Autophagy Guards Against Cisplatin-Induced Acute Kidney Injury

Autophagy is a highly conserved bulk protein degradation pathway involved in cellular homeostasis. Although emerging evidence indicates involvement of autophagy in various conditions, efforts to clarify the role of autophagy in renal tubules are beginning to be elucidated. In the present study, we examined the hypothesis that autophagy guards against acute kidney injury (AKI) by modulating several deteriorative pathways that lead to tubular cell death using a cisplatin-induced model of AKI. Cisplatin treatment of GFP-LC3 (green fluorescent protein–microtubule-associated protein 1 light chain 3) transgenic mice induced autophagy in kidney proximal tubules in a time-dependent manner. Proximal tubule–specific autophagy-deficient mice exhibited more severe cisplatin-induced AKI than did control mice, as assessed via kidney function and morphologic findings. In addition, cisplatin induced more severe DNA damage and p53 activation, concomitant with an increase in apoptotic cell number, and a massive accumulation of protein aggregates in autophagy-deficient proximal tubules. Cisplatin treatment significantly increased reactive oxygen species–producing damaged mitochondria in immortalized autophagy-deficient proximal tubular cells when compared with autophagy-retrieved control cells. In conclusion, autophagy guards kidney proximal tubules against AKI, possibly by alleviating DNA damage and reactive oxygen species production and by eliminating toxic protein aggregates. Enhancing autophagy may provide a novel therapeutic option to minimize AKI. (Am J Pathol 2012, 180:517–525; DOI: 10.1016/j.ajpath.2011.11.001)

Macroautophagy (referred to as autophagy) is a highly conserved bulk protein degradation pathway involved in the turnover of long-lived proteins, damaged organelles, and aggregation-prone proteins.1–4 In the initial step of this process, parts of the cytoplasm and cellular organelles are engulfed in a double-membrane vesicle called an autophagosome. This autophagosome fuses with lysosomes, resulting in degradation of the sequestered materials by various lysosomal hydrolytic enzymes. This degradation is followed by generation of amino acids, which are then recycled for macromolecular synthesis and energy production. Although a low level of constitutive autophagy occurring under physiologic conditions fulfills cellular homeostatic functions such as protein and organelle turnover, autophagy is up-regulated in various pathologic conditions to remove damaged cytoplasmic components, and thereby guards against the diseased conditions. Accumulating evidence has suggested that autophagic deficiency is associated with various diseases such as neurodegeneration, cancer, and liver disease.3

Although autophagy in the kidney was first described in the 1970s,5,6 efforts to clarify the role of autophagy in renal tubules are just beginning. Up-regulation of autophagy in the kidney has been observed in several animal experimental models including ischemia-reperfusion injury,7–9 cisplatin nephropathy, 10,11 cyclosporine nephropathy,12 and protein overload13; however, findings of some of these studies are contradictory insofar as the...
role of autophagy in the kidney. Although autophagy is a double-edged sword, that is, while autophagy is basically cytoprotective, in some context it could directly contribute to cell death (autophagic cell death).

To assess the role of autophagy more strictly in the kidney, we recently generated renal proximal tubule–specific autophagy-deficient mice and demonstrated that autophagy protects proximal tubular cells against ischemia-reperfusion injury. These mice enabled us to examine the role of autophagy in proximal tubules, which are exposed to multiple insults. In the present study, we used a model of cisplatin-induced acute kidney injury (AKI) in which genotoxicity, p53, activation, and reactive oxygen species (ROS) are involved in the development of nephrotoxicity. Several recent reports have demonstrated that autophagy involves stabilization of DNA and suppression of genome damage. Recent advances in the field of redox regulation of autophagy are focused on the role of mitochondria as a source of ROS and on autophagy as a means for clearance of ROS. In addition, autophagy has been demonstrated to be up-regulated in cisplatin-induced AKI and other kidney diseases; however, the precise mechanism of autophagy in cisplatin nephrotoxicity, in particular, in vivo, remains to be elucidated. We investigated the proximal tubule–specific role of autophagy in AKI using a model of cisplatin-induced nephrotoxicity and proximal tubule–specific autophagy-deficient mice.

