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Comparison of protein expressions between human livers and the hepatic cell lines HepG2, Hep3B and Huh7 using SWATH and MRM-HR proteomics: Focusing on drug-metabolizing enzymes

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ABSTRACT

Human hepatic cell lines are widely used as an in vitro model for the study of drug metabolism and liver toxicity. However, the validity of this model is still a subject of debate because the expressions of various proteins in the cell lines, including drug-metabolizing enzymes (DMEs), can differ significantly from those in human livers. In the present study, we first conducted an untargeted proteomics analysis of the microsomes of the cell lines HepG2, Hep3B, and Huh7, and compared them to human livers using a sequential window acquisition of all theoretical mass spectra (SWATH) method. Furthermore, high-resolution multiple reaction monitoring (MRM-HR), a targeted proteomic approach, was utilized to compare the expressions of pre-selected DMEs between human livers and the cell lines. In general, the SWATH quantifications were in good agreement with the MRM-HR analysis. Over 3000 protein groups were quantified in the cells and human livers, and the proteome profiles of human livers significantly differed from the cell lines. Among the 101 DMEs quantified with MRM-HR, most were expressed at substantially lower levels in the cell lines. Thus, appropriate caution must be exercised when using these cell lines for the study of hepatic drug metabolism and toxicity.

1. Introduction

The liver is the principal organ for drug metabolism, between having high expression levels of most drug-metabolizing enzymes (DMEs) and being involved in first-pass metabolism. Hepatic drug metabolism and liver toxicity have been an active research area for several decades, and are important for drug development as well. Various models have been developed for the study of drug metabolism and toxicity in the liver, such as animal models, immortal cell lines, human primary hepatocytes, human liver tissues, and recombinant enzymes [1]. Each model has its own advantages and limitations. Primary human hepatocytes are generally considered as the 'gold standard' for in vitro drug metabolism and liver toxicity studies since the primary cells retain many hepatocyte functions, especially the activity of major DMEs [2]. However, significant batch-to-batch variability, short life spans, limited availability, and high cost have hindered their applications. To address these limitations, several cell lines have been derived from human hepatoma cells, such as HepG2, Hep3B, and Huh7. These cell lines retain some morphological features and functions of hepatocytes, and have robust reproducibility, great availability, relatively low cost and high-throughput capacity. Consequently, these cell lines have been widely used as in vitro models for various studies, such as drug metabolism and toxicity [3–5], lipid metabolism and obesity [6,7], carcinogenesis and anticancer research [8–10], and virology [11,12]. For example, a study of resveratrol metabolism was successfully conducted in HepG2 cells, and the phase II DMEs UGT1A1, UGT2B7, and ST1E1 were significantly induced by resveratrol in the cells [3]. Furthermore, Hep3B cells were utilized as an in vitro model to study the mechanisms of acetaminophen-induced hepatotoxicity [5]. However, many DMEs were found to be expressed at lower levels in hepatoma cell lines in comparison with primary human hepatocytes and human liver tissues, which challenges the usefulness of these cells in the study of drug metabolism and drug-induced liver injury [13–16].

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Over recent decades, LC-MS/MS-based proteomics has emerged as a powerful technology for protein identification and quantification. Data-dependent acquisition (DDA) has been the main stay proteomics method for untargeted analysis. However, several drawbacks have been recognized for the DDA approach, such as its inherent bias to abundant peptides and the lack of reproducibility for low-abundance proteins. Recently, data independent acquisition (DIA), also termed sequential window acquisition of all theoretical mass spectra (SWATH) when analysis is performed on an AB Sciex TripleTOF-type instrument, has proven a powerful approach for both untargeted and targeted protein quantification. DIA/SWATH is capable of quantifying protein expression with high reproducibility and accuracy via overcoming the biased sampling problem seen with DDA [17]. In addition, a new targeted proteomics assay named parallel reaction monitoring (PRM) or high resolution multiple reaction monitoring (MRM-HR) has shown several advantages over conventional targeted proteomics (e.g., MRM or SRM), such as greater selectivity and robustness [18,19]. To date, these new proteomics approaches have not been utilized to determine the proteome profiles of hepatoma cell lines, much less compare them with those of human livers. The purposes of this study were to 1) compare proteome profiles between human livers and the three commonly used hepatic cell lines Hep3B, HepG2, and Huh7 using SWATH; 2) quantify selected DMEs in human livers and the cell lines using an MRM-HR strategy for evaluation of differences in expressions; 3) compare quantification results from SWATH and MRM-HR. A better understanding of the differences of protein expression between hepatic cell lines and human livers, especially differences in DMEs abundance, will lead to a more appropriate use of these cell lines for in vitro studies of drug metabolism and liver toxicity.

