Letter

Dexamethasone-induced hepatomegaly and steatosis in larval zebrafish

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ABSTRACT — Fish hepatobiliary syndrome, characterized by hepatomegaly and fatty liver, has been frequently reported in many cultured fish species and has caused a dramatic economic loss in China. Glucocorticoids are thought to be important non-nutritional factors for hepatomegaly and fatty liver development. In the present study, a dexamethasone-induced zebrafish model of fatty liver and hepatomegaly was established, and the role of glucocorticoid receptor (GR) in the development of hepatomegaly and fatty liver was investigated using developing zebrafish. Exposure of larval zebrafish at 5 days post fertilization (dpf) to dexamethasone for 24 hr caused significant increases of liver size and number of fish with hepatic steatosis at 6 dpf. The increase of liver size caused by dexamethasone was significantly reversed by treatment with RU486, a GR antagonist, and by gene knock-down with a morpholino against the GR. The dexamethasone-induced hepatic steatosis was also inhibited by treatment with RU486. Overall, the results highlight larval zebrafish as a useful model for stress-induced liver failure.

Key words: Glucocorticoid, Hepatomegaly, Liver, Steatosis, Zebrafish

INTRODUCTION

Glucocorticoids are the most effective anti-inflammatory agents. They are widely prescribed for treatment of inflammation, allergies and autoimmune disorders. Due to increasing needs in medical prescriptions and incomplete removal during wastewater treatment, increasing presence of glucocorticoids in aquatic environments has been reported in China, Europe and USA (Chen *et al.*, 2016). Hence, glucocorticoid contamination has become a major environmental issue.

In mammals, it has been reported that chronically elevated glucocorticoid levels are intrinsically tied to fatty liver development (Vegiopoulos and Herzig, 2007). Studies have shown that glucocorticoids regulate lipid metabolism and induce fatty liver and hepatomegaly (Patel *et al.*, 2011). Studies on glucocorticoids in fish have mainly focused on their effects on carbohydrate metabolism and immune function (Boone *et al.*, 2002; Salas-Leiton *et al.*,

2012). Effects of glucocorticoids on fatty liver development and hepatomegaly in fish have not been reported.

A recently emerged disease called fish hepatobiliary syndrome, characterized by fatty liver, hepatomegaly and color change, has been frequently reported in many cultured fish species and has caused a dramatic economic loss in China (Liu *et al.*, 2009). It is generally believed that overfeeding of a nutritionally unbalanced diet is one of the main reasons for the development of fatty liver and hepatomegaly. We hypothesized that glucocorticoid contamination may be another reason for the development of fatty liver and hepatomegaly in fish. A stress-induced increase in the blood cortisol level might also be a reason for the development of these liver diseases because fish are sometimes cultured at a high density.

The zebrafish liver can be confirmed by 5 dpf under a conventional microscopy. The transparency of the larvae enables *in vivo* visual observation of internal organs including the liver under a microscope. Moreover, hepat-

ic steatosis upon exposure to some toxins in larvae can be observed by oil red staining (Howarth *et al.*, 2013). The zebrafish has a single gene of the glucocorticoid receptor that is expressed in the liver and intestine by 5 dpf (Bertland *et al.*, 2007). Therefore, the larval zebrafish is an excellent model organism for studying fatty liver and hepatomegaly and the possible involvement of the glucocorticoid receptor in these toxicological endpoints.

Dexamethasone, an artificially synthesized glucocorticoid, has been used to establish models of fatty liver and hepatomegaly in mammals (Thatcher and Caldwell, 1994). It is more potent than cortisol, making it an excellent model analogue for cortisol. Therefore, dexamethasone was used to study the effects of glucocorticoids on liver size and lipid formation.

MATERIALS AND METHODS

Reagents

Reagents were of special grade and were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise stated.

Zebrafish

The long-fin wild-type strain of zebrafish was used for all experiments, and the zebrafish were maintained as previously described (Nijoukubo *et al.*, 2016). Adult zebrafish were obtained from a local pet shop. Fertilized eggs were collected from multiple pair of adult zebrafish. Embryos were reared as described previously. We used eggs from the same group of adult fish for all experiments.

