A new risk assessment method for evaluation of oxidative chemicals using catalase mutant mouse primary hepatocytes

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ABSTRACT

We examined the possibility of developing a new risk assessment method for potentially oxidative chemicals by using mouse primary hepatocytes from acatalasemic mice (Cs[b]) and the wild-type (Cs[a]) as predictive model. Chemical-induced cytotoxicities, such as hydrogen peroxide and lawsone, a main hair dye ingredient of henna, were examined. We observed the differences in cell survival between the Cs[a] and Cs[b] in a dose-dependant manner after treatment with either hydrogen peroxide or lawsone, supporting the usefulness of this newly established method for hazard identification of oxidative chemicals in a risk assessment process. More chemicals will be tested to confirm the usefulness of this method for the preliminary screening of oxidative chemicals before animal experimentation.

Keywords: Catalase-Mutant Mouse Hepatocytes; Reactive Oxygen Species; Hydrogen Peroxide; Hazard Assessment; Lawsone Cytotoxicity

1. INTRODUCTION

More and more chemicals are synthesized for industrial and consumer use, it is impossible to finish long-term rodent bioassay for detection of carcinogens and identification of hazards in all chemicals because it involves large numbers of animals and is extremely expensive. Therefore, simple and efficient pre-screening alternatives to animal experimentation are desirable [1]. Nowadays, there has been an increasing awareness that the cellular formation of highly reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or hydroxyl radicals likely cause the DNA lesions [2]. Catalase and glutathione peroxidase are the most important enzymes capable of removing intracellular H_2O_2 in biological systems [3-5], and catalase particularly plays a critical role when H_2O_2 is overproduced. Catalase-mutant acatalasemic mouse was established by Feinstein *et al.* through a large scale screening of the progeny of irradiated C3H mice [6]. A point mutation at amino acid 11 (from glutamine to histidine) of Cs[b] mouse catalase is responsible for its catalase deficiency [7,8]. In the present study, we examined the possibility of developing a new risk assessment method for evaluation of oxidative chemicals using mouse primary hepatocytes from acatalasemic mice (Cs[b]) and the wild-type (Cs[a]) as predictive model.

2. MATERIALS AND METHODS

2.1. Chemicals

Hydrogen peroxide, lawsone (2-hydroxy-1,4-naphthoquinone), kanamycin sulfate, insulin, and dexamethasone sodium phosphate, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cell counting kit (WST-8) from Dojindo Company (Osaka,

Japan). All other chemicals were obtained from Sigma Chemical Co (St. Louis, USA) unless otherwise mentioned.

2.2. Hepatocyte Isolation

Adult male wild-type (C3H/AnLCs^{a}Cs^a) and acatalasemic (C3H/AnLCs^{b}Cs^b) mice were used in this study. They were anaesthetized intraperitoneally with 9μl/g body weight of pentobarbital sodium (10% in PBS) and then the liver was first perfused at 37˚C with EGTA at a flow rate of 5.5 ml/minute for 10 minutes and thereafter with collagenase at the same flow rate for 15 minutes as described previously [9,10]. The yield of isolated hepatocytes was determined with a hemocytometer, and their viability was evaluated with the standard trypan blue exclusion method. The viability of the isolated hepatocytes was around 90%. The isolated hepatocytes (5×10^6) were seeded into 100-mm-diameter plates or 96-well plates containing serum free hepatocyte growth medium (HGM) [11,12] supplemented with 1 μM insulin (Sigma Chemical Co, St. Louis, MO), 1 μM dexamethasone sodium phosphate, 0.1 mg/ml kanamycin sulfate, and allowed to attach for 24 h before used in the experiments.

