Acute renal failure in zebrafish: a novel system to study a complex disease

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Acute renal failure in zebrafish: a novel system to study a complex disease. Am J Physiol Renal Physiol 288: F923–F929, 2005. First published December 29, 2004; doi:10.1152/ajprenal.00386.2004.—Acute renal failure (ARF) is characterized by a very high mortality essentially unchanged over the past 40 years. Simple vertebrate models are needed to improve our understanding of ARF and facilitate the development of novel therapies for this clinical syndrome. Here, we demonstrate that gentamicin, a commonly used nephrotoxic antibiotic, causes larval zebrafish to develop ARF characterized by histological and functional changes that mirror aminoglycoside toxicity in higher vertebrates and inability of zebrafish to maintain fluid homeostasis. We developed a novel method to quantitate renal function in larval zebrafish and demonstrate a decline in glomerular filtration rate after gentamicin exposure. The antineoplastic drug cisplatin, whose use in humans is limited by kidney toxicity, also causes typical histological changes and a decline in renal function in larval zebrafish. A specific inhibitor of Omi/HtrA2, a serine protease implicated in cisplatin-induced apoptosis, prevented renal failure and increased survival. This protective effect was confirmed in a mouse model of cisplatin-induced nephrotoxicity. Therefore, zebrafish provides a unique model system, amenable to genetic manipulation and drug screening, to explore the pathophysiology of ARF and establish novel therapies with potential use in mammals.

ACUTE RENAL FAILURE (ARF) in the intensive care unit setting is associated with mortality rates of 50 to 70%, which have not changed for several decades (10, 32), and proven therapies remain elusive. Current mammalian models of ARF have been challenged as to their applicability to the human syndrome and are often difficult to interpret (4, 14). The kidneys in the commonly used mouse or rat are inaccessible, and renal tubules and vessels cannot be visualized, with the exception of a very small subpopulation near the surface of the kidney (11). Cell culture systems, on the other hand, while more simple, are inadequate because of epithelial cell dedifferentiation and absence of the three-dimensional interaction of renal tubule, blood vessel, and immune cells, which plays a central role in the pathophysiology of ARF in humans (32). Furthermore, isolated cell systems are maintained in an environment containing growth factors and cytokines that do not reflect the local environment of epithelial cells in vivo in ARF. Early larval zebrafish kidneys are notable for their anatomic simplicity, consisting of a fused midline glomerulus with one nephron on each side, adjacent to the cardinal veins, while maintaining biological complexity inherent to the kidney of higher organisms. Zebrafish are translucent, facilitating microscopic observation along the entire length of the kidney. Microinjection of mRNA, DNA, and morpholinos (modified oligonucleotides with excellent antisense properties) is well tolerated. Morphological and functional changes can be correlated with protein expression after either knockdown (morpholinos) or overexpression (mRNA) (5). Their large numbers of offspring make zebrafish an important tool for drug discovery (21) and allow for the generation of transgenic fish with relative ease after injection of DNA. For these reasons, we considered that a model of ARF in zebrafish would allow us to better understand the pathophysiology of ARF, facilitate the identification of molecular mechanisms of disease, elucidate the role of specific proteins in the development of and recovery from ARF, and enable the screening of potential therapeutic agents.

