Rapid screening of glomerular slit diaphragm integrity in larval zebrafish

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1Renal Division, Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts; 2Department of Pathology and 3Division of Nephrology, Department of Medicine, Hannover Medical School, Hannover, Germany; and 4Mount Desert Island Biological Laboratories, Salisbury Cove, Maine

Submitted 4 January 2007; accepted in final form 14 August 2007

Hentschel DM, Mengel M, Boehme L, Liebsch F, Albertin C, Bonventre JV, Haller H, Schiffer M. Rapid screening of glomerular slit diaphragm integrity in larval zebrafish. Am J Physiol Renal Physiol 293: F1746–F1750, 2007.—Gene array-type experiments have identified large numbers of genes thought to be important for the integrity of the glomerular slit diaphragm. Confirmation of individual proteins has been limited by the expenses and time involved in generating transgenic or knockout mice for each candidate. We present a functional screening assay based on the clearance of a 70-kDa fluorescent dextran in another vertebrate system that is rapid and low in cost. In the pronephric glomerulus of larval zebrafish, we have demonstrated quantifiable loss of slit diaphragm integrity in a zebrafish model of puromycin aminonucleoside (PA) toxicity. In addition, after knockdown of CD2-associated protein (CD2AP) and podocin, two well-characterized genetic contributors to podocyte differentiation in mammals, we observed glomerular loss of serum macromolecules similar to that seen in mammalian kidneys with inborn mutations in these genes. Increased filtration of 70-kDa FITC-labeled dextran correlates with effacement of podocyte foot processes in ultrastructural analysis. These findings document the value of the zebrafish model in genomics and pharmacological screening applications.

podocyte; nephrotic syndrome; puromycin aminonucleoside; CD2-associated protein; podocin

THE GLOMERULAR SLIT DIAPHRAGM PLAYS A CRUCIAL ROLE IN PREVENTING LOSS OF CIRCULATING MACROMOLECULES INTO THE URINE (13). Primary genetic renal defects as well as systemic diseases can lead to a disruption of podocyte biology with subsequent proteinuria. Examples include mutations in the nephrin gene and diabetes mellitus, to name just a few. A multitude of signaling pathways converge at the structural proteins of the slit diaphragm, and we are just beginning to understand this complexity (4). The use of transgenic and knockout mice has provided great insights into diseases of the glomerulus; however, generating genetically modified mice is a time- and resource-consuming process, and the final outcome often does not reflect the expected phenotype. Whole transcriptome analysis has markedly increased the number of genes thought to play an important role in podocyte biology (12). Given the large number of candidate genes, rapid screening tools are needed to study loss- or gain-of-function effects in glomerular podocytes in vivo.

The zebrafish pronephros is a single glomerulus that is drained by a renal tubule on each side. The pronephric glomerulus is structurally very similar to its human counterpart and is fully developed 3.5 days postfertilization (dpf) (1, 2). Zebrafish are translucent during early development, which makes them uniquely suitable for in vivo studies using fluorescent markers. In addition, larval zebrafish can be injected with morpholinos (modified RNA molecules) to knockdown protein expression, or with mRNA and cDNA constructs to express proteins of interest. Finally, a single female can produce several hundred eggs per week, and larvae are small enough to be kept in 96-well plates, for instance, to study the effect of exposure to pharmacological agents (6, 7). For these reasons, we developed a screening assay of glomerular disease in zebrafish that would allow the rapid evaluation of candidate genes.

MATERIALS AND METHODS

Zebrafish stocks and injections. Zebrafish (AB) were grown and mated at 28.5°C, and embryos were kept and handled in standard E3 solution as previously described (14). Morpholinos were injected in fertilized eggs in the one- to four-cell stage using a Nanoject II injection device (Drummond Scientific, Broomall, PA). The following morpholinos were designed and ordered from GeneTools (Philomath, OR): control, 5′-CCTCTTACCTCAGTTACAATTTATA-3′; CD2-associated protein (CD2AP), 5′-CATACTCCACACACACCTCAA-CCAT-3′; and podocin, 5′-TGAGGAGATCTGTTGTAAGGAAAT-3′. Injections were carried out with concentrations ranging from 25 to 250 mM, and injection volumes ranged from 2.3 to 4.6 nl in injection buffer (100 mM KCl, 0.1% phenol red). At 50–55 h postfertilization (hpf), remaining chorions were manually removed. At 72 hpf, FITC-labeled 70-kDa dextran (Molecular Probes, Eugene, OR) was injected into the cardiac venous sinus either alone or with defined volumes of 25 mg/ml puromycin aminonucleoside (PA; Sigma-Aldrich, St. Louis, MO) or 10 mg/ml gentamicin solution (Sigma-Aldrich). For this injection, zebrafish were anesthetized in a 1:20 to 1:100 dilution of 4% Tricaine (MESAB: ethyl-m-amino benzoate methanesulfonate, 1% Na2HPO4, pH 7.0) (Sigma-Aldrich) and positioned on their backs in a 1% agarose injection mold. After the injection, fish were returned to egg water, where they quickly regained motility. The animal protocol developed a screening assay of glomerular disease in zebrafish was approved by the Harvard Medical School Standing Committee On Animals IACUC.

