Short communication

Amikacin-induced Fin Reduction is Mediated by Autophagy

I-Ting Tsai1#, Ying-Hsin Chen2#, Yau-Hung Chen*1, and Yun-Hsin Wang*3

1 Department of Chemistry, Tamkang University, 151, Yingzhuan Road, Danshui Dist., New Taipei City 25137, Taiwan, ROC
2 Department of Emergency Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
3 Division of Basic Research, Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan

Abstract: Despite its medical use, little is known about the mechanisms underlying amikacin-induced embryotoxicity, including fin reduction, in zebrafish. In this study, we examined the expression of well-known autophagy markers mTOR (target of rapamycin), atg10 (autophagy-related gene), atg12 and LC3 (mammalian homolog of Atg8) in amikacin-treated zebrafish embryos. Our results indicated that the mRNA expression level of atg12 in the amikacin-treated group was significantly increased by 1.5-fold (p<0.05) compared with the corresponding mock control group, while the expression levels of atg10 and mTOR were significantly decreased by 0.74-fold (p<0.05) and 0.58-fold (p<0.05), respectively. Western blot analysis revealed that LC3 protein expression was induced by amikacin. Taken together, these data suggest that amikacin-induced fin reduction is mediated by fin cell autophagy. (DOI: 10.1293/tox.26.79; J Toxicol Pathol 2013; 26: 79–82)

Key words: amikacin, autophagy, embryotoxicity, fin reduction, zebrafish

Amikacin is an aminoglycoside antibiotic that is commonly used to treat bacterial infections1–3. Although amikacin has been Food and Drug Administration (FDA)-approved for human use, its use has been associated with potential risks, including hearing loss and liver and renal damage4–6. In addition, amikacin treatment has been shown to lead to ototoxicity, hepatotoxicity, and nephrotoxicity in rodents and to result in fin reduction and neuramost loss in zebrafish7–9. These observations indicate that amikacin-induced toxicity is conserved among many species. In light of this fact, it is important to study the molecular mechanisms underlying amikacin-induced toxicological processes.

In rats, aminoglycoside-induced nephrotoxicity has been shown to be due to apoptosis in the renal proximal tubules, and aminoglycoside-induced ototoxicity has been linked to hair cell apoptosis10. In addition to apoptosis, autophagy has been shown to play an important role in aminoglycoside-induced ototoxicity in mouse models6. Autophagy is an evolutionarily conserved, lysosome-mediated degradation and is involved in the regulation of cell survival, differentiation, and death11,12. More recently, it was discovered that autophagy can be induced by starvation or drug treatment13–16. Furthermore, we have previously shown that zebrafish embryos displayed fin reductions following treatment with 10 ppm amikacin, including dorsal, ventral and pelvic fin shrinkage or absence9. Here, we followed the same exposure protocol (Fig. 1) and used live video to dynamically observe the teratogenic process of fin reduction following amikacin treatment. As shown in Fig. 2, vacuoles appear on the fins of amikacin-treated embryos [10 ppm, from 48 to 74 hours post fertilization (hpf)]. Pictures were taken sequentially from a 12-min live video (Suppl Movie: on-line only), and these pictures showed that the vacuoles appeared randomly in the fin and that they then gathered together to consequently cause fin reduction (Figs. 2A–2F).

What causes fin reduction? The appearance of vacuoles on fin cells seems to be the first sign of amikacin-induced fin reduction. However, vacuolization has been reported to be induced by many factors, including oxidative stress,
Amikacin-induced Fin Reduction is Mediated by Autophagy

Our previous study showed that TUNEL-positive staining did not appear on fin mesenchymal cells. We therefore concluded that apoptosis is not the main cause of fin reduction. Taken together with the observations from the live video analysis (Fig. 2), we assumed that amikacin treatment may activate other cell death pathways and consequently cause fin reduction.