MATERIALS AND METHODS

Zebrafish stocks and injection. Zebrafish (TübingenAB) were grown and mated at 28.5°C (34), and embryos were kept and handled in standard E3 solution (“egg water”; 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 10−% methylene blue) (27) buffered with 2 mM HEPES (Sigma, St. Louis, MO). In all cases, studies were done according to animal experimental procedures approved by the Institutional Animal Care and Use Committee. At 50–55 h postfertilization remaining chorions were manually removed, and zebrafish were anesthetized in a 1:20 to 1:100 dilution of 4 mg/ml Tricaine (ethyl-m-amionbenzoate methanesulphonate, 1% Na2HPO4, pH 7.0, Sigma) and positioned on their back in a 1% agarose injection mold. Using a Nanoject II injection device (Drummond Scientific, Broomall, PA), defined volumes of a 10 mg/ml gentamicin (Sigma) stock solution were injected into the cardiac venous sinus, and fish were returned to egg water, where they regained motility quickly. Defined volumes of a 1.5 mg/ml cisplatin (Sigma) stock solution were injected using the same technique. Tetramethylrhodamine-labeled 10-kDa dextran (Molecular Probes, Eugene, OR) or fluorescein-labeled inulin (Sigma) was injected at 72 h postfertilization in a similar fashion. In dose-titration experiments, green fluorescent protein-labeled 40-kDa dextran (Molecular Probes), 5 ng per injection, was added to gentamicin or cisplatin to select fish that had truly received a dose of the nephrotoxicant. Where indicated, taurine was added in a 4:1 molar ratio to gentamicin for coinjection experiments. In some experiments, to study...
Fig. 1. Zebrafish develop edema after gentamicin injection. A and B; at 55 h postfertilization (hpf), zebrafish were injected with 4.6 nl gentamicin, concentration 5 mg/ml, and placed in either 300 mosmol/lH2O egg-water solution (A) or in 300 mosmol/lH2O sucrose-egg-water solution (B) and imaged at 96 hpf. Marked pericardial and intracranial edema (white *) are found in fish maintained in 15 mosmol/lH2O solution but not in those in 15 mosmol/lH2O solution. C: the percentage of fish with gross edema observed at 72 and 96 hpf increases as a function of injected gentamicin dose. Isotonic NaCl does not cause edema. All injections of gentamicin and NaCl in these experiments were performed at 52 hpf. The standard deviation is given for the 96-hpf time point (n = 73).

Fig. 2. Gentamicin causes typical changes in mammalian aminoglycoside toxicity in larval zebrafish. A: schematic of tubular anatomy at 96 hpf. The glomerulus is shaded in green, and the pronephric tubules and pronephric ducts are shaded in blue. Planes and location of subsequent sections are indicated. B and C: histology of renal tubule of NaCl-injected zebrafish at 96 hpf as a control. Cross sections of proximal pronephric tubules (PT) at 96 hpf after NaCl injection (plane of section indicated in A) reveal only few lysosomes (black droplets). The brush borders (+) of opposing tubular cells are in close contact. Nuclei have distinct borders and 2 to 3 nucleoli. D: effect of gentamicin on histological appearance of the renal tubule. After gentamicin injection marked lysosomal phospholipidosis is noted in the proximal tubule (black +). The tubular lumen (+) and Bowman’s space (BS) are distended. E: presence of renal tubular cast (white arrow) emanating from the cloaca (CL) at 96 hpf in gentamicin-injected fish. Such casts contain red fluorescent rhodamine dextran, if dextran was injected at 72 hpf. Casts are not seen in control-injected fish. GL, glomerulus; CV, cardinal vein containing numerous nucleated erythrocytes. Scale bars = 50 μm.
UCF-101 or saline using osmotic minipumps (Durect Alzet Osmotic Pumps, Cupertino, CA). One day later, 70 μl of blood were obtained from the retroorbital venous plexus, and plasma creatinine concentration was measured using a Beckman Creatinine Analyzer. Mice were given a single intraperitoneal injection of either vehicle (saline) or cisplatin (20 mg/kg body wt). Plasma creatinine concentration was again determined 2 days after injection of cisplatin.

RESULTS AND DISCUSSION

During the first week of development, zebrafish rely primarily on their kidneys to clear surplus fluid and metabolic waste products from their bodies, as the gills are not yet fully developed (28). Clearance of water remains a main function of the adult fish kidney (15). Glomerular filtration has been observed as early as 48 h postfertilization (9) and appears temporally to coincide with hatching from the chorion. At 50–54 h postfertilization, defined volumes of gentamicin, an aminoglycoside antibiotic nephrotoxic to mammalian organisms, were injected into the cardiac venous sinus. Gross edema developed in a time- and dose-dependent fashion (Fig. 1B), reflecting the loss of the zebrafish’s ability to maintain fluid homeostasis. Injection of 150 mM NaCl, the gentamicin vehicle, at a volume of 9.2 nl did not cause edema. This suggests that water, which diffuses into the embryo along a concentration gradient, cannot be effectively removed after gentamicin injection. Edema after gentamicin injection was prevented by replacing HEPES-buffered E3 with a 300-mosmol/lH2O sucrose-egg-water solution 8 to 12 h after the injection of gentamicin (Fig. 1A). Gentamicin-injected fish that were immediately placed in the 300-mosmol/lH2O solution shrunk and usually died over the course of the ensuing 48 h (n = 20, data not shown), as did NaCl-injected fish placed in the 300-mosmol/lH2O solution (n = 18, data not shown). The observed shrinking could be explained by a greater reflection coefficient for sucrose than for electrolytes across the zebrafish outer surface. The fact that fish do not shrink if placed in sucrose solution after gentamicin injection, at a time when renal function is impaired, suggests, however, that there is little passive water loss to the environment via other, nonrenal, processes. The fish dehydration seen when kidney function is not impaired suggests that healthy larval zebrafish kidneys have obligatory free water clearance and cannot adjust urine concentration or volume adequately to respond to the needs imposed by a 300-mosmol/lH2O sucrose environment.

