.	Theoretical p*I*	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in cKDH-8/11 (fold)
1	Transitional endoplasmic reticulum ATPase	P46462	5.14	89,349.3	24	36	319.78	6.0
2	Heat-shock 90 kDa protein-alpha	P82995	4.93	84,815.3	1	1	16.41	1.6
3	78 kDa Glucose-regulated protein	P06761	5.07	72,347.3	24	42	363.86	1.6
4	Heat-shock cognate 71 kDa protein	P63018	5.38	70,871.4	31	49	459.13	3.7
5	Vimentin	P31000	5.06	53,733.0	4	11	49.48	2.1
6	Protein disulfide-isomerase A6	Q63081	4.99	48,173.7	13	26	199.72	1.6
7	Heterogeneous nuclear ribonucleoprotein F	Q794E4	5.31	45,730.1	7	19	98.57	1.5
8	Suppressor of G2 allele of SKP1 homolog	B0BN85	5.17	38,091.1	4	17	47.15	2.2
9	Elongation factor 1-delta	Q68FR9	4.95	31,330.3	3	16	34.86	1.5
10	Ubiquitin carboxyl-terminal hydrolase isozyme L3	Q91Y78	5.01	26,123.8	3	10	32.70	1.8
11	Peroxiredoxin-2	P35704	5.34	21,783.8	6	29	81.24	2.0
12	Myotrophin	P62775	5.28	12,860.8	3	43	54.11	2.5
13	Heat-shock 70 kDa protein 4	O88600	5.13	94,057.0	3	5	29.19	–1.7
14	Endoplasmin	Q66HD0	4.72	92,771.4	3	4	32.99	–1.5
15	Pyruvate kinase isozymes M1/M2	P11980	6.63	57,818.1	4	8	54.16	–2.4
16	Dihydrolipoyl dehydrogenase, mitochondrial	Q6P6R2	7.96	54,038.4	3	7	35.18	–1.6
17	Glutamate dehydrogenase 1, mitochondrial	P10860	8.05	61,416.3	3	6	43.37	–2.1
18	Glutathione reductase (fragment)	P70619	8.06	46,301.4	1	2	18.12	–1.7
19	Alpha-enolase	P04764	6.16	47,128.1	15	28	209.10	–2.1
20	Alpha-enolase	P04764	6.16	47,128.1	23	56	328.48	–1.8
21	Actin, cytoplasmic 2	P63259	5.31	41,793.1	4	14	54.02	–1.5
22	Tropomyosin beta chain	P58775	4.66	32,836.9	5	15	70.17	–1.8
23	Cell surface glycoprotein CD200 receptor 1	Q9ES58	5.45	35,533.7	1	9	13.35	–1.7
24	Eukaryotic translation initiation factor 3 subunit 1	B0BNA7	5.38	36,461.0	2	7	33.21	–1.4
25	Aldose reductase	P07943	6.26	35,797.5	9	34	127.36	–1.7
26	Annexin A1	P07150	6.96	38,829.7	6	17	78.56	–1.5
27	Glyceraldehyde-3-phosphate dehydrogenase	P04797	8.14	35,828.2	1	3	20.75	–2.0
28	Inositol monophosphatase 1	P97697	5.18	30,511.5	2	8	31.86	–1.8
29	Actin, cytoplasmic 2	P63259	5.31	41,793.1	6	20	91.39	–1.5
30	Aldose reductase	P07943	6.26	35,797.5	5	16	68.21	–1.5
31	Protein S100-A4	P05942	5.04	11,776.5	5	28	62.07	–2.0
32	Fatty acid-binding protein, adipocyte	P70623	7.72	14,708.1	4	34	51.59	–3.3

Spot numbers refer to those shown in Figure 1. These spots were cut out and 32 spots were subsequently identified by MS.

Up-regulation of vimentin might play a protectant role against apoptosis induced not only by chemotherapy, but also by TNF.

Peroxiredoxin-2 is induced by various oxidative stimuli and plays an important role in eliminating peroxides generated during metabolism to protect from oxidative damage by hydrogen peroxide. Some reports showed that up-regulation of peroxiredoxin-2 inhibited cisplatin-induced apoptosis and that peroxiredoxin-2 inhibited H_2O_2-induced cell death (19, 20). Like the other identified up-regulated proteins, up-regulated peroxiredoxin-2 may also protect cKDH-8/11 cells from TNF-induced apoptosis.

Elongation factor (EF) 1-delta is a part of the EF 1 protein complex which is active in the elongation step of protein synthesis. Sinha *et al*. reported that EF 1-delta was up-regulated in chemoresistant melanoma cell lines (21). Although the role of EF 1-delta in TNF-induced apoptosis is not clear, its up-regulation may protect cKDH-8/11 cells from apoptosis.

Twenty down-regulated proteins were identified in cKDH-8/11 cells compared with KDH-8/YK cells. Unfortunately from the literature describing them, down-regulation of these proteins seemed not to play important roles in the resistance to apoptosis induced by TNF. Up-regulation of proteins related to protection from cell apoptosis may therefore play an important role in TNF resistance.

Some of the proteins identified by proteomic differential display analysis for the rat hepatoma cell lines studied here may be involved in the mechanism of resistance to TNF-induced apoptosis, and could also be indicators of the response to ONO-4007 therapy.

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