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A transgenic rat hepatocyte - Kupffer cell co-culture model for evaluation of direct and macrophage-related effect of poly(amidoamine) dendrimers

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ABSTRACT

Increasing number of papers demonstrate that Kupffer cells (KCs) play a role in the development of drug induced liver injury (DILI). Furthermore, elevated intracellular Ca^{2+} level of hepatocytes is considered as a common marker of DILI. Here we applied an in vitro model based on hepatocyte mono- and hepatocyte/KC co-cultures (H/KC) isolated from transgenic rats stably expressing the GCaMP2 fluorescent Ca^{2+} sensor protein to investigate the effects of polycationic (G5), polyanionic (G4.5) and polyethylene-glycol coated neutral (G5 Peg) dendrimers known to accumulate in the liver, primarily in KCs. Following dendrimer exposure, hepatocyte homeostasis was measured by MTT cytotoxicity assay and by Ca^{2+} imaging, while hepatocyte functions were studied by CYP2B1/2 inducibility, and bilirubin and taurocholate transport. G5 was significantly more cytotoxic than G4.5 for hepatocytes and induced Ca^{2+} oscillation and sustained Ca^{2+} signals at 1 μM and10 μM, respectively both in hepatocytes and KCs. Dendrimer-induced Ca^{2+} signals in hepatocytes were attenuated by macrophages. Activation of KCs by lipopolysaccharide and G5 decreased the inducibility of CYP2B1/2, which was restored by depleting the KCs with gadolinium-chloride and pentoxyphylline, suggesting a role of macrophages in the hindrance of CYP2B1/2 induction by G5 and lipopolysaccharide. In the H/KC, but not in the hepatocyte mono-culture, G5 reduced the canalicular efflux of bilirubin and stimulated the uptake and canalicular efflux of taurocholate. In conclusion, H/KC provides a good model for the prediction of hepatotoxic potential of drugs, especially of nanomaterials known to be trapped by macrophages, activation of which presumably contributes to DILI.

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1. Introduction

Drug induced liver injury (DILI) represents an adverse drug reaction responsible for severe patient morbidity and mortality and in consequence for the withdrawal of drugs from the market (Kaplowitz, 2005; Chen et al., 2014). Various combinations of modes of action may be involved in DILI pathogenesis, even for a single drug (Yuan and Kaplowitz, 2013). In vivo hepatotoxic effects are not always detected in primary hepatocyte monocultures, which are however considered to be the gold standard of in vitro liver models. In view of their well-documented role in the development and progression of drug-related liver dysfunctions, the absence of non-parenchymal cells might be a reason for the insufficient recognition of DILI in vitro (Kostadinova et al., 2013; Rose et al., 2016). Immune-mediated mechanisms are considered to be major factors in the pathogenesis of DILI and growing evidence suggests that inflammation plays a role in the toxicities induced by some drugs. Kupffer cells (KCs), hepatic resident macrophages are involved in DILI by release of pro- and anti-inflammatory mediators such as cytokines, chemokines, reactive oxygen species, and nitric oxides (Shaw et al., 2010; Laskin et al., 2011). Activation of KCs, the presence of an inflammatory stress could alter the metabolism of drugs that might result in the formation of reactive intermediates only in the presence of inflammation (Goto et al., 2015). Another common adverse outcome of KC activation is the impairment of hepatic disposition of drugs and endogenous waste compounds caused by the downregulation of gene expression and internalization of members of the chemical defense system, metabolizing enzymes and transporters (Kim et al., 2011; Nguyen et al., 2015). Previously, we showed that sandwich culture of primary hepatocytes is a good model for testing direct effects of drugs on hepatocyte functions (Jemnitz et al., 2010; Szabo et al., 2013). Here we extended this model by co-culturing hepatocytes with

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KCs, which enables to differentiate direct and KC-related effects of compounds on hepatocyte viability and functions.

