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Targeting the expression of glutathione- and sulfate-dependent detoxification enzymes in HepG2 cells by oxygen in minimal and amino acid enriched medium

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ABSTRACT

Cancer cells exhibit specific metabolism allowing them to survive and proliferate in various oxygen conditions and nutrients' availability. Hepatocytes are highly active metabolically and thus very sensitive to hypoxia. The purpose of the study was to investigate the effect of oxygen on the expression of phase II detoxification enzymes in hepatocellular carcinoma cells (HepG2) cultured in minimal and rich media (with nonessential amino acids and GSH). The cells were cultured at 1% hypoxia, 10% tissue normoxia, and 21% atmospheric normoxia. The total cell count was determined by trypan blue exclusion dye and the expression on mRNA level by RT-PCR. The result indicated that the expression of glutathione-dependent enzymes (*GSTA*, *M*, *P*, and *GPX2*) was sensitive to oxygen and medium type. At 1% hypoxia the enzyme expression (with the exception of *GSTA*) was higher in minimal compared to rich medium, whereas at 10% normoxia it was higher in the rich medium. The expression was oxygen-dependent in both types of medium. Among phenol sulfotransferase *SULT1A1* was not sensitive to studied factors, whereas the expression of *SULT1A3* was depended on oxygen only in minimal medium.
It can be concluded that in HepG2 cells, the detoxification by conjugation with glutathione and, to a lower extent with sulfate, may be affected by hypoxia and/or limited nutrients' availability. Besides, because the data obtained at 10% oxygen significantly differ from those at 21%, the comparative studies on hypoxia should be performed in relation to 10% but not 21% oxygen.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in humans (Parkin et al., 2001). Cancer cells are known to exhibit specific metabolism allowing them to survive and proliferate in various oxygen and nutrients' (amino acids, glucose) availability. They grow faster than the vasculature and during intensive proliferation they distance from the blood vessels (Bertout et al., 2008; Carreau et al., 2011). Oxygen deprivation leading to hypoxia is a common feature in solid tumors. A cellular response to hypoxia may be a change in gene expression (Sonna et al., 2003). Since the liver is highly active metabolically, it is very sensitive to hypoxia. It is also the major organ involved in detoxification of xenobiotics entering the body by the gastrointestinal tract and produced during metabolism. In the present work we studied the expression of phase II detoxification enzymes: glutathione S-transferases (*GSTA*, *M*, *P*), glutathione peroxidase (*GSX-2*), and phenol sulfotransferases (*SULT1A1* and *1A3*) in human hepatocellular carcinoma cells (HepG2) at various oxygen and amino acid availability.

Glutathione S-transferases (GST; EC 2.5.1.18) are multifunctional enzymes encoded by many genes (Jakoby, 1978; Mannervik et al., 1992). They catalyze the conjugation of reduced glutathione with a large number of electrophilic compounds, including carcinogens and drugs. Showing selenium-independent glutathione peroxidase activity they inactivate products of oxidative stress (Hayes and Strange, 1995; Tew and Ronai, 1999). In addition, in the cytoplasm they non-enzymatically bind various hydrophobic compounds functioning as transport proteins (previously known as ligandins) (Hayes and Pulford, 1995; Oakley, 2011). The most abundant mammalian GSTs are the class alpha, pi, and mu. GST alpha (GSTA) is the most common in hepatocytes. It is expressed by genes located in a cluster mapped to chromosome 6 and is highly specific for organic peroxides (cumene hydroperoxide, phospholipid hydroperoxide, fatty acid hydroperoxides (Hayes and Pulford, 1995; Yang et al., 2001).

Glutathione S-transferase pi (GSTP) is expressed in many extrahepatic tissues and its overexpression was observed in the majority of human tumors and tumor cell lines (Aliya et al., 2003; Hokaiwado et al., 2008; Konohana et al., 1990). In humans it is encoded by a single *GSTP1* gene located on chromosome 11 (Konohana et al., 1990). GSTP is an important enzyme in cancer development and chemotherapeutic resistance (Hayes and Pulford, 1995). Under normal conditions GSTP

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shows an anti-apoptotic activity as selective inhibitor of c-Jun N-terminal kinase (JNK), but under conditions of oxidative or chemical stress, it dissociates from the JNK complex and induces a pro-apoptotic signaling pathway (Adler et al., 1999; Laborde, 2010).

GSTM1, which in humans is expressed by a gene located on chromosome 1, shows similar effects on apoptosis as GSTP but it interacts with apoptosis signal-regulating kinase 1 (ASK1) (Cho et al., 2001; Xu et al., 1998). Suppressing ASK1 activity GSTM1 participates in the regulation of stress-activated signals. It shows high activity with o-quinones and products of lipid peroxidation (Townsend and Tew, 2003).

Glutathione peroxidase 2 (GSHPx-2 or GPX2; EC 1.11.1.9) is a member of the selenium-dependent glutathione peroxidase family (Chu et al., 1993). It plays a major role in antioxidant defense by reducing hydrogen peroxide and various organic hydroperoxides. In humans GPX2 is encoded by *GPX2* gene located on chromosome 14 and expressed mainly in the gastrointestinal tract and liver (Chu et al., 1996; Wingler and Brigelius-Flohe, 1999). *GPX2* is up-regulated in many cancer cells, supports growth of established tumors, and inhibits cultured cancer cell migration counteracting COX-2 expression and PGE2 production (Brigelius-Flohe and Kipp, 2009; Suzuki et al., 2013).