Chemical exposure and observation of hepatomegaly

Zebrafish larvae (5 dpf) were exposed to either dimethyl sulfoxide (DMSO) or different concentrations of dexamethasone dissolved in DMSO in 3 mL of Zebrafish Ringer solution (38.7 mM NaCl, 1.0 mM KCl, 1.7 mM HEPES–NaOH pH 7.2, 2.4 mM CaCl₂) in 3.5 cm diameter polystyrene Petri dishes (Nijoukubo *et al.*, 2016). Twenty-four hours later, zebrafish were placed dorso-laterally in 3% carboxymethyl cellulose/Zebrafish Ringer solution, and images of 6 dpf larvae were taken under a microscope. Images were imported into Adobe Photoshop 7.0. The region of the liver in the image was manually drawn, and the pixel value of the target region was defined as the liver size.

Gene knock-down with morpholinos

Splice blocking-type morpholino of the zebrafish glu-

cocorticoid receptor (zfGR) (5'-CTGCTTCATGTATTT-TAGGGTTCCG-3') (Mathew *et al.*, 2007) was obtained from Gene Tools (Philomath, OR, USA). Standard control morpholino (Std-MO) recommended by Gene Tools was used as a negative control (Nijoukubo *et al.*, 2016). Both morpholinos were diluted in Ca²⁺-free ZR solution to the concentrations indicated in Table 1. A Narishige IM-300 microinjector (Tokyo, Japan) with a fine glass needle was used to inject 2 nL of morpholino into the yolk of 1- to 4-cell stage embryos. These morphants were exposed to dexamethasone at 5 dpf and used for observations at 6 dpf.

Oil red staining

Zebrafish larvae at 7 dpf were fixed overnight in 4% paraformaldehyde with PBS at 4°C. After fixation, samples were washed twice in PBS, followed by oil red O staining with freshly filtered 0.5% oil red O in 60% 2-propanol for 1-2 hr. Samples were quickly washed a few times in 60% 2-propanol and then finally washed once in PBS (Raldúa *et al.*, 2008).

Statistical analysis

All values are shown as means ± standard error of the mean. Data were analyzed with one-way ANOVA followed by the Tukey-Kramer post hoc test. *P*-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

As shown in Fig. 1, exposure of the 5 dpf larval zebrafish to 12.5 and 25.0 µg/mL dexamethasone for 24 hr caused significant increases of liver size. This effect was attenuated by the GR receptor antagonist mifepristone (RU486); co-exposure of the larval zebrafish to RU486 (1.5 and 3 µg/mL) and the dexamethasone reduced dexamethasone-induced increase of liver size, suggesting that

Table 1. Effect of a glucocorticoid receptor antagonist on dexamethasone-induced hepatic steatosis.

Treatment	(-) %	(+) %	(++) %
Control	50	40	10
D12.5	0	40	60
D12.5+ RU 0.75	10	30	60
D12.5+ RU 1.5	50	50	0
D12.5+ RU 3.0	60	40	0

Twenty fish were used in each group. The degree was assessed on the basis of degree of redness in the liver, as indicated in Fig. 3. D12.5: 12.5 µg/mL dexamethasone, RU 0.75, RU 1.5, RU 3.0: RU486 (0.75-3.0 µg/mL).

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Fig. 1. Effect of RU486 on dexamethasone-induced hepatomegaly. Larval zebrafish at 5 dpf were exposed to dexamethasone (12.5 and 25.0 $\mu\text{g/mL}$) or co-exposed to dexamethasone (12.5 and 25.0 $\mu\text{g/mL}$) and RU486 (1.5 and 3.0 $\mu\text{g/mL}$) for 24 hr. D12.5, 12.5 $\mu\text{g/mL}$ dexamethasone; D25, 25 $\mu\text{g/mL}$ dexamethasone; RU1.5, 1.5 $\mu\text{g/mL}$ RU486, RU3, 3 $\mu\text{g/mL}$ RU486. $n = 10$. Values with different letters are significantly different ($p < 0.05$).

