Biochimica et Biophysica Acta 1794 (2009) 615–626

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

A proteomic approach to characterizing ciglitazone-induced cancer cell differentiation in Hep-G2 cell line

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ARTICLE INFO

Article history:
Received 19 August 2008
Received in revised form 15 December 2008
Accepted 9 January 2009
Available online 29 January 2009

Keywords:
Biomarker
Cancer
Cell differentiation
Diagnostics
Oncoproteomics
Mitochondria
Stress protein

ABSTRACT

Drug induced cell differentiation represents a promising experimental model for proteomic analysis of cancer cells. In fact, by modulating and monitoring neoplastic cell differentiation it could be possible to identify cytodifferentiation related protein expression changes that can be subsequently utilized *in vivo* as potential cancer biomarkers. One main advantage of this approach is the significant reduction of biological variability normally observed in clinical biomarker research, with important implications also in prognosis and therapy. At this regard, a new class of differentiating agents is emerging, the so called PPAR-ligands, which however are characterized by a debated mechanism of action that has not been yet studied through a proteomic approach. To this aim, we investigated ciglitazone-induced differentiation of a human hepatocarcinoma HepG2 cell line, by monitoring biochemical and cellular parameters of cytodifferentiation and modifications of cellular protein profiles through 2-DE and MALDI-TOF analysis. Independent of the hypothesized mechanism of action of this intriguing PPARγ agonist, results indicated that ciglitazone is a strong differentiating agent for the HepG2 cell line and that this process is associated with modifications of protein expression related to cell antioxidant systems, the cell cycle apparatus, signal transduction pathways, cellular stress and invasiveness. At last, considering these and other published data, a proteomic profile related to the cancer aggressiveness is beginning to emerge.

1. Introduction

From a clinical perspective, hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and is the third leading cause of cancer mortality worldwide. Unfortunately, the prognosis of HCC remains unfavorable because of its late diagnosis, especially in patients afflicted by chronic liver diseases. Curing HCC, usually through surgery, is possible in fewer than 5% of all patients. Median survival time from diagnosis is 4–8 months and is shorter for patients with cirrhosis. Diagnosis is generally determined by NMR and/or ultrasound data that showed the presence of neoplastic nodules generally greater than 2 cm. All these factors relating to the detection and diagnosis of HCC reinforce the assertion that the discovery of predictive tumor markers must be a priority of biomedical research and not only in liver cancers [1]. Numerous proteomic studies on hepatocarcinoma have been conducted through comparative analysis of normal and neoplastic liver cells without obtaining definitive results because of wide biological variability of clinical samples [2–5]. For these considerations, our approach tried to obtain information by modulating the differentiation grade of an experimental model of hepatocarcinoma by adopting a new differentiating agent [6,7].

At this regard, it could be useful also to underline that hepatocellular carcinoma can represent an interesting experimental model to analyze a proteomic profile of undifferentiated vs. differentiated cancer cells in general. In fact, by using differentiating agents, it could be easy to monitor functional and structural differences during drug-induced cancer cell differentiation [3]. This approach of investigating different dynamic aspects related to cancer differentiation could shed some light not only on the molecular mechanisms at the basis of this neoplasia, but also on potential new diagnostic, prognostic, and therapeutic markers [8].

Importantly, a further aim of this study was to analyze the molecular mechanisms underlying the differentiating properties of the ciglitazone, a well-known PPAR (peroxisome proliferators-activated receptor)-γ ligand, on human hepatocarcinoma. Ciglitazone was one of the first thiazolidinediones to be synthesized. This particular class of drugs is utilized in the pharmacotherapy of type-2 diabetes mellitus and metabolic syndrome because of its peculiar insulin-sensitizing activity [9–11]. Thiazolidinediones seem to act as selective agonists for nuclear PPARγ although some biochemical, pharmacological and, above all, toxicological data seem to partially contradict this mechanism of action. Interestingly, thiazolidinediones have been shown to act as differentiating factors in various forms of

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doi:10.1016/j.bbapap.2009.01.006

experimental cancer although there is also contradictory data for this peculiar biological activity. In fact, some studies have demonstrated the antiproliferative and differentiating activity of this class of molecules [12–16], while several other studies have demonstrated carcinogenic effects in similar experimental models [17–18]. Other conflicting data also exist about PPAR ligands differentiating molecular mechanism (i.e., the anticancer effect of thiazolidinediones is not always dependent on the specific PPAR subtype distribution in tumor tissues) while they seem to correlate to other extraperoxisomal activities of these drugs (i.e., an inhibitory effect on oxidative mitochondrial metabolism) [12,15,16,18]. For these pathogenetic and pharmacotoxicological considerations, it would be extremely important to search for a signal transduction pathway at the basis of such complex drug-induced cellular reorganization. At this purpose, a proteomic approach represents an optimal method to rapidly identify some molecules that associate with this peculiar cellular phenotype differentiation [19,20].

Our data confirmed that tumor cell lines can be used to ameliorate the level of 2-DE reproducibility and can improve the data obtained with the usual cell biology approaches. In fact, the ability to work with a homogeneous pool of cells and to modulate their stage of differentiation permits experimenters to significantly simplify the oncoproteomic approach for analyzing the protein expression profile that characterize the cancerous cells with respect to normal cells. Specifically, results showed that ciglitazone-induced differentiation implied that a significant modification of protein expression patterns relates to cellular antioxidant system and oncogenes/oncosuppressors balance with significant implication in terms pathophysiology of cancer cells.