Materials and Methods

Animals

GFP-LC3 (green fluorescent protein–microtubule-associated protein 1 light chain 3) transgenic mice have been described previously. Mice were crossed with C57BL/6N Crj mice over 10 generations, and were maintained as heterozygotes for the GFP-LC3 transgene. C57BL/6N Crj mice over 10 generations, and were main-

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Drug Administration

Cisplatin (Sigma-Aldrich Corp., St. Louis MO) was dissolved in saline solution at a concentration of 1 mg/mL. Mice received a single intraperitoneal injection of either cisplatin (15, 17.5, or 20 mg/kg body weight) or vehicle (saline solution).

Antibodies

Antibodies used included the following: antibodies for megalin; phospho-histone H2AX (Ser139); p53; actin, which genotoxicity, p53, activation, and reactive oxygen species (ROS) are involved in the development of nephrotoxicity. Several recent reports have demonstrated that autophagy involves stabilization of DNA and suppression of genome damage. Recent advances in the field of redox regulation of autophagy are focused on the role of mitochondria as a source of ROS and on autophagy as a means for clearance of ROS. In addition, autophagy has been demonstrated to be up-regulated in cisplatin-induced AKI and other kidney diseases; however, the precise mechanism of autophagy in cisplatin nephrotoxicity, in particular, in vivo, remains to be elucidated. We investigated the proximal tubule–specific role of autophagy in AKI using a model of cisplatin-induced nephrotoxicity and proximal tubule–specific autophagy-deficient mice.

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press reflects Cre activity, exhibited LacZ-positive tubules in the cortex and the outer medulla, whereas LacZ-positive tubules were absent in female mice. Therefore, only male mice were used in the present study. The mice were allowed free access to water and standard mouse chow. All animal experiments were approved by the institutional committee of the Animal Research Committee of Osaka University and the Japanese Animal Protection and Management Law (No. 25).

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primary antibodies at 4°C overnight. Except for p53, sections were then incubated with Alexa 488- and Alexa 555-conjugated secondary antibodies for 30 minutes at room temperature. Immunofluorescence images were obtained using a laser confocal microscope (Leica TCP SP5; Leica Microsystems GmbH, Wetzlar, Germany). For double staining for p53 and megalin, p53 was first visualized using a horseradish peroxidase–diaminobenzenide compound (Nichirei Corp., Chuo-ku, Tokyo, Japan), followed by detection of megalin using alkaline phosphatase and NBT/BCIP Stock Solution (Roche Diagnostics, Ltd., Basel, Switzerland). In all quantitative or semiquantitative analyses of histologic staining, at least 10 high-power fields were reviewed for each tissue by two nephrologists (T.K. and Y.T.) in a blinded manner.

**Liver Injury**

Liver sections, cells were reacted with 5 μg/mL Proteinase K (Roche Diagnostics) for 15 minutes at 37°C with or without cisplatin (50 μg/mL). ROS production was detected using MitoSOX Red (Invitrogen Corp.). According to the manufacturer's instructions, cells were reacted with 5 μmol/L MitoSOX Red for 10 minutes at 37°C with or without cisplatin (50 μmol/L).

**TUNEL Staining**

Apoptotic cells were detected using the TUNEL assay with an in situ apoptosis detection kit (Takara Bio Inc., Tokyo, Japan). In brief, paraffin-embedded renal tissue sections were deparaffinized and incubated with 10 μg/mL Proteinase K (Roche Diagnostics) for 15 minutes at room temperature. The sections were then exposed to terminal deoxynucleotidyl transferase labeling reaction mixture for 90 minutes, followed by incubation with anti-FITC–horseradish peroxidase conjugate for 30 minutes in a 37°C humidified chamber. Finally, the sections were reacted with diaminobenzenide.

**Biochemical Measurements**

The serum urea nitrogen concentration was measured using the BUN-test-Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the creatinine concentration was determined using the CRE-EN Kainos Kit (Kainos Laboratories, Inc., Tokyo, Japan).