2. Materials and methods

2.1. Chemicals and reagents

Human liver microsomes (pooled from 200 donors with mixed-gender) were purchased form Sekisui XenoTech (Kansas City, KS). Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), and 100 × antibiotics mixture containing 100 IU/mL penicillin and 100 μg/mL streptomycin (P/S) were products of Invitrogen (Carlsbad, CA, USA). HyClone™ RPMI-1640 medium was purchased from Thermo Scientific (Waltham, MA). Urea and dithiothreitol were purchased from Fisher Scientific Co. (Pittsburgh, PA). Trifluoroacetic acid, formic acid, and acetonitrile were from Sigma-Aldrich (St. Louis, MO). Iodoacetamide and ammonium bicarbonate were the products of Acros Organics (Morris Plains, NJ). TPCK-treated trypsin was obtained from Worthington Biochemical Corporation (Freehold, NJ). Water Oasis HLB columns were from Waters Corporation (Milford, MA). Bovine serum albumin (BSA) standard was purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Cell culture

HepG2 cells (HB-8065™) were purchased from ATCC (Manassas, VA, USA). Hep3B and Huh7 cell lines were kindly provided by Drs. Theodore H. Welling III and Lei Yin (University of Michigan), respectively. Cells were cultured in DMEM supplemented with FBS, 1% P/S and 2 mM glutamine. The concentrations of FBS were 10% for HepG2 and Hep3B cells, and 5% for Huh7 cells. Cell passages were performed with 0.25% trypsin-EDTA (Gibco, Life Technologies) when cells reached 80–90% confluence.

2.3. Microsomes preparation from HepG2, Hep3B, and Huh7 cells

Cells pellets were collected following typsin-EDTA digestion and centrifugation, and were homogenized in phosphate buffered saline (PBS, pH 7.4) on ice using an ultrasonic probe (10 s × 4 times). S9 fractions were obtained after the homogenates were centrifuged at 4 °C at 10,000 g for 30 min. The supernatants (S9 fractions) were transferred to a Beckman ultracentrifuge tube and centrifuged at 300,000 g (80,000 rpm) for 20 min. Microsomes were obtained by resuspending the pellets in PBS using a tissue grinder. Protein concentrations of the microsomes were determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). The microsome samples were stored at −80 °C until use.

2.4. Proteomics sample preparation

Protein digestion was conducted according to a previously reported Lys-C/Trypsin combinatorial digestion protocol with some modifications [20]. An aliquot of 100 μg protein of microsomes was mixed with the internal standard 0.5 μg BSA in an Eppendorf Protein LoBind tube. A 10-fold volume of pre-cooled acetone was added. The mixture was briefly vortexed and incubated at −20 °C for at least 2 h, followed by centrifugation at 17,000 g for 15 min at 4 °C. The supernatants were discarded, and the precipitated proteins were added with 200 μL ice-cold 80% ethanol for a washing step. The mixture was centrifuged again at 17,000 g for 15 min at 4 °C. The supernatants were removed and the precipitated proteins were air-dried at room temperature. The dried proteins were resuspended in 100 μL of freshly prepared 4 mM dithiothreitol in 8 M urea solution containing 100 mM ammonium bicarbonate. Samples were briefly vortexed and sonicated, then incubated at 37 °C for 45 min. After samples were cooled down to room temperature, 100 μL of 20 mM iodoacetamide freshly prepared in 8 M urea/100 mM ammonium bicarbonate solution was added. The mixture was incubated at room temperature for 30 min in the dark for alkylation. Following the incubation, urea concentration was adjusted to 6 M by adding 64.6 μL of 50 mM ammonium bicarbonate, and lysyl endopeptidase (Wako Chemicals, Richmond, VA) was added for the first digestion step (protein to lysyl endopeptidase ratio = 100:1) at 37 °C for 6 h. Samples were then diluted with 50 mM ammonium bicarbonate to decrease urea concentration to 1.6 M urea, followed by the second step of digestion with trypsin at a protein to trypsin ratio of 50:1 for an overnight incubation at 37 °C. Digestion was terminated by the addition of 1 μL trifluoroacetic acid. Digested peptides were extracted and purified using Waters Oasis HLB columns according to the manufacturer's instruction. Peptides eluted from the columns were dried in a SpeedVac SPD1010 concentrator (Thermo Scientific, Hudson, NH), and reconstituted in 80 μL of 3% acetonitrile solution with 0.1% formic acid. The peptide samples were centrifuged at 17,000 g for 10 min at 4 °C, and half of the supernatant was collected and supplemented with 1 μL of the synthetic iRT standards solution from Biognosys AG (Cambridge, MA) prior to LC-MS/MS analysis.

2.5. LC-MS/MS-based protein quantification

Proteomic analysis was carried out on a TripleTOF 5600 + mass spectrometer (AB Sciex, Framingham, MA) coupled with an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). LC separation was performed via a trap-elute configuration, which includes a trapping column (ChromXP C18-CL, 120 Å, 5 μm, 0.3 mm cartridge, Eksigent Technologies, Dublin, CA) and an analytical column (ChromXP C18-CL, 120 Å, 150 × 0.3 mm, 5 μm, Eksigent Technologies, Dublin, CA). The mobile phase consisted of water with 0.1% formic acid (phase A) and acetonitrile containing 0.1%

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