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Identification of major cellular proteins synthesized in response to interleukin-1 and interleukin-6 in human hepatoma HepG2 cells

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Abstract

Interleukin-1 (IL-1) and interleukin-6 (IL-6) are principal proinflammatory cytokines inducing the acute phase response of various tissues, including liver. Cultured human hepatoma HepG2 cells were stimulated with IL-1 (10 ng/ml) and IL-6 (10 ng/ml). After 24 h the cells were collected and disrupted by sonication in a lysis buffer containing 8 M urea. The extracted cellular proteins were separated by 2D polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brilliant Blue R-250 and the protein spots showing different intensities in comparison to control (unstimulated) cells were excised and subjected to analysis by LC-MS/MS. Alternatively, proteins were stained with SYPRO Ruby. These differentially expressed proteins include seven up-regulated and two down-regulated intracellular proteins of various functions. The identification of three cytokine-responsive proteins was confirmed by biosynthetic labeling with [^{35}S]methionine after incubation of HepG2 cells, and by western blot with specific antisera.
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1. Introduction

The systemic inflammatory reaction, known as the acute phase response, is induced by many noxious environmental factors but in all cases the cytokines, such as interleukin-1-beta (IL-1β) and interleukin-6 (IL-6) are involved [1,2]. The liver cell response to inflammatory cytokines is manifested by a characteristic change in the profile of synthesized plasma proteins (acute phase reactants) and has been extensively studied both in primary hepatocyte cultures and in established cell lines (for details and references see [1,3–5]). The action of IL-1 and IL-6 on induced protein synthesis may be either synergistic (e.g. serum amyloid A) or antagonistic (e.g. fibrinogen) [6], and involves a cross-talk between signaling cascades initiated by IL-1 and IL-6 [7,8].

Paradoxically, much less is known about cytokine-induced changes in the metabolic rates of the hepatocyte intra-cellular proteins, although the human liver proteome has been extensively investigated [9]. Here we report the results of studies concerning the effects of IL-1 and IL-6 on the cellular proteome of human hepatoma HepG2 evaluated by two-dimensional polyacrylamide gel electrophoresis (2D electrophoresis) and capillary chromatography linked to mass spectrometry. Gene expression in hepatoma cells, even as highly differentiated as HepG2, is certainly not identical to normal hepatocytes [10], but is easily reproducible in consecutive experiments and represents a reliable model for studying the response of liver cells to cytokines [4].

In order to simulate the in vivo situation we used relatively low doses of cytokines. Moreover, IL-6 was added to cultured

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cells 3 h after IL-1, in agreement with sequential appearance of these cytokines in the inflamed tissue [11], and as recommended by Uhlar and Whitehead [5] for cultured HepG2 cells. To verify the obtained results, biosynthetic labeling with [^{35}S]methionine, followed by 2D electrophoresis and autoradiography was carried out. Moreover, cytokine-induced changes in the level of three proteins were confirmed by western blot with specific antisera. After 24 h exposure of HepG2 cells to IL-1 and IL-6 we identified seven proteins that are up-regulated (one of them, manganese-dependent superoxide dismutase, occurring in three isoforms) and two that are down-regulated. Functionally, these proteins are involved either in cellular metabolic processes, or participate in gene expression machinery. Our results permit to identify some proteins of HepG2 cells that have so far been unknown as being modulated by the inflammatory cytokines.

2. Materials and methods

2.1. Tissue culture

Human hepatoma HepG2 cells (obtained from ATCC, Rockville, MD, USA) were cultured in Petri dishes (100 mm diameter) in DMEM enriched with 5% fetal bovine serum (FCS) until they have reached approximately 70% confluency. One day before the experiment the medium was changed to DMEM with 0.5% FCS. At time 0 human recombinant interleukin-1 beta (IL-1) (a generous gift of Dr C.A. Dinarello, Denver, CO, USA) was added to the final concentration of 10 ng/ml culture medium, followed by 10 ng/ml of human recombinant interleukin-6 (IL-6, ICN, Aurora, OH, USA) 3 h later. After further 21 h of culture, the cells were collected by scraping and washed twice by centrifugation with 10 mM Tris-buffered 250 mM sucrose. The control culture was treated in exactly the same manner but the cytokines were not added.