Histological analysis of gentamicin-injected embryos at 96 h postfertilization demonstrates lysosomal phospholipidosis, flattening of the brush border, accumulation of debris in the tubular lumen, as well as tubular and glomerular distention (Fig. 2). Renal tubular casts can be seen extruded from the cloaca after gentamicin injection but not in NaCl-injected fish (Fig. 2E). There is peritubular accumulation of leukocytes with occasional infiltration into the glomerulus (Fig. 3), a prominent finding in ARF of different etiologies (19). These findings are typical features of ARF in humans in the setting of aminoglycoside antibiotic therapy.
coside therapy (1). Glomerular and tubular distention and the finding of casts indicate the presence of tubular obstruction after gentamicin-induced tubular injury with subsequent inability to adequately filter and excrete fluid.

Glomerular filtration rate (GFR) is a quantitative measure of renal function. Clearance of a substance A is a good estimate of GFR to the extent that this substance is completely filtered in the glomerulus and not significantly secreted or reabsorbed by

Fig. 4. Clearance of dextran and inulin is decreased after gentamicin injection and tubular transport function is disturbed. A and B: at 72 hpf, gentamicin- and control-treated larval zebrafish were injected with 10-kDa rhodamine-labeled dextran, and each fish’s individual fluorescence intensity was measured at baseline and after 1, 5 (or 6), and 24 h. A: visual comparison of rhodamine dextran clearance after gentamicin and NaCl injection in 2 individual fish. B: decline of fluorescence intensity averaged over 16 (control) and 20 (gentamicin) fish. Values are means ± SE. *P < 0.02 gentamicin vs. control. C: normal uptake of dextran into the tubular cells is seen after NaCl injection (middle, white arrow), whereas this uptake is not apparent after gentamicin treatment (left). The higher power image on the right demonstrates vesicular uptake under control conditions, highlighting the tubule. D: zebrafish were injected at 55 hpf with 2.3 nl of 5 mg/ml gentamicin (n = 12), followed by injection of fluorescein-labeled inulin at 72 hpf. Compared with NaCl-injected zebrafish as control (n = 8), clearance is markedly decreased. E: effect of taurine to mitigate gentamicin toxicity. Zebrafish at 55 hpf were coinjected with 4.6 nl of a 4:1 molar ratio of taurine to gentamicin, followed by injection of 10-kDa rhodamine-labeled dextran. Clearance of dextran is markedly improved when taurine, an antioxidant β-amino acid, is cojected with gentamicin (n = 15).

