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Differential methotrexate hepatotoxicity on rat hepatocytes in 2-D monolayer culture and 3-D gel entrapment culture

Jian Yin [a], Qin Meng [a,*], Guoliang Zhang [b], Yonghong Sun [c]

[a] Department of Chemical Engineering and Biochemical Engineering, Zhejiang University, Zhejiang 310027, PR China
[b] Institute of Biological and Environmental Engineering, Zhejiang University of Technology, Zhejiang 310032, PR China
[c] Key laboratory of Chinese Medicine Screening, Exploitation and Medicinal Effectiveness Appraise for Cardio-Cerebral Vascular & Nervous System of Zhejiang Province, PR China

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ABSTRACT

It has been reported that 3-D cultures of hepatocytes or HepG2 cells were less susceptible to methotrexate (MTX) than their 2-D counterparts. Such a mechanism was addressed in this study by investigation of MTX hepatotoxicity in gel entrapped (3-D) rat hepatocytes vs. traditional monolayer culture (2-D). Similarly, gel entrapped hepatocytes showed higher drug resistance to MTX than hepatocyte monolayers in whatever culture medium with or without modification by hormone supplements (dexamethasone, glucagon and insulin). It was also found that medium modification by hormones greatly increased drug resistance of hepatocyte monolayers but has only a slight effect on 3-D cultured hepatocytes. These differential MTX toxicities regarding culture medium and culture models were assumed to correlate with multidrug resistance associated protein 2 (Mrp2). The involvement of Mrp2 was confirmed directly by the fact that MTX intracellularly accumulated less in gel entrapped hepatocytes than in hepatocyte monolayer but could be enhanced by Mrp2 inhibitors accompanied by reduced drug resistance. Furthermore, the expression of Mrp2 on gene level and transportation activity together with bile-duct-like structure were more significantly evidenced in 3-D gel entrapment culture than in 2-D monolayer culture. In conclusion, the highly preserved Mrp2 in 3-D gel entrapped hepatocytes determines its high drug resistance to MTX. Gel entrapped hepatocytes could be useful for investigation of hepatic transportation and hepatotoxicity.

1. Introduction

Methotrexate (MTX), a folic acid anti-metabolite drug in cancer treatment, triggered hepatotoxicity in animals and humans [1–5]. This hepatoxicity was reportedly attributed to either decrease in intracellular nicotinamide adenine dinucleotide phosphate (NADPH) enhancing sensitivity of hepatocytes to oxidative stress [6] or long-term accumulation of MTX in hepatocytes causing formation of MTX-polyglutamate [7]. Most of such mechanistic studies on MTX hepatoxicity were carried out *in vivo*.

Limited *in vitro* studies on MTX toxicity were reported using either 2-D hepatocyte monolayers or 3-D cultured hepatocytes [8,9]. Interestingly, differential hepatotoxicities of MTX were observed between the two cultures [8,9]. The 3-D culture in either rat hepatocyte spheroids [8] or polystyrene scaffolds of HepG2 cells [9] claimed more drug resistance to MTX treatment than corresponding 2-D monolayer cultures. Accordingly, high drug resistance to other drug treatments in 3-D culture was observed in skin cells [10], human bone marrow cells [11] and breast cancer cells [12]. Unfortunately, none of the *in vitro* studies mentioned above investigated the mechanism on the higher drug resistance of cells in 3-D culture than in 2-D culture. Walker et al. [8] suggested the poor penetration of drugs into the 3-D hepatocyte spheroids as a major reason for its higher drug resistance, and thus showed negative in applying such hepatocyte culture on drug hepatotoxicity.

On the other hand, the metabolism of MTX *in vitro* has been well studied in isolated perfused liver. MTX does not undergo any phase I or II metabolism except minor hydroxylation [13]. Instead, MTX is largely excreted into bile via mediation of membrane transporters, in particular an ATP-dependent efflux transporter named multidrug resistance associated protein 2 (Mrp2) located on canalicular membrane of hepatocytes [14]. Although other membrane transporters like multidrug resistance protein 1 (Mdr1) [15] reportedly mediate biliary excretion of MTX, Mrp2 is the major mediator in rat hepatocytes [14]. It has been suggested that Mrp2 mediated the drug resistance of malignant cells to MTX [16]. The hepatic uptake and biliary excretion via membrane transporters of xenobiotics which

* Corresponding author at: Department of Chemical Engineering and Biochemical Engineering, College of Materials Science and Chemical Engineering, Zhejiang University, 38 Zheda Road, Hangzhou, Zhejiang 310027, PR China.
Tel.: +86 571 87953193; fax: +86 571 87951227.
E-mail address: mengq@zju.edu.cn (Q. Meng).

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are named as phase III drug metabolism [17] play an important role in drug performance [18,19] and hepatotoxicity [20].

However, the possible mechanism of differential MTX toxicity in variously cultured hepatocytes has never been addressed. We assumed that the higher drug resistance of 3-D cultured hepatocytes to MTX was caused by its higher expressions on membrane transporters, in particular Mrp2. Thereby, another 3-D culture of hepatocytes by gel entrapment was included in this paper and compared with 2-D hepatocyte monolayers whereas MTX toxicity, Mrp2 expression and the morphology of bile-duct-like structure were individually examined in each culture.