**In Vitro Analysis**

Atg5-deficient proximal tubular cells were isolated from 3-week-old Atg5<sup>F/F</sup>-KAP mice using the CELLection Biotin Binder Kit (Invitrogen Corp.) and biotinylated *Lotus tetragonolobu* agglutinin (Vector Laboratories) as described previously. Immortalized cell lines were established by transformation using pEF321-T, an SV-40 large T antigen expression vector. cDNA from Atg5 mice was subcloned from pCI-neo-mApg5 (plasmid 22956; Addgene, Inc., Cambridge, MA). The subcloned cDNA fragment was cloned into pMRX-IRES-bsr. Stable cell lines restoring Atg5 were generated using a retroviral expression system with Plat-E cells as described previously. Stable transformants were selected using blasticidin. Cell viability was assessed via trypan blue staining. Mitochondrial ROS production was detected using MitoSOX Red (Invitrogen Corp.). According to the manufacturer’s instructions, cells were reacted with 5 μmol/L MitoSOX Red for 10 minutes at 37°C with or without cisplatin (50 μmol/L).

**Western Blot Analysis**

Western blot analysis was conducted as described previously. In brief, whole-cell lysates were extracted in lysis buffer (Cell Signaling Technology), and protein concentration was determined using the Pierce BCA Protein Assay Reagent (Thermo Scientific Pierce Protein Research Products, Rockford, IL). An equal amount (15 μg) of protein lysates was loaded in each lane and separated using 12% SDS-PAGE. Then the gels were transferred onto polyvinylidene difluoride membranes. Membranes were incubated with anti-LC3, Atg5, and β-actin primary antibodies at 4°C overnight. After incubation with horseradish peroxidase–conjugated secondary antibodies, detection was performed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

**Statistical Analysis**

All results are given as mean ± SE. Statistical analyses were conducted using JMP software (SAS Institute, Inc., Cary, NC). Multiple-group comparisons were performed using analysis of variance with post-testing using the Tukey-Kramer test. *P* < 0.05 was considered statistically significant.

**Results**

**Cisplatin Induces Autophagy in Kidney Proximal Tubules in Vivo**

To confirm the induction of autophagy in proximal tubular cells after cisplatin administration, we used transgenic mice expressing GFP-LC3. GFP-positive dots reflect autophagosomes because transgene GFP-LC3 under the control of constitutive CAG promoter produces nearly equivalent amounts of endogenous LC3. A single intraperitoneal administration of 15 mg/kg cisplatin induced an obvious accumulation of GFP-LC3 dots as early as 6 hours, and they increased in a time-dependent manner, peaking at day 3, whereas GFP-LC3 dots were rarely observed in the vehicle-treated GFP-LC3 transgenic mice (Figure 1, A and B). Co-immunostaining with megalin, a marker of proximal tubular cells, indicated that GFP-LC3 dots were localized in the proximal tubules. These results demonstrate that cisplatin induces autophagy in proximal tubules in vivo.

**Autophagy Deficiency Affects Kidney Morphology and Function after Cisplatin Administration**

After proving that cisplatin induces autophagy in proximal tubular cells, we examined the effect of autophagy deficiency on kidney morphology and function during cisplatin nephrotoxicity using the proximal tubule–specific Atg5-deficient mice (*Atg5<sup>F/F</sup>-KAP* mice). PAS staining demonstrated that, compared with control *Atg5<sup>F/F</sup>* mice, *Atg5<sup>F/F</sup>-KAP* mice exhibited severe loss of brush border, tubular dilatation, and cast formation at 3 days.
after the 15-mg/kg dose of cisplatin (Figure 2A). Semi-
quantitative assessment of the severity of the tubular injury demonstrated that Atg5<sup>F/F</sup>:KAP mice exhibited significantly higher injury than did Atg5<sup>F/F</sup> mice (3.9 ± 0.3 versus 1.7 ± 0.3, respectively; P < 0.05; Figure 2B).