Image analysis. The 70-kDa FITC-labeled dextran-injected zebrafish larvae were transferred into individual wells of a 96-well plate (Fisher, Pittsburgh, PA). Sequential images of live fish were generated using a Zeiss inverted microscope (Axiovert 200) connected to an AxioCam MRm charge-coupled device camera, and images were taken with fixed exposure times and gain using the Axio Vision release 4.5 SP1 software package. The maximum fluorescence inten-

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sities of grayscale images of the cardiac area or the pupil of the fish were measured using NIH’s ImageJ application and reported in relative units of brightness. For heart fluorescence measurements, fish with significant amounts of FITC-dextran trapped in the yolk sac were excluded from the analysis. For retinal blood vessel measurements, no fish was excluded from the analysis.

Histology and microscopy. At 96 and 120 hpf, larval zebrafish were fixed and embedded following an electron microscopy protocol for zebrafish as previously described (8). Thin sections of 0.5 and 1 μm were generated using a Leica RM2165 rotary microtome and stained with 0.5% toluidine blue in a 1% sodium tetraborate solution. Histological images were obtained using a Nikon Eclipse E1000 microscope with a SPOT RT camera.

RESULTS AND DISCUSSION

PA or morpholinos targeting CD2AP or podocin are associated with edema of larval zebrafish. Administration of a single intravenous dose of 50–150 mg/kg PA in rats leads to severe, reversible proteinuria and glomerular ultrastructural changes similar to human minimal change disease (10). In 2.5 dpf, larval zebrafish injection of 50–100 mg/kg PA is associated with edema in only a minority of fish (data not shown). Development of edema is reliably observed after injection of 250–350 mg/kg. Although this calculated dose is higher than in mammalian systems, the delivered dose is likely lower, since some back leak of injectate into the surrounding water was typically observed. For the current study in 80-hpf zebrafish, we chose injection of PA at a dose of 350 mg/kg and noted pericardial edema within 18 hpi (hours postinjection) compared with controls (Fig. 1, a and b), which increased in severity until 48 hpi.

In mice, targeted disruption of CD2AP leads to a severe nephrotic syndrome with apoptosis of podocytes and glomerulosclerosis beginning at 3 wk of age (11). After injection of a morpholino directed against the ATG initiation site of zebrafish CD2AP ortholog, larval zebrafish developed notable pericardial effusion at 48 hpf (Fig. 1c) as well as yolk sac edema and cerebral ventricular distension.

A morpholino specific for the ATG initiation site of the zebrafish ortholog for podocin similarly was associated with pericardial effusion and yolk sac edema at 96 hpf (Fig. 1d). Our observations support phenocopy findings recently described for a morpholino directed against an exon splice donor site of zebrafish podocin (5). Injection of 420 fmol of the CD2AP or podocin ATG-morpholino resulted in obvious edema in >75% of larvae compared with <1% in control morpholino-injected fish. All CD2AP- and podocin morpholino-injected fish died at 5–7 days of age, whereas control fish that were injected with a scrambled sequence morpholino in the same concentration appeared normal.

Effacement of podocyte foot processes after injection of PA or morpholinos directed against CD2AP. After injection of PA on day 3 after fertilization, there was marked effacement of foot processes 48 h later (Fig. 1g, inset and details 1–3) compared with the ‘beans-on-a-string’ appearance of wild-type zebrafish podocytes (Fig. 1f, details 1 and 2). Four of 17 podocytes evaluated in PA-injected fish were completely effaced (Fig. 1g), 9 had moderate to severe effacement, and 2 were mildly affected. Two podocytes could not be categorized, because their contact with the vascular loop was not visible. These findings are similar to changes seen in mammalian models of PA toxicity. We did not observe changes in tubular morphology previously reported after PA injection (9). However, in proximal pronephric tubule cells we noted dark vacuoles, suggestive of reabsorption granules, compared with wild-type tubule cells (Fig. 2). Electron microscopy of 100-hpf CD2AP morpholino-injected fish revealed moderate to severe effacement in 12 of 14 podocytes (Fig. 1h, details 1–3) and mild or complete effacement in one podocyte each compared with wild-type zebrafish (Fig. 1f). In contrast, the tubular brush border of CD2AP-injected fish was completely preserved, as in controls (Fig. 2), and the tubular epithelium exhibited resorption vesicles typical for functionally intact and active epithelium. These observations document that the injection of a morpholino directed against the ATG initiation site of zebrafish CD2AP results in a podocyte phenotype similar to early changes seen in CD2AP knockout mice (11).