2. Materials and methods

2.1. Chemicals

MTX and Cyclosporine A (CsA) were from Shanghai Sangon Biological Technology & Services Co. Ltd. (Shanghai, China). Probenecid (PB) was obtained from Leawell International Ltd. (Guangzhou, China). Dexamethasone and insulin were purchased from Hangzhou Haotian Biotechnology Co. Ltd. Glucagon was from Shenzhen Hybio Engineering Co. Ltd. Methyl thiazolyl tetrazolium (MTT) were purchased from Hangzhou Huadong Medicine Group Company (Hangzhou, China). Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Eng. Material Co. Ltd. (Hangzhou, China). Williams' E basal medium and L-glutamine were from Gibco (Gaithersburg, USA) and Amresco Inc. (Solon, OH, USA), respectively. 5-Carboxyfluorescein diacetate (CFDA) was from Sigma–Aldrich (St. Louis, MO, USA). The remaining chemicals were from local chemical suppliers and were all of reagent grade.

2.2. Hepatocytes isolation and culture

Hepatocytes were isolated from male Sprague–Dawley rats (weighing 200–250 g) by the two-step collagenase perfusion method as previously described [21]. Cell viability was assessed by trypan blue exclusion, and hepatocytes with a viability of greater than 85% were used. Hepatocytes were cultured in Williams' E medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% (v/v) fetal bovine serum. For medium modification experiments, supplements including 1 μM of dexamethasone, 0.2 U/ml of insulin, 4 ng/ml of glucagon were added to the regular medium.

For the monolayer culture, the wells of 24-well plates were pre-coated with 0.16 mg/l of collagen (type I). Freshly isolated rat hepatocytes were seeded at a density of 2×10^5 cells/well, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

For the gel entrapment culture, freshly isolated hepatocytes were mixed with the collagen solution and loaded into hollow fibers by injection as described before [21]. Briefly, hepatocytes were inoculated into a 3:1 (v/v) mixture of type I collagen (3 g/l) and four-fold concentrated Williams' E medium at pH of 7.4. Hepatocytes suspension at cell density of 1×10^6 cells/ml was loaded into the lumen of long fibers at a length of 35 cm. Hollow fibers were circuitously put into 12 cm diameter dishes and maintained in a 5% CO_2 incubator for collagen gelation. Ten minutes later, hollow fibers were cut into four equal pieces and put into 6 cm culture dishes full of 6 ml prewarmed culture medium before put into the 5% CO_2 incubator for hepatocyte cultures.

2.3. Exposure of hepatocytes to MTX

MTX was dissolved in culture medium directly before use.

After 4 h of seeding, hepatocyte monolayers in both regular and modified medium were washed with phosphate buffered saline and then exposed to MTX (1, 5 and 10 mM). Medium was changed every 48 h and cell viability was recorded at 24, 72 and 120 h, respectively.

For the gel entrapment culture, hepatocytes in both regular and modified medium were treated by MTX (1, 5 and 10 mM). Medium was changed at every 48 h of culture and cell viability was detected at 24, 72 and 120 h, respectively.

2.4. The effect of Mrp2 inhibitor on MTX toxicity

After 72 h of pre-incubation, hepatocytes in either monolayer or gel entrapment cultures were washed with phosphate buffered saline, and incubated in medium containing 10 mM of MTX for 3 h with co-treatment by vehicle or Mrp2 inhibitors of CsA at 2 μM or PB at 200 μM. Cell viability and glutathione (GSH) of hepatocytes in each culture were detected. PB and CsA were dissolved in ethanol and 1 M NaOH, respectively, and then both were diluted in culture medium before use. The final concentration of ethanol in CsA treatment group was less than 0.1% which had no adverse effect on cell culture.

2.5. Analysis of intracellular accumulation of MTX

After 72 h of pre-incubation, hepatocytes in monolayer and gel entrapment were washed with phosphate buffered saline and then incubated in medium containing 10 mM of MTX with or without Mrp2 inhibitors (CsA, 2 μM or PB, 200 μM). After 3 h of treatment, medium was removed and hepatocytes were washed with ice-cold phosphate buffered saline for three times to terminate the uptake or excretion of MTX. Methanol at 500 μl was added to extract MTX at 37 °C for 1 h in 1.5-ml centrifuge tubes before centrifugation at $12{,}000 \times g$ for 10 min. The supernatant was transferred to another centrifuge tube and stored at −20 °C until usage. Analysis of MTX was performed with HPLC (Hewlett Packard 1100, USA) assay as described previously by Li et al. [22]. The detection wavelength was set at 260 nm using linear calibration curves for MTX at a concentration range of 0–200 μM.

2.6. Cell viability assay

The MTT assay was performed as described previously by Alley et al. [23]. Briefly, after incubation with the corresponding drugs, hepatocytes of gel entrapment were excluded from the hollow fibers with a 5-ml syringe to 24-well plates and washed with 500-μl phosphate buffered saline. Then, to each well was added 500-μl phosphate buffered saline and 150 μl MTT solution (5 mg/ml dissolved in phosphate buffered saline) and incubated for 3 h at 37 °C. At the end of incubation, the solution was removed and the formazan crystals were dissolved in 1.5 ml of acidified-isopropanol for 1 h at 20–25 °C. The absorbance was measured using a 96-well microplate reader at a wavelength of 570 nm on a spectrophotometer [24]. Same procedure was conducted on monolayer hepatocytes for determination of cell viability. The cell viability was expressed as a relative percentage to untreated group in each culture.

2.7. GSH assay

After incubation with the corresponding drugs, cells cultured in 24-well plates were initially rinsed with 500 μl of phosphate buffered saline and then scraped off the wells with a rubber policeman, while hepatocytes of gel entrapment were excluded from the hollow fibers with a 5 ml syringe to 24-well plates and washed with 500 μl of phosphate buffered saline. Cell samples were suspended in 500 μl of 5,5′-dithiobis(2-nitrobenzoic acid)-phosphate buffered saline mixer and sonicated in a sonicator at 40 kHz and 900 W twice for 5 s and centrifuged at 12,000 rpm for 10 min to precipitate cellular fragments. GSH in the cell supernatants was determined at a