We next assessed kidney function at 3 days after administration of various dosages of cisplatin. Kidney function was significantly deteriorated in the 15-mg/kg dose of cisplatin-treated Atg5<sup>F/F</sup>:KAP mice compared with that of cisplatin-treated Atg5<sup>F/F</sup> mice, as assessed using serum urea nitrogen concentrations (105 ± 4 mg/dL versus 63 ± 13 mg/dL, respectively; P < 0.05) and serum creatinine concentrations (1.0 ± 0.2 mg/dL versus 0.4 ± 0.1 mg/dL, respectively; P < 0.05) (Figure 2C). Similar data were obtained when cisplatin was administered at a dose of 17.5 mg/kg; however, the 20-mg/kg dose of cisplatin caused such severe damage that differences between Atg5<sup>F/F</sup>:KAP mice and Atg5<sup>F/F</sup> mice were not observed as assessed using kidney function and morphologic findings (see Supplementary Figure S1 at http://ajp.amjpathol.org).

**Figure 1.** Induction of autophagy in proximal tubules of GFP-LC3 transgenic mice after administration of cisplatin. GFP-LC3 transgenic mice were intraperitoneally injected with either vehicle (saline solution) or cisplatin (15 mg/kg). At the indicated time points, renal tissues were collected. A: Kidney sections were immunostained for megalin, a marker of proximal tubules (red) and for DAPI (blue) for counterstaining. B: The number of GFP-LC3 dots per proximal tubule at each time point was counted in at least 10 high-power fields (×1058). GFP-LC3 dots were observed 6 hours after cisplatin treatment and increased in a time-dependent manner, peaking at day 3. Data are given as mean ± SE. Original magnification, ×1058. Images are representative of multiple experiments.

**Autophagy Deficiency Augments DNA Damage, p53 Activation, and Apoptosis after Cisplatin Administration**

We next investigated the extent of DNA damage in the proximal tubule–specific autophagy-deficient kidney, one of the major mechanisms in cisplatin nephrotoxicity. DNA damage in renal tubular cells was assessed via immunostaining for γ-H2AX. Administration of 15 mg/kg cisplatin increased γ-H2AX–positive nuclei significantly in the proximal tubules in Atg5<sup>F/F</sup>:KAP mice compared with Atg5<sup>F/F</sup> mice, whereas they were rarely observed in vehicle-treated Atg5<sup>F/F</sup>:KAP or Atg5<sup>F/F</sup> mice (Figure 3A; see also Supplemental Figure S2A at http://ajp.amjpathol.org). Inasmuch as severe DNA damage leads to p53 activation and apoptotic cell death, we performed immunostaining for p53 and TUNEL staining. The number of p53-positive proximal tubular cells significantly increased in Atg5<sup>F/F</sup>:KAP mice compared with Atg5<sup>F/F</sup> mice at 3 days after cisplatin injection (Figures 3B; see also Supplemental Figure S2B at http://ajp.amjpathol.org). The number of TUNEL-positive apoptotic tubular cells increased significantly in Atg5<sup>F/F</sup>:KAP mice compared with Atg5<sup>F/F</sup> mice at 3 days after cisplatin injection (Figure 3C).