Quantification of glomerular slit diaphragm integrity in vivo. In the developing zebrafish, larval size selectivity of the glomerular filter is not acquired until after the third day of development, and previous attempts to measure glomerular filtration at 72 hpf with a 70-kDa dextran did not succeed (5). We reasoned that any assay to quantify glomerular filtration would have to extend to days 4 and 5 of larval development, when mature slit diaphragms are present. Therefore, we injected 70-kDa FITC-labeled dextran into the cardiac venous sinus at 80 hpf. After injection, individual fish were placed in 96-well plates and images were taken of their hearts and eyes 1, 18, and 48 hpi. The maximum fluorescence intensity was measured with NIH’s ImageJ software, and changes in intensity relative to the 1-hpi (heart) or 18-hpi (eye) measurements were calculated. Figure 3A demonstrates the decrease of fluorescent signal over the 48 h following the injection of PA and FITC-dextran.

Previous experience with clearance studies in a larval zebrafish model of acute kidney injury (3) led us first to determine clearance based on intensity measures obtained at the heart. In this analysis of heart images, we identified CD2AP or podocin morpholino-injected fish that cleared the fluorescent dextran faster than any scrambled control morpholino-injected fish (Fig. 3B, a and b). Coinjection of FITC-dextran with PA at 72 hpf also was associated with increased clearance of the fluorescent marker 18 and 48 h later (Fig. 3Bc). The results of the cardiac imaging analysis were more uniform than those after morpholino injection (Fig. 3B).

We observed that fluorescent dextran trapped in the yolk at the site of injection close to the heart made some intensity measurements unreliable. To improve the quantitative analysis, we chose the retinal blood vessels as a site distant from the heart for further analysis and used the intensity at 18 hpi as the baseline for 70-kDa dextran taken up into the circulation (Fig. 3C). Strikingly, control morpholino-injected fish experienced an increase in intensity from 18 to 48 hpi (Fig. 3D), which is likely related to continued uptake of 70-kDa dextran from the site of injection without (or with very little) renal clearance. In contrast, in all CD2AP or podocin morpholino-injected fish, fluorescence intensity measurements over the eye were reduced (CD2AP, \( P = 3.7 \times 10^{-13} \); podocin, \( P = 1.97 \times 10^{-12} \), as was the case after injection of PA (\( P = 1.76 \times 10^{-6} \)) (Fig. 3D).

It could be argued that a decrease in fluorescence intensity in fish with edema may be a consequence of an increase in volume of distribution rather than increased glomerular filtration and renal clearance. To address this possibility, we coinjected FITC-dextran with gentamicin at 80 hpi in a modific-
Fig. 1. Chemical injury with puromycin or knockdown of CD2-associated protein (CD2AP) is associated with pericardial edema formation and effacement of glomerular foot processes. a: Control. b–d: Development of edema at 96 h postfertilization (hpf) after injection of puromycin amino-nucleoside (PAN; b), CD2AP morpholino (c), or podocin morpholino (d). Arrowhead indicates pericardial edema. e: Quantitative assessment of podocyte damage (wild type, n = 18; PAN, n = 17; CD2AP, n = 14). f: Electron micrograph of normal glomerular vascular loop morphology and foot process development at 96 hpf in wild-type larvae. Details 1 and 2 show “beads-on-string” pattern. g: Electron micrograph of 96-hpf zebrafish after injection of 250 mg/kg PAN at 55 hpf. Detail 1 represents mild effacement; detail 2, moderate to severe; and detail 3, mild at left and moderate to severe at right. Inset shows a podocyte with complete effacement. h: Electron micrograph of 100-hpf CD2AP morpholino-injected (CD2AP) zebrafish. Effacement of podocyte foot processes in details 1–3 is moderate to severe. Asterisks indicate glomerular basement membrane; E, vascular endothelial cell; P, podocyte. Scale bars, 500 nm.
tion of the tubular injury model recently reported by our group (3). We found that clearance of fluorescence from the circulation was similar to that of controls injected with FITC-dextran alone ($P = 0.226$) (Fig. 3Bd), despite development of significant edema in gentamicin-injected fish. These data indicate that after injection of PA, or injection of CD2AP or podocin morpholinos, clearance of 70-kDa dextran is due to increased glomerular filtration and not to dilution in a larger volume of distribution.

