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Cell line-specific oxidative stress in cellular toxicity: A toxicogenomics-based comparison between liver and colon cell models

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ABSTRACT

Imbalance between high reactive oxygen species formation and antioxidant capacity in the colon and liver has been linked to increased cancer risk. However, knowledge about possible cell line-specific oxidative stress-mechanisms is limited. To explore this further, gene expression data from a human liver and colon cell line (HepG2/Caco-2), both exposed to menadione and H_2O_2 at six time points (0.5–1–2–4–8 and 24 h) were compared in association with cell cycle distribution. In total, 3164 unique- and 1827 common genes were identified between HepG2 and Caco-2 cells. Despite the higher number of unique genes, most oxidative stress-related genes such as CAT, OGG1, NRF2, NF-κB, GCLC, HMOX1 and GSR were differentially expressed in both cell lines. However, cell-specific regulation of genes such as KEAP1 and GCLM, or of the EMT pathway, which are of pathophysiological importance, indicates that oxidative stress induces different transcriptional effects and outcomes in the two selected cell lines. In addition, expression levels and/or -direction of common genes were often different in HepG2 and Caco-2 cells, and this led to very diverse downstream effects as confirmed by correlating pathways to cell cycle changes. Altogether, this work contributes to obtaining a better molecular understanding of cell line-specific toxicity upon exposure to oxidative stress-inducing compounds.

1. Introduction

Oxidative stress may occur in almost any tissue and is believed to play an important role in carcinogenesis. To sustain a proper regulation of biological processes, a physiological balance between the formation of reactive oxygen species (ROS) and the antioxidant network is essential (Forman et al., 2010; Sauer et al., 2001). Small amounts of ROS produced under normal physiological conditions have a protective role in the cell, however, overproduction may lead to accumulation in the intracellular environment resulting in oxidative stress, which subsequently leads to damage to various cell structures (Reuter et al., 2010). Spontaneous mutations that are then induced by oxidative stress may lead to carcinogenesis (Klaunig et al., 2010), and various cancers have been found to be in a constant state of oxidative stress, which suggests a role for oxidative stress in cancer promotion as well (Tudek et al., 2010). As portals of entry for xenobiotics, the liver and the gastrointestinal tract are continuously exposed to multiple chemicals, and as such are prone to oxidative damage induced by different types of oxidative compounds. As a consequence, the imbalance between ROS formation and antioxidant capacity in the colon and liver has been linked to increased cancer risk (Benhar et al., 2002). ROS-induced mechanisms have actually been related to different chronic liver diseases and hepatocellular carcinoma (HCC), and are induced by various risk factors for liver cancer such as hepatitis B and C or aflatoxin-B1 (Llovet et al., 2003). In addition, patients with inflammatory bowel diseases, accompanied by oxidative stress (Pavlick et al., 2002), are at increased risk for developing colorectal cancer (Itzkowitz and Yio, 2004).

Both liver and colon are equipped with defense mechanisms to limit oxidative stress induced damage. The nuclear factor E2-related

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMPO, 5,5-dimethyl-1-pyrolline N-oxide; ARE, antioxidant response elements; CDF, chip definition files; DEGs, differentially expressed genes; DTW, dynamic time warping; EMT, epithelial-mesenchymal transition; ESR, electron spin resonance; FDR, false discovery rate; HCC, hepatocellular carcinoma; HCA, Hierarchical Clustering Analysis; IBD, inflammatory bowel disease; NER, nucleotid excision repair; ROS, reactive oxygen species; RMA, Robust Multi-array Average; STEM, Short Time-series Expression Miner.

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factor 2 (NRF2) is a key regulator in the oxidative stress response and is expressed in a wide number of tissues, including liver and colon (Aleksunes and Manautou, 2007). Under non-stressful physiological condition, NRF2 is kept in the cytosol by KEAP1 (Sun et al., 2011). Oxidative stress may modify KEAP1 directly to cause their dissociation and consequently, NRF2 can escape proteosomal degradation and translocates to the nucleus to activate the antioxidant response element (ARE) which facilitates the transcriptional machinery in protecting the cell against oxidative stress (Kay et al., 2010). Glutathione biosynthesis is regulated by this cascade which activates its rate-limiting enzymes GCLC and GCLM and is believed to be involved in multiple liver diseases, as well as in chemo-resistance in HCC (Lu, 2013). Furthermore, nuclear factor-κB (NF-κB) is translocated to the nucleus after induction by oxidative stress to activate genes involved in inflammation and immune responses, apoptosis and proliferation (Braun et al., 2006).

When these first line defense mechanisms fail in preventing oxidative stress-induced cellular damage, other processes such as DNA damage repair, cell cycle arrest or programmed cell death can be activated to prevent the formation of fixed mutations. However, when ROS levels are excessively elevated in cells, oxidative stress and consequently chronic inflammation will be induced. Attracted immune cells will constantly generate new ROS resulting in chronic oxidative stress which will induce fixed DNA mutations and will contribute to carcinogenesis by activating oncogenes and/or inactivating tumor suppressor genes (Iwanaga et al., 2008; Kundu and Surh, 2012).