2. Materials and methods

2.1. Materials and chemicals

All chemicals were from Sigma Chemical Co. (Milan, Italy) unless otherwise indicated. Tissue culture media and supplements were obtained from Lonza Group Ltd, Basel, Switzerland. All reagents and equipment for 2-DE were purchased from Bio-Rad (BioRad Laboratories, Milan, Italy).

2.2. Cell culture and treatment

The human hepatocellular carcinoma cell line, HepG2, was obtained from the Interlab Cell Line Collection (ICLC, National Institute for Cancer Research, Genoa, Italy). Cells were grown at 37 °C, under a humidified atmosphere of 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza Group Ltd, Switzerland). Exponentially growing cells were incubated with different concentrations of ciglitazone (1, 10, 30, 50 μM), predissolved in 0.1% DMSO as a vehicle.

2.3. Determination of cell viability

Cell number and viability were assessed by trypan blue exclusion assay. An aliquot of trypan blue solution was mixed with the cells suspension and allowed to stand for 2 min at room temperature. Viability was assessed by cells ability to exclude trypan blue. The cell suspension was then analyzed using a Neubauer chamber and by counting the number of viable (unstained) and dead (stained) cells. The counting chamber was viewed by using an inverted binocular microscope (Olympus Italia Srl, Milan, Italy) (400× magnification). To limit potential pitfalls of trypan blue exclusion assay (i.e., cell lysis in some cell culture models), we usually adopt also the lactate dehydrogenase (LDH) release assay to monitor suitability. LDH release is a useful marker because it is released upon cell death and is stable over short periods of time (5% loss per day), so that the concentration of LDH in the medium provides an estimate of the total number of dead cells. Data obtained with trypan blue exclusion were always congruent with cytotoxicity data obtained with LDH release (data not shown).

Cell growth was expressed as AUC considered after 96 h of culture. Cytotoxic activity was expressed as vitality index (percent of viable cells divided by the total number of dead and viable cells) calculated at 96 h of drug treatment as means ± SEM from four experiments, each performed in duplicate.

2.4. Cell cycle analysis and apoptosis evaluation

The cell cycle distribution was determined by flow cytometry analysis of DNA content (EPICS XL-MCL Flow cytometer, Coulter Electronics, Miami, FL, USA) using propidium iodide stained cells. Briefly, after incubation with vehicle and ciglitazone (50 μM for 48 h), trypsinized cells (2×10^6 cells/ml) were washed twice with ice-cold PBS, resuspended in 50 μl PBS containing 2% fetal calf serum, and fixed at 4 °C for 30 min with 1 ml 80% cold ethanol. Before analysis, cells were washed three times at 4 °C and incubated with 0.5 ml PBS and 0.5 ml DNA-prep stain (Coulter Reagents, Miami, FL, USA) containing RNase (4 KU/ml) with propidium iodide (50 mg/ml) and maintained at room temperature for 30 min in the dark. DNA histograms were analyzed using Multicycle AV software (Phoenix, San Diego, CA, USA) to evaluate proportion of cells in G0/G1, S, G2/M phases. Four independent experiments were performed in duplicate for each condition. The proportion of hepatocarcinoma cells undergoing apoptosis was evaluated cytofluorimetrically, by analyzing the hypoploid peak. Ciglitazone showed to greatly influence cell cycle at 48 h of culture. Moreover, after this period, classic in vitro interferences begin to grow up above all in control cell cultures (i.e., contact inhibition and/or nutrient wasting) which could jeopardize cell cycle analysis.

2.5. Differentiation markers

Hepatocarcinoma differentiation markers (albumin, transferrin, and cholinesterase) were measured in culture supernatants by means of a Hitachi 917 automated analyzer and an appropriate reagent kits supplied by the same instrument company (Roche Diagnostics AG, Rotkreuz, Switzerland). α-fetoprotein was determined by means of Axsym analyzer and appropriate reagent kit supplied by the same instrument company (Abbott Laboratories, Abbott Park, IL, USA). Levels of different hepatocarcinoma differentiation markers, measured in culture supernatants and normalized for 10^6 viable cells, are expressed as means ± SEM, calculated from four experiments, each performed in duplicate.

2.6. 2-DE analysis

From each cell culture, the cell pellet was dissolved in a cell lysis buffer containing 8 M urea, 50 mM DTT, 4% CHAPS, 0.2% ampholine, pH 3–10, with a protease inhibitor cocktail (Complete, Mini; Roche, Basel, Switzerland), and sonicated twice on ice for 30 s at 20 W with a sonifier model VC50 Vibracell (Sonics & Materials Inc., U.S.A.). The lysate was centrifugated at 20,000 g for 15 min at 4 °C followed by a BioRad Protein Assay (BioRad Laboratories, Milan, Italy) for measuring protein concentration. Treated and untreated samples, containing the same protein amount (500 μg), were rehydrated overnight, at room temperature on 11 cm IPG gel strips, utilizing 3–10 NL range. Proteins were focused on Protean IEF Cell (BioRad Laboratories, Milan, Italy), at 20 °C, using a low initial voltage and then by applying a voltage gradient of 8000 V with a limiting current of 50 mA per strip for approximately 35,000 V/h total. After isoelectric focusing (IEF), IPG gel