Sulfotransferases constitute a superfamily of isoforms encoded by at least 10 functional *SULT* genes (Weinshilboum et al., 1997). In humans, phenol sulfotransferases SULT1A1 and SULT1A3 (EC 2.8.2.1) are encoded by neighboring genes on chromosome (Dooley, 1998). All SULTs catalyze the transfer of a sulfonate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to various acceptor substrates decreasing their biological or toxic activity. In particular SULT1A1, a thermostable form, has a broad tissue distribution and is important in detoxification of xenobiotics (including many drugs), as well as endogenous substrates such as estrogens and iodothyronines (Blanchard et al., 2004). SULT1A3, a thermolabile form, is the major tyrosine, dopa, catecholamines, and products of their peroxidation sulfonating enzyme (Dajani et al., 1999). It is expressed principally in the gastrointestinal tract and at high level in the fetal but not adult liver (Richard et al., 2001). Phenol sulfotransferases are present in human hepatocellular carcinoma cells (HepG2), and as it has been shown, among the 11 tested SULTs only SULT1A3 exhibits activity toward nitrotyrosine, the product of tyrosine nitration formed during oxidative/nitrative stress (Ischiropoulos, 1998; Yasuda et al., 2007).

Hypoxia is a common feature of tumor tissues and oxygen level in the tumor surroundings never exceeds 10% (tissue normoxia) (Carreau et al., 2011). However, the majority of studies on hypoxia in tumor cell lines are conducted at 21% oxygen (atmospheric normoxia), and the tension never reached in vivo. Therefore, our studies were conducted on HepG2 cells cultured at oxygen levels that reflected the in vivo conditions (1%, 10% oxygen), and comparatively at 21% oxygen. The cells were cultured in minimal medium without nonessential amino acids and glutathione, and in rich medium with all the above compounds.

2. Material and methods

2.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was purchased from American Type Culture Collection (ATCC) and maintained in rich (RPMI 1640 with L-Gln and GSH, CytoGen) or minimal medium (MEM SH30024, free of glycine, L-Ala, L-Cys, L-Ser, L-Asp, L-Asn, L-Glu, L-Pro, pyruvate, and GSH, Thermo Sci.) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (20 mM), in tissue culture flasks (50 ml), in 37 °C/5% CO_2 humidified incubator, and cultured until 80–90% confluence was reached. After harvesting by the treatment with 0.25% trypsin–0.02% EDTA in PBS the cells were seeded in tissue culture flasks (4×10^5 cells) for 120 h in hypoxic chamber with oxygen controller (Coy Laboratory Products INC, USA) at 1% oxygen (chronic hypoxia), 10% oxygen (tissue normoxia), and 21% oxygen (atmospheric normoxia).

2.2. Determination of the total cell number

After 120 h incubation at various oxygen conditions the cells were washed twice with PBS, trypsinized and harvested. The total cell number was assessed by trypan blue exclusion dye assay using automated cell counter (Countess Invitrogen). Cell viability ranged from 94 to 98% in all settings. Each experiment was repeated 3 times.

2.3. RT-PCR analysis

Total RNA was isolated according to the manufacturer's protocol using TRI-Reagent (MRC, Cincinnati OH). The expression of mRNA for each studied protein was determined by reverse transcription polymerase chain reaction (RT-PCR) followed by DNA electrophoresis in agarose gel. The specific primers for studied genes and for beta-2-microglobulin (*B2M*, a housekeeping gene) were designed using PRIMER 2.01 software, and are shown in Table 1.

The expression of each mRNA was expressed in semi-quantitative way as the ratio of the optical density band of studied enzyme to the optical density of beta-2-microglobulin band (housekeeping gene). Each assay was performed in duplicates and repeated twice. System UVI-KS4000, Syngen Biotechnology was used for densitometric analysis of RT-PCR. Results were expressed as means ± SD. Quantitative comparison between studied groups was performed by Student's t test using Statistica 9.0 (StatSoft). Differences were considered significant at $P < 0.05$. The data were analyzed by two-way analysis of variance (ANOVA). Pearson's correlation test was performed to find out the correlation between the expression, oxygen level and type of medium. The correlation coefficient (r) values were represented at 0.05 level of significance.

3. Results

3.1. The effect of oxygen on the total HepG2 cell number

The total cell number of HepG2 was increasing with the level of oxygen independently of the medium used (Table 2). At 1% oxygen it was approximately 4–5-fold lower than at 10% oxygen, and 8–10-fold lower than at 21% oxygen (non-physiological condition). In rich medium the differences between the total cell number at 10% and 1% and at 10% and 21% oxygen were significant ($P < 0.05$). Only at 21% oxygen the total cell number was significantly higher in rich than in minimal medium ($P < 0.05$).

Table 1
Sequences of oligonucleotides used in PCR reaction.

Gene	Primer	Primer sequence
GSTA	Forward	5′-GATTCTGGTTTTCAGGGCCTTC-3′
	Reverse	5′-ACATTGCCAGCAAATACAACCT-3′
GSTM	Forward	5′-GCTCCTGGAATACACAGACTC-3′
	Reverse	5′-CACAGGTTGTGCTTGCGGG-3′
GSTP	Forward	5′-AGTGCCTTCACATAGTCATC-3′
	Reverse	5′-GGCTCACTCAAAGCCTCCTG-3′
GPX2	Forward	5′-GGTGCCATCATTCTGTGAAG-3′
	Reverse	5′-CCATCAACATTGAGCCTGAC-3′
SULT1A1	Forward	5′-CCTTGACCTTCTGATCCAAC-3′
	Reverse	5′-GGTAAGCCAGATTCTGGACA-3′
SULT1A3	Forward	5′-CCTTGAGGTCAATGATCCAGG-3′
	Reverse	5′-GACACTTCTCCAGCCATGAAC-3′
B2M	Forward	5′-GATGCTGCTTACATGTCTCG-3′
	Reverse	5′-CCAGCAGAGAATGGAAAGTC-3′