Autophagy consists of several sequential steps, including initiation, autophagosome formation, autophagosome-lysosome fusion and degradation. Experimental evidence accumulated from previous studies has shown that many genes and proteins contribute to each specific step of autophagy. For example, the autophagy-related gene (atg) Atg10 (protein) is known to be essential for the formation of the Atg12-Atg5 conjugate, the formation of which is essential to autophagosome formation. Inhibition of mTOR (mammalian target of rapamycin) is known to be important during the initiation step of autophagy. To test whether amikacin-induced fin reduction is due to autophagy, we first compared the expression levels of atg10, atg12 and mTOR between mock control and amikacin-treated groups (100 embryos each group) using real-time PCR (all primer sequences are listed in Table 1). As shown in Fig. 3, the mRNA levels of atg10, atg12 and mTOR were significantly increased in the amikacin-treated group compared to the mock control group.

Table 1. Primers Used in This Study

| Primer  | Sequence (5’ → 3’)          |
|---------|----------------------------|
| β-actin F | CAGCAAGCAGGAGTAGATGAGT   |
| β-actin R | TTGAATCTCTTTGAGCCATT    |
| ATG10 F | ATGACCGGTAGAGAAAGCCT |
| ATG10 R | AGCCTTCCATCAAGGCCCCTGA |
| ATG12 F | TATGTTAACATGCTGGTTCGCC |
| ATG12 R | CTAGTTTGCCGTICACCTCCGA |
| mTOR F | TTATCGTGCTGCTGTCGAGCT |
| mTOR R | AAGTGGGCCCTTATCGCTGT |

Fig. 2. Fin reductions in zebrafish embryos after amikacin treatment. Images were captured from live videos of zebrafish embryos after treatment with 10 ppm amikacin. (A) Initial position of the vacuole in the fin cells. (B–E) Position of the vacuole 3–12 min later. Arrow indicates the position of the vacuole. Scale bar: 0.2 mm

Fig. 3. Relative quantification of mRNA expression using the comparative C_T method. One hundred embryos derived from the mock control (0 ppm) and amikacin-treated (10 ppm) groups were collected, and their total RNA were isolated using a standard procedure. Approximately 25 μg of total RNA from each group was used for cDNA synthesis; 1% of cDNA was used for each quantitative PCR reaction. Quantitative PCR was performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers (Table 1). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicate, and the fold-changes in expression were derived using the comparative CT method. β-actin was used as the endogenous control for relative quantification. The amikacin-treated group was significantly different from the corresponding mock group (C_T: cycles of qPCR; relative fold to control group = 2^ΔΔCT).
levels of \textit{atg12} in the amikacin-treated group were significantly increased by 1.5-fold (p<0.05) compared with the corresponding mock control group. In contrast, the mRNA levels of \textit{atg10} and mTOR in the amikacin-treated group were significantly decreased by 0.74-fold (p<0.05) and 0.58-fold (p<0.05), respectively, compared with the corresponding mock control group. These observations revealed that amikacin affected fin morphology by mediating the gene expression of mTOR, \textit{atg10} and \textit{atg12}.

The LC3 protein is the mammalian homolog of Atg8. The cytosolic form of LC3-I has been shown to be cleaved to form the membrane form of LC3-II during the induction of autophagy. These two proteins have been used as markers to study autophagy. Thus, we monitored the expression of LC3-I and LC3-II in the fin reduction zebrafish embryos by performing Western blotting and immunofluorescence analyses. Western blotting and antibody labeling were performed as previously described using the following antibodies: LC3 (autophagosomal marker; 1:200, Novus, Littleton, CO, USA) and GAPDH (loading control; 1: 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The results indicated that the LC3-positive cells co-localized with the vacuoles in the amikacin-treated embryos (Figs. 4A and 4A’ vs 4B and 4B’). Western blot analysis revealed that the expression levels of LC3-II remained unchanged, but the expression levels of LC3-I were upregulated (Fig. 4C).