Fig. 5. Cisplatin causes changes in the zebrafish pronephric tubule typical of those seen in mammalian kidneys. After injection of cisplatin (4.6 nl, concentration 1.5 mg/ml) at 48 hpf, zebrafish with mild (B) or severe (C) decreases in inulin clearance can be distinguished by the level of residual fluorescence at 96 hpf, 24 h after the injection of fluorescein-labeled inulin. A: there is essentially no residual fluorescence in NaCl-injected control fish 24 h after inulin administration for comparison. Intensities in A–C have been adjusted identically to render the fish in A visible. A mild decrease (B) in clearance correlates with vacuolization of tubule cells (white ^), partial loss of the brush border (white arrow), and minimal tubular distention (top right). Fish with severe decreases in inulin clearance (C) have near complete loss of the brush border (black arrow), and where it is preserved its height is decreased (bottom). Cells are flattened, and the tubular lumen is distended. *, Tubular lumen; CV, cardinal vein. Scale bars = 10 μm.
the tubule. Clearance can be calculated by measuring the plasma concentration of a substance A before \( [P_1] \) and after \( [P_2] \) a period of time \( t \), assuming that the overall volume of distribution \( V \) is constant and the kidney is the path of excretion (30): 
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C = \frac{V \cdot (\log [P_1] - \log [P_2])}{t}
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Glomerular filtration of 10-kDa dextran has previously been observed in zebrafish at 40–48 h postfertilization (9). In humans, clearance of dextran molecules of this size is rapid, indicating high levels of filtration and minimal reabsorption relative to the filtered load (2). The polysaccharide inulin has been the reference substance used to measure renal function since its introduction into renal physiology by Shannon and Smith (29). Inulin has an advantage over dextran in that it is freely filtered but not taken up or secreted by the tubule. To quantify the change in zebrafish renal function, fish were injected with gentamicin or isotonic NaCl solution at 50–54 h postfertilization, followed at 72 h postfertilization by an injection of 25–50 ng of 10-kDa rhodamine-labeled dextran or fluorescein-labeled inulin into the cardiac venous sinus. Using a fluorescence microscope, images of individual fish were obtained after the dextran injection and 1, 5, and 24 h later. Fluorescence intensity was measured over the heart, which is a predominantly vascular compartment (Fig. 4A). The rate of decline in fluorescence intensity due to filtration and excretion of the dextran by the kidney differed significantly between gentamicin- and vehicle-injected fish (Fig. 4B). In addition, the small amount of proximal tubule cell dextran reuptake, normally present 5 h after dextran injection, was not observed in gentamicin-injected fish (Fig. 4C). The lower rate of fluorescence decline in the gentamicin (4.6 nl, concentration 5 mg/ml) group represents a greater than 75% reduction in renal clearance. This is likely to be an underestimation, as the small component of dextran reuptake by functional proximal tubule cells reduces the clearance in the control group. Clearance of inulin was reduced by 67% after injection of a lower dose of gentamicin (2.3 nl, concentration 5 mg/ml) compared with injection of NaCl (Fig. 4D).

Lysosomal phospholipidosis is a hallmark of early gentamicin toxicity in rodents and humans (3, 23), and it is present in zebrafish 48 h after injection of gentamicin. Gentamicin toxicity increases malondialdehyde content in the mammalian renal cortex (33) as a result of oxidation of polyunsaturated fatty acids. It also inhibits lysosomal phospholipase A and C (16). Taurine is an intracellular β-amino acid, which can act as

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**Fig. 6.** Nuclear condensation and shedding of tubular cells after cisplatin injection. Propidium iodine staining reveals nuclear condensation (thin white arrow) in cisplatin-injected fish (A) but not NaCl-injected control fish (B). *, Tubular lumen. C: tubular cast emanating from the cloaca at 5 dpf in cisplatin-treated fish. The white arrow indicates an embedded tubular cell.

**Fig. 7.** Effect of cisplatin and cisplatin plus Ucf-101 on zebrafish and mouse renal function. A: zebrafish at 55 hpf were injected with 4.6 nl of 1.5 mg/ml cisplatin (\( n = 13 \)) or 150 mM NaCl (\( n = 12 \)), followed by injection of inulin at 72 hpf and imaged over the ensuing 24 h. Values represent means ± SE. *\( P < 0.02 \) control vs. cisplatin. B: after coinjection (\( n = 14 \)) of 4.6 nl cisplatin (concentration 1.5 mg/ml) and 1 mM Ucf-101 or cisplatin alone (\( n = 11 \)) at 55 hpf, fish were followed and classified according to the degree of edema (+ pericardial only, ++ pericardial, cerebral, and yolk-sac edema, +++ panedema with 5–6 volume expansion). C: Ucf-101 (\( n = 6 \)) or vehicle (\( n = 6 \)) was infused using an osmotic minipump (alzet) into BALB/c mice. One day after the initiation of infusion, the mice were injected with 20 mg/kg body wt cisplatin. Plasma creatinine levels were analyzed before and 2 days after the administration of cisplatin. Values represent means ± SE. *\( P < 0.05 \) vs. cisplatin alone.
a reactive oxygen species scavenger in oxidant-induced injury (17). It has been successfully used in a rat model to alleviate the gentamicin-induced rise in creatinine and the severity of the observed phospholipidosis (12). In zebrafish, coinjection of taurine with gentamicin in a 4:1 molar ratio reduces the development of gross edema and prevents the gentamicin-induced reduction in dextran clearance (Fig. 4E).