Where oxidative stress-related mechanisms described so far, seem to be quite generic, cell type-specific signaling pathways in cellular damage and carcinogenesis-induced by oxidative stress may underlie risks for chronic inflammation and carcinogenesis in particular target organs. For contributing to cancer prevention and treatment of tissue-specific cancers, it thus is of primary importance to investigate such cell type-specific differences at the molecular level. Since oxidative stress-related effects will differ in time, examining and comparing temporal changes in different cell types is of additional relevance. In previous *in vitro* studies, these oxidative stress-related mechanisms in response to different oxidants were extensively investigated using such a time series gene expression approach (Briede et al., 2010; Deferme et al., 2013). These cellular models readily allow for time-dependent analysis of whole genome gene expression, so here, we compare oxidant-induced gene expression changes and cell cycle distribution data from these previous performed *in vitro* studies in a human hepatoma cell line (HepG2 cells) and a human colon adenocarcinoma cell line (Caco-2 cells). Since both these cell lines respond to oxidative stress in activating antioxidant machineries (Briede et al., 2010; Deferme et al., 2013), are able to carry out biotransformation of xenobiotics and are permeable for different types of compounds (Artursson et al., 2001; Jennen et al., 2010), these cells are a convenient and reproducible *in vitro* alternative for *in vivo* toxicity testing. Both cell lines were exposed to menadione, a polycyclic aromatic quinone generating superoxide after redox cycling mediated by quinone oxidoreductase (NQO1), and H_2O_2 which can oxidize transition metals using the Fenton reaction to create hydroxyl radicals and is metabolized by catalase. Using a range of bioinformatics tools, unique and common genes/pathways will be identified as well as temporal expression profiles of differentially expressed genes (DEGs) investigated. In particular, oxidative stress-related pathways such as the NRF2/KEAP1 and the NF-κB pathway will be examined, since both regulate the transcription of a wide array of genes involved in the protection against different cell type-specific pathologies (Aleksunes and Manautou, 2007; Lee et al., 2005; Sun and Zhang, 2007). Therefore, it is of interest to investigate whether oxidative stress-induced transcription of these genes differs between different cell types.

2. Material and methods

2.1. Cell culture

HepG2 and Caco-2 cells (ATCC, LGC logistics, UK) were cultured in 6-well plates as previously described (Briede et al., 2010; Deferme et al., 2013). When cells were 80% confluent, the medium was replaced with medium containing 100 μM menadione in both cell lines (Sigma–Aldrich, Zwijndrecht, The Netherlands) or 50 μM H_2O_2/Fe^{2+} in HepG2 cells and 20 μM H_2O_2 in Caco-2 cells (VWR int, UK). These non-cytotoxic concentrations were selected as previously described using MTT and ESR spectroscopy (Briede et al., 2010; Deferme et al., 2013). An exposure time series was applied in both cell lines (0.5, 1, 2, 4, 8 and 24 h) and time-matched control cells (only medium) were treated in an identical manner without addition of oxidants.

2.2. Cell cycle distribution

Analyses of cell cycle profiles were performed as previously described (Staal et al., 2007). Cells were stained with propidium iodide and cell cycle profiles were analyzed using ModFit LT for Mac (version 2.0).

2.3. Quantitative PCR and whole genome gene expression

First RNA was extracted using QIAZOL in combination with MiRNeasy mini kits (Qiagen, Westburg, The Netherlands) and quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) as previously described (Deferme et al., 2013).

Quantitative PCR was performed in biological duplicates for both treated and untreated HepG2/Caco-2 cells and calculated as previously reported (Livak and Schmittgen, 2001; Staal et al., 2007) ($n = 2$). RT-PCR was run on the MyiQ Single-Color RT-PCR Detection System (Bio-Rad Laboratories). Forward and reverse primers of Beta-Actin (used as reference), HMOX1, BCL2, GCLC, MAFG and NQO1 can be found in supplementary data 1.

2.3.1. Whole genome gene expression

cRNA from treated and untreated HepG2 cells was prepared using Affymetrix synthesis and labeling kits as described before (Affymetrix, Santa Clara, CA) (Jennen et al., 2010). cRNA targets of control and exposed were individually hybridized on high-density oligonucleotide genetitan chips (Affymetrix Human Genome U133 Plus PM GeneTitan 24 arrays) as previously described (Deferme et al., 2013). Two oxidant exposures and time matched control samples during six different time points in biological triplicate ($n = 3$) resulted in a total of 72 single-color arrays.

Exposed and time matched control RNA samples from Caco-2 cells were two-color labeled and hybridized on the same array and scanned according to the manual for G4110B 22K/G4112F 44K Agilent Human Oligo Microarray (Agilent Technologies, Santa Clara, CA) as previously described (Briede et al., 2010). Two oxidant exposures and time matched control samples during six different time points in biological duplicates and technical duplicates (dye swap) resulted in a total of 48 two-color arrays.

2.4. Re-annotation and normalization

In HepG2 cells, data from 72 arrays were obtained, and Robust Multi-array Average (RMA) normalized and re-annotated to custom CDF files using the array analysis tool (http://arrayanalysis.org/). In Caco-2 cell, images of 48 hybridizations were processed with ImaGene 6.0 software (BioDiscovery Inc., Los Angeles, CA) to