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A proteomics profile analysis was performed on a human hepatocyte carcinoma cell line (HepaRG) by the FD-LC-MS/MS method. One hundred and fifty-eight proteins were newly identified for the first time and 10 were specific to human hepatocytes. These proteins are a "proteomics fingerprint" that can be used to characterize HepaRG cells.

HepaRG is a human hepatocellular carcinoma cell line composed of hepatocyte- and biliary-like cells. Differentiated HepaRG is the only accepted cell line that is capable of being infected in vitro by the hepatitis B virus (HBV) in a similar way to cultured primary hepatocytes. In addition, HepaRG retains a drug metabolic capacity comparable to that of primary human hepatocytes. Therefore, HepaRG is regarded as a model for drug metabolism studies. Several differential proteomics analyses on HepaRG, including hepatitis B virus-infected vs. non-infected cells and on membrane proteins in differentiated vs. non-differentiated cells, have identified 44, 108–118 and 210–307 proteins as differentially expressed proteins. However, there has not been a comprehensive profile analysis of the proteins expressed in HepaRG cells that characterize mammalian hepatocytes from the standpoint of the proteome.

Proteomics analysis is usually performed by conventional methods, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and shotgun proteomics analysis using LC/MS/MS. However, these methods require pretreatment steps, such as precipitation, cleanup with the extraction column, or enzymatic protein digestion before LC/MS/MS (shotgun proteomics analysis), which tend to remove proteins, thereby resulting in low sensitivity and low reproducibility, and also imprecise information on the expressed proteins.

In contrast, the fluorogenic derivatization (FD)-LC-MS/MS method is a quantitative proteomic analysis method that does not require any sample pretreatment procedure; proteins are derivatized by fluorogenic reagents such as 7-chloro-N-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) and derivatized proteins are separated by HPLC. This is followed by quantification based on peak height, fractionation of the protein peaks, enzymatic digestion of the isolated proteins, and the final identification of the proteins using HPLC and tandem MS. Because this approach has been used for differential proteomics analysis in many biological samples, such as human breast and colorectal cancer cells, and mouse liver, FDL-MS/MS should be suitable for a profile proteomics analysis of HepaRG cells. Such analysis should identify the expressed proteins that have been lost during the pretreatment steps of conventional proteomics analysis methods. Therefore, the FDL-LC-MS/MS method was used in the present study. To separate the DAABD-labeled proteins, we tried using columns of core shell particles (Aeris WIDEPORE XB, Phenomenex, Torrance, CA, USA) that have recently become commercially available.

Kirkland et al. developed 2.7-µm columns of core-shell particles composed of a 1.7-µm solid core enriched by a 0.50-µm porous layer. Compared with a column with totally porous particles of less than 2 µm, the 2.7-µm columns showed equally efficient separation with much lower column pressure drops and were used for the separation of low-molecular-weight compounds, including drugs. A column of this wide-pore type of core-shell material recently became commercially available (Aeris WIDEPORE XB) and has been shown to have highly efficient separation of several proteins and monoclonal antibodies. Because there have been no reports on the separation of proteins in bio-samples by a single analysis, we used the Aeris WIDEPORE XB-C8 column (250 × 4.6 mm i.d.; TSKgel Amide-80, Phenomenex, Torrance, CA, USA) to achieve efficient separation of the expressed proteins in HepaRG cells. The composition of the eluents was referenced to data taken from the separation of proteins of human hepatocytes on a column of non-porous materials (Prep FF-C18, 250 × 4.6 mm i.d.; Intakt, Kyoto, Japan).

We investigated the separation efficiency under various flow rates (0.10–0.60 mL/min) (Supplementary Fig. 1). The number of peaks between the first and act peaks (identified as actin, as shown in Supplementary Fig. 1) in every chromatogram was compared to evaluate the separation efficiency. The number of peaks was 290, 304, 299, 306, 285, and 269 at flow rates 0.10, 0.15, 0.20, 0.30, 0.40, and 0.60 mL/min, respectively. The lower flow rates (0.10–0.30 mL/min) showed higher separation efficiency (290–306 peaks) as compared with the higher flow rates (0.40–0.60 mL/min) at a column temperature of 60 °C. These results agree with a trend reported by Fekete et al. when using the standard insulin on an Aeris WIDEPORE XB-C18 column. Moreover, the peak height increased as the flow rate decreased (Supplementary Fig. 1). This observation suggests that lower flow rates show higher sensitivity than higher flow rates. The same trend was also observed when the column temperature was set at 50 °C. The numbers of peaks were 327, 315, and 311 at
flow rates 0.10, 0.15, and 0.20 mL/min, respectively. Second, as
for the column temperatures (30–70 °C) (Supplementary Fig. 2),
higher column temperatures (50, 60, and 70 °C) showed higher
separation efficiency (311, 299, and 302 peaks, respectively) than
the lower column temperatures (208 and 289 peaks for 30 and
40 °C, respectively) at a flow rate of 0.20 mL/min. Taking these
results into consideration and using a single column to separate
the HepaRG extract, a flow rate of 0.10 mL/min and a column
temperature of 50 °C were considered optimal.