In this study, we showed that decreases in the expression levels of mTOR and the accumulation of LC3-I correspond with the reported gene expression profile of the initiation step of autophagy. These observations revealed that amikacin affected fin morphology by mediating the expression of mTOR and LC3-I, and consequently lead to the initiation of autophagy. In addition, our data showed that the expression of an autophagosome marker, \textit{atg10}, is decreased following amikacin treatment, which is inconsistent with well-known gene expression profiles during autophagosome formation. This inconsistency might be due to (1) amikacin being mainly involved in the initiation of autophagy, but not autophagosome formation, or (2) amikacin possibly regulating \textit{atg10} expression at the protein level. Moreover, a transgenic zebrafish line (LC3:GFP) has been created that should provide a convenient tool for assaying amikacin-induced autophagic activity during embryogenesis \textit{in vivo}.

In conclusion, amikacin-induced fin malformations can easily be dynamically observed \textit{in vivo} using the present model, which may provide novel insights into the adverse effects of amikacin.

Acknowledgments: This project was supported by the National Science Council (NSC 100-2313-B-032-001), and Tri-Service General Hospital (TSGH-C97-3-S03, DV101-04).

References

1. McCracken Jr GH. Aminoglycoside toxicity in infants and children. Am J Med. 80: 172–178. 1986. [Medline] [CrossRef]
2. Duff P. Antibiotic selection in obstetrics: making cost-effective choices. Clin Obstet Gynecol. 45: 59–72. 2002. [Medline] [CrossRef]
3. Ji B, Lefrançois S, Robert J, Chauffour A, Truffot C, and Jarlier V. \textit{In vitro} and \textit{in vivo} activities of rifampin, streptomycin, amikacin, moxifloxacin, R207910, linezolid, and PA-824 against \textit{Mycobacterium ulcerans}. Antimicrob Agents Chemother. 50: 1921–1926. 2006. [Medline] [CrossRef]
4. Forge A, and Schacht J. Aminoglycoside antibiotics. Audiol Neurootol. 5: 3–22. 2000. [Medline] [CrossRef]
5. Martínez-Salgado C, López-Hernández FJ, and López-Novoa JM. Glomerular nephrotoxicity of aminoglycosides. Toxicol Appl Pharmacol. 223: 86–98. 2007. [Medline] [CrossRef]
6. Taylor RR, Nevill G, and Forge A. Rapid hair cell loss: a mouse model for cochlear lesions. J Assoc Res Otolaryngol. 9: 44–64. 2008. [Medline] [CrossRef]
7. Aran JM, Chappert C, Dulon D, Erre JP, and Aurousseau C. Uptake of amikacin by hair cells of the guinea pig cochlea.
and vestibule and ototoxicity: Comparison with gentamicin. Hearing Res. 82: 179–183. 1995. [Medline] [CrossRef]
8. Begg EJ, and Barclay ML. Aminoglycosides-50 years on. Br J Clin Pharmacol. 39: 597–603. 1995. [Medline]
9. Chen YH, Tsai IT, Wen CC, Wang YH, Cheng CC, Hu SC, and Chen YH. Fin reduction is a novel and unexpected teratogenic effect of amikacin-treated zebrafish embryos. Toxicol Mech Methods. 22: 151–158. 2012. [Medline] [CrossRef]
10. El Mouedden M, Laurent G, Mingeot-Leclercq MP, Taper HS, Cumps J, and Tulkens PM. Apoptosis in renal proximal tubules of rats treated with low doses of aminoglycosides. Antimicrob. Agents Chemother. 44: 665–675. 2000. [Medline] [CrossRef]
11. Mizushima N, and Levine B. Autophagy in mammalian development and differentiation. Nature Cell Biol. 12: 823–830. 2010. [Medline] [CrossRef]
12. Rabinowitz JD, and White E. Autophagy and metabolism. Science. 330: 1344–1348. 2010. [Medline] [CrossRef]