Most molecular mechanisms of DILI disturb the balance of calcium homeostasis of hepatocytes, which in turn alters several intracellular signaling that lead to cell death (Shaw et al., 2010; Tolosa et al., 2012). Cell injury can result in release of Ca^{2+} from intracellular stores, enhanced Ca^{2+} influx and diminished Ca^{2+} extrusion at the plasma membrane (Díez-Fernández et al., 1996; Kheradpezhouh et al., 2014). Accordingly, increase of intracellular Ca^{2+} level is considered as a common marker of hepatocyte damage, so monitoring the intracellular Ca^{2+} concentration provides a versatile tool for evaluation of hepatotoxic potential of drugs (Amaral et al., 2013; Tolosa et al., 2012). In order to investigate the change of intracellular Ca^{2+} level of hepatocytes in response to drug treatment, we used a recently established transgenic rat strain stably expressing the green fluorescent protein-calmodulin-based genetically encoded Ca^{2+} indicator, GCaMP2. High protein and mRNA GCaMP2 expression were detected in several tissues including the liver (Szebényi et al., 2015a). Suitability of the Ca^{2+} indicator for direct functional in vitro and in vivo investigations was also verified (Szebényi et al., 2015b). Genetically engineered Ca^{2+} indicators can overcome drawbacks of Ca^{2+} sensitive dyes such as relatively low reproducibility and toxicity. Since hepatocytes efficiently extrude traditional Ca^{2+} indicators like Fura-2 or Fluo-4, transgenic models are especially appropriate for liver studies (Apáti et al., 2013; Szebényi et al., 2015b). In this work we applied hepatocytes and KCs isolated from the liver of transgenic rats in order to estimate hepatotoxic potential of PAMAM dendrimers by monitoring Ca^{2+} response following exposure.

Increasing number of studies report extensive biomedical applications of polyamidoamine (PAMAM) dendrimers in drug or gene delivery and imaging because of their advantageous physico-chemical properties (Jain et al., 2011; Labieniec-Watala and Watala, 2015; Peng et al., 2012) however, their bio-safety is a critical issue. Toxicity of polycationic dendrimers has been demonstrated (Nyitrai et al., 2013; Sun et al., 2015; Thiagarajan et al., 2013), and it has also been proved that minor alterations in surface chemistry modify the adverse effects (Lin et al., 2015; Nyitrai et al., 2012; Pryor et al., 2014). The fact that many of the nanomaterials are trapped by KCs raises the possibility that some of the initial processes resulting in hepatotoxicity take place within the KCs (Kettiger et al., 2013). Accordingly; nanomaterials may exert an influence directly on hepatocytes and also through KCs (Xue et al., 2014). For that reason, co-culturing hepatocytes with KCs provides a better approach for evaluating hepatotoxic potential of nanomaterials than studies based on hepatocyte mono-cultures (Nel et al., 2013; Nguyen et al., 2015).

In this study, PAMAM dendrimers with amine (G5) and carboxyl (G4.5) groups were used to compare the effect of surface charge on the hepatotoxic potential of dendrimers. In addition, the amino groups of the positively charged G5 were neutralized with polyethylene-glycol (G5 Peg) coating. Hepatocytes were maintained in mono and co-cultures with KCs at physiological (H/KC 6:1) and pathological (H/KC 2:1) cell ratio, and were exposed to cationic (G5), anionic (G4.5) and neutral (G5 Peg) PAMAM dendrimers at a sub-toxic concentration. Cytotoxicity assays and Ca^{2+} imaging were used to study the homeostasis of hepatocytes, while functional changes were assayed by CYP2B1/2 inducibility, bilirubin and taurocholate transport measurements.