In larval zebrafish, injection of cisplatin (4.6 nl, concentration 1.5 mg/ml) causes cellular vacuolization, flattening and loss of the brush border, distension of the tubular lumen, and a marked decrease in cell height in the proximal pronephric tubule (Fig. 5). These findings of cisplatin toxicity are very similar to those observed in mammals (8). The response of individual fish to the same injected dose varies (Fig. 5, B vs. C), which is also similar to observations in rodent models of cisplatin toxicity. Apoptosis, a feature of cisplatin toxicity, is an event that, due to its transient nature, is difficult to capture in fixed renal tissues after injury. Fewer than three nuclei with features typical for apoptosis are detected 2 days after cisplatin administration per one square millimeter section of mouse kidney (26). Our analysis of propidium iodine-stained zebrafish nephrons reveals nuclear condensation patterns, typical of apoptotic nuclei (Fig. 6, A and B). Moreover, we found tubular casts with embedded cells emanating from the cloaca, resulting from shedding of tubular cells and cellular disintegration products into the lumen (Fig. 6C).

Omi/HtrA2 is a nuclear-encoded mitochondrial serine protease that is released to the cytoplasm in response to apoptotic stimuli. In the cytoplasm Omi/HtrA2 activates both caspase-dependent as well as caspase-independent apoptosis (7, 31). The proteolytic activity of Omi/HtrA2 is necessary and essential for its normal function, which is inhibited specifically by the Ucf-101 compound (7). The catalytic domain of Omi/HtrA2 is conserved in Drosophila, zebrafish, mouse, and humans (Supplemental Fig. S1 is available online at http://ajprenal.physiology.org/cgi/content/full/00386.2004/DC1). We reported that Omi/HtrA2 protease activity is upregulated in injured mouse kidneys (13). Recently, we found that inhibition of Omi/HtrA2 with Ucf-101 in cultured mouse kidney proximal tubular cells or HK-2 cells decreased cisplatin-induced apoptosis (6). Injection of cisplatin induces a 73% reduction in renal clearance (Fig. 7A). Coadministration of 1 mM Ucf-101 with cisplatin markedly reduced the rate of edema development, decreased the number of fish in which edema was found, and markedly increased survival rates compared with fish injected with cisplatin alone (Fig. 7B). In a mouse model of cisplatin nephrotoxicity, in which a single dose of cisplatin (20 mg/kg) was administered intraperitoneally on day 0 and plasma creatinine was measured on day 2, the rise in serum creatinine, a measure of decline in renal function, was significantly reduced with coadministration of Ucf-101 (1 μl/h from an osmotic pump; Fig. 7C).

We conclude that, in response to gentamicin or cisplatin, larval zebrafish develop renal failure with the typical features observed in higher organisms. The biological response to renal injury due to these agents appears to be evolutionarily conserved from teleosts to humans, from the simple pronephros to the complex human kidney. The cisplatin-induced decline in renal function can be prevented with Ucf-101 in larval zebrafish. This protective effect could be replicated in a mammalian system of cisplatin-induced acute renal injury. Thus Ucf-101 is a potential candidate for the prevention of cisplatin-induced renal injury in a large number of cancer patients receiving a life-saving, but nephrotoxic, therapy.

With zebrafish a wide array of genetic methods and drug screening techniques is now available to elucidate the pathophysiology of ARF due to gentamicin, cisplatin, or other etiologies. In addition, targets for intervention can be identified and potential therapeutic agents screened, which, if effective, will then be tried in mammals, as we exemplified for Ucf-101. We developed a method that permits quantitative monitoring of the clearance function of the kidney in large numbers of organisms, facilitating the study of physiology and pathophysiology of the nephron. Currently, we are able to inject more than 100 fish/h, which represents a significant increase over the number of rodents that can be processed in the same amount of time. Automation of the determination of fluorescence intensity will further increase the throughput capacity. In contrast to other species (20), combining knockdown of several genes by use of morpholinos and overexpression of others by injection of mRNA is feasible in zebrafish, rendering it an important tool for asking complex questions in the study of renal disease. Our model is of particular interest in this regard, as the phenotypic readout occurs during the first 4 days after fertilization, a time period when the aforementioned techniques of genetic manipulation are highly effective in modulating protein expression. Thus the zebrafish provides a valuable and unique model for studying renal injury under conditions where the complexity of the organism is preserved but the accessibility of the kidney is markedly improved compared with currently available models. Use of this model may aid in clearing the stagnant waters of ARF in humans.

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