Glomerular filtration is dependent not only on the size of the filter but also on renal blood flow. Decreased cardiac output could lead to lower renal blood flow and glomerular filtration. Heart rates in podocin and CD2AP morphants at 72 hpf were slightly lower (mean: 120 and 155 beats/min, respectively) than in control morpholino-injected fish (mean: 168 beats/min), and heart rates of puromycin- and gentamicin-injected fish at 96 hpf both had a mean of 144 beats/min for the group. These findings indicate that changes in heart rate are unlikely to be responsible for the higher filtration of 70-kDa dextran that was observed.

In the current study we have demonstrated that chemical glomerular injury by injection of PA leads to effacement of foot processes and edema within 24 h of application. Similarly, a morpholino targeting the ATG initiation site of zebrafish CD2AP or podocin leads to effacement of foot processes. We relied on the clearance of a 70-kDa FITC-labeled dextran to determine the functional integrity of the slit diaphragm. Fluorescence intensity measurements in the zebrafish pupil were superior to measurements of the heart: they were a better reflection of the amount of fluorescent marker delivered into the vascular system, were not confounded by marker remaining at the injection site, and separation of controls and treated

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**Fig. 3.** Clearance of 70-kDa dextran is increased after chemical injury with puromycin or knockdown of CD2AP or podocin. Larval zebrafish were injected with 70-kDa FITC-labeled dextran at 80 hpf, and fluorescence intensity of individual fish was measured at baseline and 18 and 48 h later. A: representative image series of a fish coinjected with FITC-labeled 70-kDa dextran and puromycin at 80 hpf. B: residual fluorescence intensity measured over the heart in relation to the intensity at 1 h. CD2AP morpholino-injected (a; shaded lines) and podocin morpholino-injected larvae (b; shaded lines) are compared with control morpholino-injected fish (average, solid line; ±SD, dashed lines). Larvae coinjected with puromycin (c; shaded lines) or gentamicin (d; shaded lines) and 70-kDa dextran are compared with larvae injected only with 70-kDa dextran alone (average, solid line; ±SD, dashed lines). C: fluorescence intensity in retinal vessels in zebrafish embryos 18 and 48 h following injection of 70-kDa FITC-labeled dextran. Increase or reduction in fluorescence intensity over the pupil was calculated in individual fish relative to the 18-hpi time point. Representative fluorescence images of individual eyes are given for each treatment group. D: bar graphs summarizing the changes for each experimental group. Values are means ± SE. Control-MO, control morpholino injected, $n = 20$; CD2AP-MO, CD2AP morpholino injected, $n = 62$; podocin-MO, podocin morpholino injected, $n = 53$; puromycin, puromycin and dextran coinjected, $n = 21$; gentamicin, gentamicin and dextran coinjected, $n = 29$. *$P < 0.0001$. 

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**Fig. 2.** Tubular morphology is preserved after chemical injury with puromycin or knockdown of CD2AP. Proximal pronephric tubule segments of 96-hpf (WT and PA) and 100-hpf zebrafish (CD2AP), corresponding to glomeruli imaged in Fig. 1. Arrowheads indicate intracellular vacuoles. WT, wild type; PA, puromycin aminonucleoside-injected fish; CD2AP, CD2AP morpholino-injected fish.
groups benefited from ongoing uptake of marker at the site of injection.

In conclusion, edema formation in larval zebrafish after exposure to pharmacological agents or alteration of protein expression indicates possible glomerular pathology. Loss of slit diaphragm integrity can be quantified after gene knockdown and chemical injury. In contrast to tissue culture models of glomerular filtration, the three-dimensional relationship of capillary network, glomerular basal membrane, and surrounding podocytes is maintained in the zebrafish larva. The glomerular effect of changes in protein expression levels and consequences of applied pharmacological agents can now be studied in vivo.

GRANTS

This work was supported by American Heart Association Fellow-to-Faculty Transition Award AHA0575042N (to D. M. Hentschel), National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-39773, DK-72381, and DK-74099 (to J. V. Bonventre), Deutsche Forschungsgemeinschaft Emmy Noether Fellowship Schi 587/2 (to M. Schiffer), Mount Desert Island Biological Laboratory New Investigator Awards (to D. M. Hentschel and M. Schiffer), and a Thyssen-Foundation Travel Grant (to M. Schiffer).

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