Fig. 2. Effect of GR-MO on dexamethasone-induced hepatomegaly. D25, 25.0 $\mu\text{g/mL}$ dexamethasone; Std-MO, standard morpholino; zfGR-MO, zebrafish GR morpholino. $n = 10$. Values with different letters are significantly different ($p < 0.05$).

Fig. 3. Degree of steatosis in 6 dpf zebrafish larvae. A, negative (-); B, mild (+); C, severe (++). Arrowheads indicate liver and asterisks indicate the swim bladder that was stained with oil red nonspecifically. Scale bar, 500 μm .

dexamethasone-induced hepatomegaly was mediated by GR.

To further confirm the involvement of zfGR in dexamethasone-induced hepatomegaly, gene knock-down with morpholino antisense oligonucleotides against zfGR was conducted. As shown in Fig. 2, dexamethasone at 25.0 $\mu\text{g/mL}$ caused a significant increase of liver size compared to the control. Exposure of larval zebrafish developed from standard-morpholino-injected embryos to 25.0 $\mu\text{g/mL}$ dexamethasone also caused a significant increase of liver size compared to the control. However, larval zebrafish developed from zfGR-MO-injected embryos showed no significant difference in liver size after exposure to 25.0 $\mu\text{g/mL}$ dexamethasone. These

results provide further evidence that dexamethasone-induced hepatomegaly is mediated by GR.

It has been proposed that corticosteroid-induced hepatomegaly is caused by excessive fat accumulation in the liver (Micuda *et al.*, 2007). Oil red staining was performed to detect fat accumulation in larval zebrafish after 24-hr exposure to dexamethasone. The degree of steatosis of larval zebrafish after oil red staining was graded as negative (-), mild (+) and severe (++) based on the shades of red in the liver (Fig. 3). As shown in Table 1, 50% of the larvae had negative steatosis in the control group. Treatment with 12.5 $\mu\text{g/mL}$ dexamethasone decreased the proportion of larvae with negative steatosis from 50% to 0%. Instead, the proportions of larvae with mild steato-

sis and severe steatosis were increased to 40% and 60%, respectively. Co-exposure of larvae to RU486 and dexamethasone caused dose-response decreases in the percentages of larva with mild and severe steatosis, suggesting that dexamethasone-induced hepatic steatosis was also mediated by zfGR.

In the present study, we demonstrated for the first time that dexamethasone induced hepatomegaly and fatty liver in fish and also larval fish, primarily via the zfGR. Both concentrations of RU486 inhibited hepatomegaly induced by dexamethasone (25.0 µg/mL) to 14.5-15.0%. Knock-down by zfGR-MO also reduced 25.0 µg/mL dexamethasone-induced hepatomegaly to 39.1%. In fish, cortisol is the major corticosteroid that is released in response to stress. The plasma concentrations of teleost cortisol rise dramatically during stress (Ellis *et al.*, 2012). In intensive aquaculture, fish are subject to constant stress such as stress from handling, transport, and high stocking density and stress caused by toxicants, which will elevate the plasma cortisol concentration. A stressor-induced elevation of cortisol might also contribute to the development of fatty liver and hepatomegaly.

The GR is believed to be maintained as an inactive cytoplasmic complex with cofactors, such as heat shock proteins and immunophilins. Following ligand binding that subsequently dissociate the cofactors, the GR translocates to the nucleus, where it binds glucocorticoid response elements (GREs), as a dimer, to promote the transcription of responsive genes (Chivers *et al.*, 2004). Using the responsiveness of gene expression and morphological changes to GR agonists, use of larval zebrafish as an *in vivo* bioassay system in monitoring environmental glucocorticoid contaminants has been suggested (Chen *et al.*, 2016, 2017). Our studies raise the possibility that larval zebrafish could be used as a convenient model system to study the mechanisms of the development of hepatomegaly and steatosis caused by a glucocorticoid as a representative non-alcoholic fatty liver disease in humans. Thus, further studies are needed to determine whether these changes may lead to liver failure in zebrafish during development. Detailed information on the mechanism of glucocorticoid-induced liver disease is required for comparison with mammalian studies (Vegiopoulos and Herzig, 2007; Patel *et al.*, 2011).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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