**Autophagy Protects Proximal Tubular Cells by Eliminating ROS-Producing Mitochondria during Cisplatin Treatment**

To examine the role of autophagy on cell survival and mitochondrial injury after cisplatin treatment in vitro, we established immortalized Atg5-deficient renal proximal tubular cells (Atg5-negative PTCs) isolated from Atg5<sup>F/F</sup>:KAP mice. As a control, we generated Atg5-positive PTCs by stably transfecting pMRX-IRES-Atg5-bsr (Atg5-expressing retroviral vector cassette) to Atg5-negative PTCs. Western blot analyses confirmed retrieval of Atg5 expression and recovery of autophagic activity assessed
via LC3-I–LC3-II transition (Figure 4A). Treatment with cisplatin for 12 hours tended to decrease cell survival of Atg5-positive PTCs (Figure 4B). Of interest, Atg5-negative PTCs were vulnerable to cisplatin toxicity when compared with Atg5-positive PTCs. Because cisplatin was reported to induce mitochondrial dysfunction and increase ROS production via the disrupted respiratory chain, we investigated whether autophagy could guard against the mitochondrial ROS production in proximal tubular cells during cisplatin nephrotoxicity. Cisplatin-treated Atg5-negative or Atg5-positive PTCs were stained with MitoSOX Red, which exhibits red fluorescence when oxidized by ROS. Exposure to 50 μmol/L cisplatin for 12 hours significantly increased intracellular MitoSOX Red oxidation in Atg5-negative PTCs when compared with Atg5-positive PTCs (Figure 4, C and D). These results suggested that autophagy protects proximal tubular cells by eliminating ROS-producing mitochondria during cisplatin treatment.

**Formation of Protein Aggregates in Autophagy-Deficient Proximal Tubules after Cisplatin Administration**

Inasmuch as some studies have indicated that autophagy deficiency leads to accumulation of p62-positive and ubiquitin-positive protein aggregates in several organs, we performed immunohistochemical analysis for p62 and ubiquitin. Mass accumulation of p62-positive and ubiquitin-positive aggregates was observed in proximal tubular cells of Atg5<sup>F/F</sup>-KAP mice at 3 days after administration of 15 mg/kg cisplatin, whereas they were rarely detected in cisplatin-treated Atg5<sup>F/F</sup> mice (Figure 5, A and B).

**Discussion**

Findings of the present study provide clear and convincing evidence that autophagy has a protective role against AKI by alleviating DNA damage and mitochondrial injury in proximal tubule–specific autophagy-deficient mice. Furthermore, we observed that the autophagy-deficient proximal tubular cells exhibited aberrant activation of p53 signaling and subsequent increase of apoptosis, and accumulation of protein aggregates during cisplatin-induced AKI, which will provide new knowledge about the mechanism of the renoprotective role of autophagy.

Although previous studies have suggested up-regulation of autophagy in the cisplatin-induced model of AKI, it has been controversial whether autophagy has protective effects against cisplatin-induced AKI. One study has suggested a cytoprotective and prosurvival role for autophagy during cisplatin treatment of proximal tubular cells by demonstrating that blockade of autophagy using pharmacologic inhibitors (3-methyladenine or bafilomycin) or short hairpin RNA for beclin inhibition of autophagy-deficient proximal tubular cells exhibited elevated expression of p62 and ubiquitin-positive protein aggregates in several organs, we performed immunohistochemical analysis for p62 and ubiquitin. Mass accumulation of p62-positive and ubiquitin-positive aggregates was observed in proximal tubular cells of Atg5<sup>F/F</sup>-KAP mice at 3 days after administration of 15 mg/kg cisplatin, whereas they were rarely detected in cisplatin-treated Atg5<sup>F/F</sup> mice (Figure 5, A and B).

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clear evidence that the proximal tubule-specific autophagy-deficient mice exhibited more severe injury after cisplatin treatment as assessed by kidney function and morphologic findings. We believe that our observation settles the controversy about the role of autophagy during cisplatin nephrotoxicity; that is, autophagy has a protective role against cisplatin-induced AKI.

Clinical use of cisplatin, one of most potent chemotherapeutic agents, has been substantially restricted because of its nephrotoxic effects. Renal microenvironmental changes after cisplatin treatment is a complex process, and the mechanism for cisplatin-associated nephrotoxicity has not been completely elucidated; however, many theories have been developed, including 1) DNA...
Atg5 (and LC3-I-LC3-II transition were confirmed in Pd differences (*with Atg5 significantly decreased viability of Atg5 cell survival demonstrated that higher dosages of cisplatin treatment mitochondrial ROS production in proximal tubular cells after cisplatin treatment. This study ways via intrinsic mitochondrial damage or extrinsic death and apoptosis are exacerbated in cisplatin-treated proximal tubule–specific autophagy-deficient kidneys compared with control kidneys, which supports the hypothesis that autophagy protects renal tubular cells against cisplatin nephrotoxicity by attenuating DNA damage.