2.3. Confirmation of the Genotype of Catalase Mutant Mouse Hepatocytes

Genomic DNA was purified from primary cultured hepatocytes of Cs^a and Cs^b using Wizard Genomic DNA Purification Kit (Promega Corporation, WI) and was used as a template for PCR amplification of the catalase gene segment encompassing the Cs^b mutation site. The primers, 5'-CGGGTGGAGACCAGACCGCT-3' and 5'-TGCGAGGCCCGCTGCTCCTT-3', were used to target a 141 bp fragment of the mouse catalase gene [13]. PCR amplification was performed for 35 cycles (30 s at 98˚C for denaturation, 20 s at 58˚C for annealing, and 40 s at 68˚C for extension) in a final volume of 20 μl with 50 ng of genomic DNA, 50 nM primers, and 0.05 U/ml LA *Taq* DNA polymerase under the conditions recommended by the manufacturer (TaKaRa Biochemicals, Japan). Amplified products were purified by ethanol precipitation, digested with restriction enzyme *Nde*I, and subjected to agarose gel electrophoresis (4% NuSieve GTG agarose). DNA bands were visualized by staining with ethidium bromide.

2.4. Cell Viability Assay

Primary hepatocytes seeded into 96-well plates at a density of 1.2×10^4 per well were used to evaluate the cytotoxic effect of chemicals. Various concentrations of test compounds (H$_2$O$_2$ and lawsone were added to the cells and incubated at 37˚C for 24 h. Cell viability was measured using the WST-8 assay, based on the reduction of the tetrazolium salt to a water-soluble formazan product by the cellular dehydrogenase [14]. Absorbance was measured at 450 nm by microplate reader. The survival of cells exposed to chemicals was expressed as a percentage of cell survival in the negative control group based upon the following formula: Survival (%) = (Absorbance of sample − Absorbance of blank)/(Absorbance of negative control − Absorbance of blank). At least three tests were performed in each experiment.

2.5. Statistic Analysis

Significant differences ($p < 0.05$) among groups were determined by two-way analyses of variance (two-way ANOVA) by SPSS 15.0 statistical program package (SPSS Inc., Illinois, USA).

3. RESULTS AND DISCUSSION

3.1. The Cs^b Catalase Mutant Gene Is Susceptible to *Nde*I Digestion

Since a point mutation at amino acid 11 (from glutamine to histidine) of Cs^b mouse catalase gene is responsible for the deficient catalase activity [7,8], there is a recognition site for *Nde*I that can cut the targeted 141 bp fragment of Cs^b band into 2 fragments of 108 and 33 bp. **Figure 1** showed the genomic PCR products with or without the enzymatic digestion of *Nde*I. The 141-bp target region in the wild-type mouse Cs^a catalase gene was not cleaved by the enzyme, however, all the 141-bp products of the Cs^b catalase mutant gene were cleaved to 2 fragments of 108 and 33 bp (the 33 bp fragment is not visible here) by *Nde*I. The results agreed with that of previous reported [13].

3.2. Difference in Cytotoxic Effects Induced by Oxidant H$_2$O$_2$ on Mouse Primary Hepatocytes

In **Table 1**, catalase-mutant mouse Cs^b hepatocytes showed a significantly higher susceptibility to H$_2$O$_2$ in comparison with the wild-type Cs^a, and the cytotoxic effects of H$_2$O$_2$ on both cell groups were dose dependent.

Since a point mutation at amino acid 11 of Cs^b mouse catalase gene is responsible for catalase deficiency, H$_2$O$_2$ exposed to Cs^b hepatocytes could not be fully decomposed, which markedly affected the cell survival compared to that of the wild-type Cs^a. Therefore, based on the cell viability, we can use the Cs^a and Cs^b as predictive models to assume whether H$_2$O$_2$ involves in the chemical-induced cytotoxicity, which would be helpful for the preliminary screening of the potential oxidative chemicals.
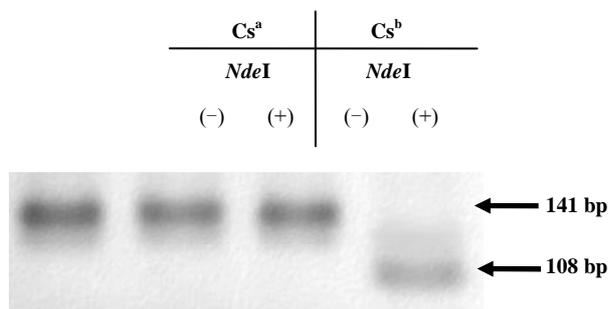
	Cs^a		Cs^b	
	*Nde*I		*Nde*I	
	(−)	(+)	(−)	(+)

← 141 bp

← 108 bp

Figure 1. The genomic PCR products with or without the enzymatic digestion *of Nde*I.