In accordance with previous reports, when the column
length was varied (250 mm, 250 + 250 mm, and 250 + 250 + 250
mm) at the flow rate 0.20 mL/min and the column temperature
was set at 60 °C, the number of peaks increased as the column
length increased (299, 334, and 350 peaks, respectively, data not
shown). Furthermore, the number of peaks (350) at the flow rate
of 0.20 mL/min and at 60 °C was higher than that (328) at the
flow rate of 0.10 mL/min and a column temperature of 50 °C,
with a column length of 750 mm. Finally, for the proteomics
profiling analysis of the HepaRG cell extract, a 750 mm
connecting column was selected with the column temperature set
at 60 °C and the flow rate was changed from 0.4 to 0.2 mL/min
between 20 and 25 min to shorten the analysis time. The injection
volume was changed to 50 µL (20 µg protein) to increase the
number of proteins, as shown in Fig. 1.

On the chromatogram, 532 peaks appeared during the 10-h
analysis (Fig. 1). When each peak was fractionated, and the
isolated proteins enzymatically digested and subjected to nano-
LC/MS/MS, the number of identified proteins was 254.

The proteins were classified using the DAVID
(http://david.abcc.ncifcrf.gov/) software, according to the Gene
Ontology terms for their cellular components (Supplementary
Table 1).22 The results showed that the proteins originated from
mitochondrion (n = 73; 28.7%), endoplasmic reticulum (n = 36;
14.2%), cytoskeleton (n = 35; 13.8%), ribosome (n = 20; 7.9%),
nucleolus (n = 17; 6.7%), and cytosol (n = 78; 30.7%).

As summarized in Supplementary Table 2, 158 expressed
proteins were shown for the first time that have not been found in
previous papers in which HepaRG cells were analyzed for
hepatitis B virus infections vs. non-infections by the 2D-PAGE
proteomics method4 or for membrane proteins expressed on
differentiated vs. non-differentiated cells by the shotgun
proteomics method.5,6 These proteins may be proteins that were
removed by precipitation or other cleanup procedures employed in
the shotgun or 2D-PAGE analysis methods. The number of
proteins recovered in the present experiment that were localized
in mitochondrion, endoplasmic reticulum, cytoskeleton, ribosome,
nucleus, and cytosol was 49, 22, 15, 13, 11, and 34, respectively.
Division of these numbers by the identified proteins in each
component (Supplementary Table 1) gives the percentage of
recovered proteins for each component as 67.1, 61.1, 42.9, 65.0,
64.7, and 43.6% respectively, showing that the FD-LC-MS/MS
method identifies more proteins in each component than the
conventional methods. Furthermore, it should be stressed that
the present method clearly identifies the proteins based on protein
isolation, and the identity is not deduced from the tremendous
variety of peptide fragments from large amounts of mixed
proteins, as in the case of shotgun proteomics analysis.

Of the 254 proteins identified in the present experiment, 10 proteins
were specific to human hepatocytes, according to the
classification by Slany et al. (Fig. 1 and Supplementary Table 1):
h1, arginase-1; h2, haptoglobin; h3, serotransferrin; h4,
phosphoenolpyruvate carboxykinase [GTP]; h5, glyoxylate
reductase/hydroxypropyruvate reductase; h6, carbamoyl-phosphate
synthase [ammonia]; h7, delta-1-pyruvole-5-carboxylate
derhydrogenase; h8, acyl-coenzyme A synthetase ACSM2B; h9,
apolipoprotein-L2; and h10, liver carboxylesterase L.

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profile data for other cells is accumulated, it should allow us to
develop a “proteomics fingerprint” for mammalian cells.

Notes and references

a Laboratory of Proteomics Analysis, Research Institute of
Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi,
Nishitokyo-shi, Tokyo 202-8585, Japan. E-mail: k-imai@musashino-
acad.jp; Fax: +81-42-468-9787; Tel.: +81-42-468-9787

b Research Division, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado,
Gotemba, Shizuoka 412-8513, Japan

t Electronic Supplementary Information (ESI) available: Details of
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Profile analysis leads to proteomics fingerprint!

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