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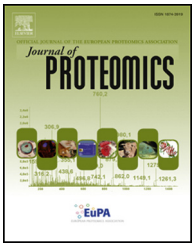

Quantitative proteomic analysis of hepatocyte-secreted extracellular vesicles reveals candidate markers for liver toxicity

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ABSTRACT

Extracellular vesicles have created great interest as possible source of biomarkers for different biological processes and diseases. Although the biological function of these vesicles is not fully understood, it is clear that they participate in the removal of unnecessary cellular material and act as carriers of various macromolecules and signals between the cells. In this report, we analyzed the proteome of extracellular vesicles secreted by primary hepatocytes. We used one- and two-dimensional liquid chromatography combined with data-independent mass spectrometry. Employing label-free quantitative proteomics, we detected significant changes in vesicle protein expression levels in this *in vitro* model after exposure to well-known liver toxins (galactosamine and *Escherichia coli*-derived lipopolysaccharide). The results allowed us to identify candidate markers for liver injury. We validated a number of these markers *in vivo*, providing the basis for the development of novel methods to evaluate drug toxicity. This report strongly supports the application of proteomics in the study of extracellular vesicles released by well-controlled *in vitro* cellular systems. Analysis of such systems should help to identify specific markers for various biological processes and pathological conditions.

Abbreviations: EV, extracellular vesicle; LC–MS, liquid chromatography coupled to mass spectrometry; MP, microparticle; ALT, alanine transaminase; CES, carboxylesterase; MVB, multivesicular body; LPS, lipopolysaccharide; galN, D-galactosamine; AMRT, accurate mass measurement–retention time pair; GO, Gene Ontology; DDA, data-dependent analysis; DIA, data-independent analysis; ACN, acetonitrile; RFU, relative fluorescence unit

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Biological significance

Identification of low invasive candidate marker for hepatotoxicity. Support to apply proteomics in the study of extracellular vesicles released by well-controlled *in vitro* cellular systems to identify low invasive markers for diseases.

1. Introduction

The qualitative and quantitative analysis of (sub)proteomes is an important step toward better understanding of diverse biological functions, and is one of the greatest challenges in the field of proteomics. Mass spectrometry quantitation is already widely applied in comparative studies of protein expression but the majority of the relative quantitative methods use isotopic labeling. Such analytical schemas involve multiple sample preparation steps to incorporate the label either metabolically or chemically [1–5]. One important limitation of labeling approaches is that the number of available tags might not be sufficient for the simultaneous discrimination of multiple analytes [6,7]. Recently, label-free LC–MS quantitation methods have been increasingly employed to compare the levels of various proteins under different conditions. Some quantitative, label-free LC–MS-based strategies for the profiling of complex protein mixtures have been reported. These strategies rely either on spectral counting methods [8,9] or on the direct measurement of signal intensity [10–14]. Label-free LC methods for the quantitative analysis of proteins have been recently reviewed [15,16]. In contrast to label-based techniques, label-free methods are not restricted by the number of samples; however, more care has to be taken to minimize experimental variation, mainly involving the sample preparation stage.

The successful application of quantitative proteomics in biomedicine is difficult because of the complexity and dynamic range of protein samples derived from various tissues and body fluids. Recently, extracellular cell-secreted vesicles (EVs) [17] were recognized as a novel biological material with reduced protein complexity and created interest as a potential source of disease biomarkers. These vesicles fall mainly into two groups, depending on their size, origin, and the mechanism of their release: the endosome-derived vesicles named "exosomes" and the vesicles shed from plasma membranes, referred to as ectosomes or microparticles (MPs). Exosomes are intraluminal vesicles (40–150 nm) produced by inward budding of the limiting membrane of multivesicular bodies (MVB), which are the central organelles of the endocytic and secretory pathways [18]. There is a growing body of evidence that there are at least 2 different kinds of MVBs. One class that ends up in the lysosomes and another class that fuses with the plasma membrane. The latter type is responsible for releasing the exosomes into the extracellular space [19]. As a consequence of their endosomal origin, exosomes contain proteins involved in membrane transport, fusion, and MVB biogenesis, including CD9, CD63, CD81, Rab GTPases, annexins, flotillin, Alix and Tsg101. MPs are a population of vesicles that vary in size (0.1–1.0 μm), and are formed by outward budding of the cell plasma membranes in response to different stimuli. These vesicles are shed by different cell types and express a subset of cell surface proteins that depend on the cells of origin [20,21]. Although the cell biology of these two types of EVs is different, both types circulate in the adjacent extracellular space and appear in biological fluids after their release from the cells. They have been identified in human, rodent, and fetal calf sera [22–27]. They are released both by the cells of haematopoietic and non-haematopoietic origin [28,29], quiescent and activated [30], and non-transformed and tumor cells [31]. Because of their involvement in the intercellular signaling, the examination of their protein components in the healthy and diseased individuals may provide valuable markers for determining the site, type, and an extent of injury in various pathological conditions.

Our group reported the secretion of EVs by the primary hepatocytes in culture [32]. In the current report, we identified novel putative markers for liver injury in an *in vitro* model. We used two well-known hepatotoxins, galactosamine (galN), which causes liver injury resembling acute viral hepatitis [33], and *Escherichia coli*-derived lipopolysaccharide (LPS) promoting liver inflammation and damage [34–38]. Finally, by using an animal model for acute liver injury, we showed that similar protein alterations can be detected in the EVs isolated from sera. Our results provide the basis for the creation of novel, non-invasive tools to assess liver toxicity supporting the use of EVs as a biological source of disease biomarkers.

2. Experimental procedures

2.1. Reagents

All media and reagents for tissue culture were purchased from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and mainly acquired from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies were purchased from the following vendors: anti-Clusterin (clone 0.T.19) and anti-CPS1 (clone OCH1E5) from Abcam (Cambridge, UK), anti-Hsp70 (clone BRM-22) from Sigma Chemical Co. (St. Louis, MO), anti-Hsp90 (clone 68) and anti-AIP1/Alix (clone 49) from BD Biosciences (Mountain View, CA). Rabbit polyclonal antibodies were purchased from the following vendors: anti-FRIL1 (clone D-9) was purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA), anti-SLC27A2, anti-SULT1, and anti-Tsg101 from Abcam (Cambridge, UK). Goat anti-CES3 (clone M-14) and anti-COMT were from Santa Cruz Biotech., Inc. and Abcam, respectively. Horseradish peroxidase (HRP)-conjugated secondary antibody was from GE Healthcare (Buckinghamshire, UK).

2.2. Animal experimentation

All the animal experimentation was conducted in accordance with the Spanish Guide for the Care and Use of Laboratory Animals (RD 1201/2005 — BOE 21/10/05). Eight male 14-week-old Sprague–Dawley rats (body weight 300–400 g) were maintained in an environmentally controlled room at 22 °C on a 12 h light/

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