13. Abe A, Yamada H, Moriya S, and Miyazawa K. The β-carboline alkaloid harmol induces cell death via autophagy but not apoptosis in human non-small cell lung cancer A549 cells. Biol Pharm Bull. 34: 1264–1272. 2011. [Medline] [CrossRef]
14. Barth JMI, Szabad J, Hafen E, and Köhler K. Autophagy but not apoptosis in human non-small cell lung cancer A549 cells. Biol Pharm Bull. 34: 1264–1272. 2011. [Medline] [CrossRef]
15. Din FA, Valanciute A, Houde V, Zibrova D, Green KA, Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19: 5720–5728. 2000. [Medline] [CrossRef]
16. Troncoso R, Vicencio JM, Parra V, Nemchenko A, Kawashima Y, del Campo A, Torbο B, Battiprolu PK, Aranguez P, Chiong M, Yakar S, Gillette TG, Hill JA, Abel ED, LeRoith D, and Lavandero S. Energy-preserving effects of IGF-1 antagonize starvation-induced cardiac autophagy. Cardiovasc Res. 93: 320–329. 2012. [Medline] [CrossRef]
17. Orsolic N, and Sirovina D. Končić MZ, Lacković G, and Gregorović G. Effect of Croatian propolis on diabetic nephropathy and liver toxicity in mice. BMC Complement Altern Med. 12: 117. 2012. [Medline] [CrossRef]
18. Banaee M, Sureda A, Mirvaghefi AR, and Ahmadi K. Biochemical and histological changes in the liver tissue of rainbow trout (Oncorhynchus mykiss) exposed to sub-lethal concentrations of diazinon. Fish Physiol Biochem.; doi 10.1007/s10695-012-9714-1. [Medline]
19. Aki T, Nara A, and Uemura K. Cytoplasmic vacuolization during exposure to drugs and other substances. Cell Biol Toxicol. 28: 125–131. 2012. [Medline] [CrossRef]
20. Pyo JO, Nah J, and Jung YK. Molecules and their functions in autophagy. Exp Mol Med. 44: 73–80. 2012. [Medline] [CrossRef]
21. Tanida I. Autophagosome formation and molecular mechanism of autophagy. Antioxid. Redox Signal. 14: 2201–2214. 2011. [Medline] [CrossRef]
22. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19: 5720–5728. 2000. [Medline] [CrossRef]
23. Tanida I, Ueno T, and Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 36: 2503–2518. 2004. [Medline] [CrossRef]
24. Chou CY, Hsu CH, Wang YH, Chang MY, Chen LC, Cheng SC, and Chen YH. Biochemical and structural properties of zebrafish Capsulin produced by Escherichia coli. Protein Expr Purif. 75: 21–27. 2011. [Medline] [CrossRef]
25. Chen YH, and Lin JS. A novel zebrafish mutant with wavy-notochord: an effective biological index for monitoring the copper pollution of water from natural resources. Environ Toxicol. 26: 103–109. 2011. [Medline] [CrossRef]
26. Tsai IT, Yang ZS, Lin ZY, Wen CC, Cheng CC, and Chen YH. Flavone is efficient to protect zebrafish fins from UV-induced damages. Drug Chem Toxicol. 35: 341–346. 2012. [Medline] [CrossRef]
27. Chen YH, Lin YT, and Lee GH. Novel and unexpected functions of zebrafish CCAAT box binding transcription factor (NF-Y) B subunit during cartilage development. Bone. 44: 777–784. 2009. [Medline] [CrossRef]
28. Lee GH, Chang MY, Hsu CH, and Chen YH. Essential roles of basic helix-loop-helix transcription factors, Capsulin and Musculin, during craniofacial myogenesis of zebrafish. Cell Mol Life Sci. 68: 4065–4078. 2011. [Medline] [CrossRef]
29. Ding YJ, and Chen YH. Developmental nephrotoxicity of aristolochic acid in a zebrafish model. Toxicol Appl Pharmacol. 261: 59–65. 2012. [Medline] [CrossRef]
30. He C, Bartholomew CR, Zhou W, and Klionsky DJ. Assaying autophagic activity in transgenic GFP-LC3 and GFP-Gabarap zebrafish embryos. Autophagy. 5: 520–526. 2009. [Medline] [CrossRef]