Table 1. Comparison of H_2O_2-induced cytotoxic effect on primary hepatocytes[#].

Treatment H_2O_2[##]	Cs^a	Cs^b
None	100	100
3.75 mM	74.2 ± 2.5	18.3 ± 0.4
7.5 mM	54.0 ± 3.8	15.6 ± 1.6
15 mM	36.0 ± 4.1	12.8 ± 1.1

[#]Data are expressed as percentage of mean ± SD. At least three tests were performed in each experiment. Differences within and among groups were evaluated by two-way ANOVA; [##]Concentrations of H_2O_2 were expressed as final concentrations in the reaction cultures. P1 < 0.01(comparison between groups of Cs^a and Cs^b). P2 < 0.001 (comparison among groups of H_2O_2 concentrations).

3.3. Comparison of Lawsone-Induced Cytotoxicity on Mouse Primary Hepatocytes

Then, we further tested the cytotoxic effect of lawsone, a main color ingredient of hair dye henna, on mouse primary hepatocytes. Lawsone has been reported causing mutagenicity/genotoxicity both *in vitro* and *in vivo* [15] by the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP), a scientific advisory body to the European Commission in matters of consumer protection. **Figure 2** demonstrated that in comparison with the wild-type Cs^a hepatocytes, the survival of catalase-mutant mouse Cs^b hepatocytes was significantly reduced by lawsone treatment, showing 86.1% in Cs^a, 38.0% in Cs^b even at 100 μM lawsone exposure. Both cell groups dose-dependently detected cytotoxic effects of lawsone.

Catalase is an important component of the cellular defenses against the toxicity induced by H_2O_2 and its products [4]. Wang *et al.* reported that the catalase activity in liver of Cs^b mice were 39.6% of that in wild-type Cs^a [16]. Lawsone was reported to generate H_2O_2 in phosphate buffer system [17]. The present study found lawsone treatment markedly reduced cell viabilities in
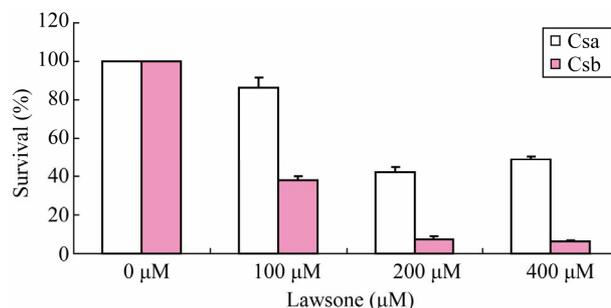
Figure 2. Comparison of lawsone-induced cytotoxic effect on primary hepatocytes. Data are expressed as percentage of mean ± SD. At least three tests were performed in each experiment. There were significant differences between Cs^a and Cs^b (P < 0.001) and among exposure concentrations of lawsone (P < 0.001) evaluated by two-way ANOVA.

catalase-mutant mouse Cs^b hepatocytes in comparison with in wild-type Cs^a, suggesting that H_2O_2 was increasingly produced in the process of lawsone cytotoxicity.

4. CONCLUSIONS

The present study has attempted to develop a new assessment method for evaluation of oxidative chemicals using mouse primary hepatocytes from catalase-mutant mouse Cs^b and the wild-type Cs^a as predictive model. The differences in cell survival between the Cs^a and Cs^b in a dose-dependant manner support the usefulness of this newly established method for hazard identification of oxidative chemicals in a risk assessment process. More chemicals will be tested to confirm the usefulness of this method for the preliminary screening of oxidative chemicals before animal experimentation.

It is known that most primary cell cultures have limited lifespan. The primary cultured hepatocytes employed in our study can be used for 5 - 6 days, during which the hepatocytes are supposed to maintain most of their *in vivo* functional characteristics in culture [18,19], and they are expected to be possible predictive models for large scale screening of oxidative chemicals if the issue of long-term storage of primary hepatocytes can be solved.

5. ACKNOWLEDGEMENTS

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