2. Materials and methods

2.1. Materials

Dendrimers ($G5\text{-}NH_2$ and G4.5-COONa) were purchased from Dendritech Inc. (Dendritech.com, USA) and were purified as described previously (Nyitrai et al., 2012). PAMAM – mPEG1000 conjugate (G5 Peg) was synthesized by reacting aqueous solution of PAMAM G5 dendrimer (31.5 μl, 43.8 nmol, 4.05 *w*/w %) (Sigma-Aldrich, Hungary) with N-hydroxysuccinimide functionalized methoxy polyethylene glycol (mPEG-NHS, MW 1000, Nanoncs Inc., New York, NY, USA) (200 eq, 8.76 mg, 8.76 μmol) dissolved in 0.1 M $NaHCO_3$ buffer (0.5 ml, pH 8.5) at room temperature for 2 h. The unreacted PEG derivative was removed from the solution by ultrafiltration (3000 MWCO) in Amicon Ultracel – 3 K centrifugal filter unit. Matrigel was from BD Biosciences (Bedford, MA, USA). Percoll, bilirubin, bovine serum albumin, dimethyl sulfoxide (DMSO), type IV collagenase, all cell culture media and reagents were purchased from Sigma-Aldrich (Hungary). [^{3}H] Taurocholic acid (10 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources unless stated otherwise in the text.

2.2. Isolation of primary rat hepatocytes and Kupffer cells

Primary rat hepatocytes and KCs were isolated from male Wistar rats (200–250 g, Charles River, Hungary) or from male transgenic rats stably expressing the GCaMP2 (Toxicoop, Hungary) by a two-step collagenase perfusion technique (Seglen, 1976). The protocol was approved by the Institutional Animal Care and Use Committee (Permit Number: 22.1/2728/3/2011). Animals were housed in polycarbonate cages under a 12 h light–dark cycle with food and water ad libitum. All surgeries were performed under diethyl ether anesthesia, and all efforts were made to minimize suffering. For separation of hepatocytes from the non-parenchymal cells, the cell suspension obtained after collagenase perfusion was centrifuged at 100 ×g, 2 min, 4 °C. The supernatant enriched in non-parenchymal cells was used for the KC isolation. The cell pellet containing the hepatocytes was subjected to a 45% Percoll (Sigma–Aldrich) density gradient centrifugation to remove non-viable cells. Cell viability (>90%) was determined by the trypan blue exclusion. Hepatocytes were plated on collagen-coated 24-, or 6-well plates (Greiner Bio-One, Hungary) at a density of 2.0×10^5 cell/cm^2 in Williams' Medium E supplemented with 10% FCS and 100 nM insulin. KCs were isolated as described previously (Gopalakrishnan and Harris, 2011) with slight modifications. In brief, the supernatant from the initial centrifugation steps was further centrifuged at 100 ×g, 2 min, 4 °C, and the resulting supernatant at 350 ×g, 10 min, 4 °C to sediment the non-parenchymal fraction. The cells, suspended in Williams' Medium E, were layered on a density cushion of 25%/50% Percoll gradient and centrifuged at 900 ×g, 20 min, 4 °C. The cells floating at the boundary of the two Percoll layers were collected, washed with Williams' Medium E and seeded on collagen-coated 24-, or 6-well plates at a density of 1.0×10^5 KC/cm^2 (for hepatocyte-KC co-cultures of 2:1 ratio), or 0.33×10^5 KC/cm^2 (for hepatocyte-KC co-cultures of 6:1 ratio). 20 min after seeding non adherent cells were removed by washing the plates with phosphate buffered saline so KCs were separated from the other non-parenchymal cell types by their different adherence ability on cell culture plates. The attached cells were identified by immunostaining with CD163 (AbD Serotec, MCA342A647), a macrophage-specific antigen (data not shown). For co-cultures, hepatocytes were seeded on top of the KC layer at a density of 2.0×10^5 cell/cm^2. 24 h after seeding, cultures were overlaid with Matrigel to achieve sandwich configuration. The cells were cultured in Williams' Medium E supplemented with 10% FCS and 100 nM insulin.

2.3. Cell viability experiments

The MTT assay was used to establish the cytotoxicity of PAMAM dendrimer derivatives. 24 h after seeding, hepatocytes were treated with various concentrations (1, 2, 4, 10 μM) of G5, G4.5 dendrimers for 1, 3, 24 and 72 h. Cultures subjected to the vehicle (0.1% DMSO) were used as controls. Next, MTT (1 mg/ml) was added to each well and incubated at 37 °C for 2 h. The supernatant was discarded and the formazan precipitates were dissolved in DMSO. After dissolution, absorbance was measured at 540 nm. Viability data are expressed as mean ± SD as a

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