2.2. Protein extraction and separation by 2D electrophoresis

The cell pellet, containing approximately 5×10^6 cells, was placed on ice, suspended in the lysis buffer containing 8 M urea, 2% CHAPS, 50 mM DTT (both from Sigma-Aldrich, St. Louis, MO, USA) and 0.2% Ampholyte pH 3–10 (Fluka, Buchs, Switzerland) and sonicated three times for 5 s. To remove the insoluble debris, the suspension was centrifuged at $40\,000 \times g$ for 30 min at +4 °C. Protein content in the supernatant was measured with the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA).

Isoelectric focusing was carried out after active rehydration of the immobilized pH 3–10 gradient strips (IPG strips, Bio-Rad, Hercules, CA, USA). The proteins were subjected to focusing at increasing voltage from 250 to 4000 V. Depending on the method of subsequent staining 50–1000 μg of proteins were loaded on a 7 cm strip.

The focused proteins were further separated by SDS-PAGE after a two-step equilibration of the strip in order to reduce disulphide bonds with 130 mM DTT followed by alkylation of thiol groups with 135 mM iodoacetamide. The electrophoresis according to Laemmli [12] was carried out for approximately 2 h in the 12% separating gel.

2.3. Detection and identification of proteins

The gels were stained either with Coomassie Brilliant Blue (CBB) R-250 (Sigma-Aldrich, St. Louis, MO, USA) or with SYPRO Ruby (Bio-Rad, Hercules, CA, USA). After staining, the gel images were scanned and the data acquired by the white light illuminator Fluor-S MultiImager (Bio-Rad, Hercules, CA, USA). Subsequently, analyses of gel images were performed with the PD Quest 2-D Analysis Software Version 6.2 (Bio-Rad, Hercules, CA, USA). The process included: spots imaging and quantitation, qualification, comparison, as well as placing gels in the match sets for comparison and statistical analysis. The software system gave position, shape and density information for each of the detected spots. Spot detection and matching were performed simultaneously by the software and manually. For mass spectrometric analysis those matched spots were taken, which differed between the stimulated and control cells by more than 50% of intensity in three consecutive cell culture experiments.

Sample preparation for mass spectrometry was performed according to the protocol described elsewhere [13]. Briefly, the spots of interest were excised from the CBB stained gel with a scalpel, chopped into cubes (ca. 1 × 1 mm), dehydrated with acetonitrile and re-swollen in 13 ng/μl trypsin in 50 mM NH_4HCO_3 (sufficient to cover the gels). After overnight incubation at room temperature an equal volume of acetonitrile was added and samples were incubated under shaking for 30 min at 37 °C. Extraction of the peptides was repeated twice with 5% formic acid/acetonitrile (50:50) solution (in a volume sufficient to cover gel pieces). The supernatants were combined together, and the extracts were evaporated to dryness in a vacuum centrifuge. Dried samples dissolved in 6 μl 0.1% acetic acid were separated by the Ultimate LC microchromatography system (LC Packings/Dionex, Amsterdam, The Netherlands) on the reversed-phase capillary column C18 (2–3 μm bead size and 100 Å pore size, 15 cm length, 75 μm ID).

The chromatographic column was coupled directly to the Esquire 3000 Quadrupole Ion-Trap Mass Spectrometer (Bruker Saxonia, Leipzig, Germany). The instrument operated in the positive-ion mode. In each mass scan, the highest peak within the range of 400–1400 *m/z* has been automatically fragmented, provided its intensity has exceeded the threshold value. The obtained spectra were analyzed using Data Analysis Software (Bruker Daltonics, Bremen, Germany) and Mascot database search engine (ver. 1.9, Matrix Science, London, UK). Database searches, using combined PMF and MS/MS datasets, were performed against the SWISS-PROT sequence database. Search parameters included: taxonomy, human; modifications, carbamidomethyl (permanent) and methionine oxidation (variable); enzyme, trypsin; up to one missed cleavage, peptide charges 2+ and 3+, mass tolerance 2 Da for precursor mass and 0.3 Da for fragment mass. The probability

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