Another important finding is that autophagy deficiency in renal proximal tubular cells enhanced cisplatin-induced ROS production from mitochondria, thereby resulting in cell death. Cisplatin is proposed to induce mitochondrial dysfunction and increase ROS production, an important factor contributing to nephrotoxicity. Several recent reports have demonstrated that autophagy has an essential role in the control of mitochondrial quality by eliminating damaged mitochondria, which is referred to as mitophagy. Indeed, we have recently demonstrated that morphologically abnormal mitochondria gradually increased in the proximal tubule–specific autophagy-deficient mice. In the present study, exposure to cisplatin substantially increased mitochondrial ROS production in Atg5-deficient proximal tubular cells compared with Atg5-expressing control cells, demonstrating that autophagy eliminates ROS-producing damaged mitochondria during cisplatin nephrotoxicity, presumably through mitophagy. In relation to DNA damage, increased mitochondrial ROS may target and modify genomic DNA, which may eventually enhance the susceptibility to genomic stress by cisplatin-DNA adducts.

It is noteworthy that autophagy deficiency induced accumulation of p62-positive and ubiquitin-positive aggregates in proximal tubular cells after cisplatin treatment. Similar observations have been demonstrated in chronic settings in autophagy-deficient liver, neurons, heart, and kidneys and in the acute setting in autophagy-deficient kidney after ischemia-reperfusion injury. Emerging evidence has revealed that unfolded proteins, which could be produced under various stresses including ROS, are common targets of both proteasomal and autophagy-mediated degradation, although these two systems interact and share respective roles in clearance of the unfolded proteins remains unclear. Excessive unfolded proteins, which cannot be cleared with two mechanisms, can accumulate and form large aggregates. Formation of protein aggregates is at least in part mediated by ubiquitin-binding protein p62.

Figure 4. The effects of autophagy deficiency on cell viability and mitochondrial ROS production in proximal tubular cells after cisplatin treatment in vitro. A: Western blot analysis of Atg5 and LC3. Retrieval of Atg5 and LC3-I-LC3-II transition were confirmed in Atg5 PTCs. B: Analysis of cell survival demonstrated that higher dosages of cisplatin treatment significantly decreased viability of Atg5 PTCs compared with Atg5 PTCs. C and D: Exposure to 50 μmol/L cisplatin for 12 hours significantly increased intracellular MitoSOX Red oxidation in Atg5 PTCs compared with Atg5 PTCs. Data are given as mean ± SE. Statistically significant differences (*P < 0.05) are indicated. C: Original magnification, ×200. Atg5 (+), Atg5 PTCs; Atg5 (−), Atg5 PTCs. Images are representative of multiple experiments.

damage and subsequent p53 activation, ii) apoptotic pathways via intrinsic mitochondrial damage or extrinsic death receptor activation, and iii) oxidative stress. This study may answer some of the questions about how autophagy attenuates cisplatin-induced signaling pathways that lead to tubular cell injury or death.

One of the remarkable findings of this study is that the autophagy-deficient proximal tubules exhibited a substantial increase in DNA damage during cisplatin nephrotoxicity, as assessed via immunostaining for γ-H2AX, a marker of DNA damage. One of the proposed mechanisms in cisplatin nephrotoxicity is that cisplatin cross-links genomic DNA after aquation into a highly reactive form, which leads to distortion of the duplex structure. The DNA damage signal is then relayed to activation of p53, which then induces apoptotic cell death of renal tubular cells, in part via mitochondrial injury. Of note, several recent reports have demonstrated that autophagy involves suppression of genome damage and that autophagy dysfunction leads to tumorigenesis. Our data demonstrate that DNA damage, p53 activation, and apoptosis are exacerbated in cisplatin-treated proximal tubule–specific autophagy-deficient kidneys compared with control kidneys, which supports the hypothesis that autophagy protects renal tubular cells against cisplatin nephrotoxicity by attenuating DNA damage.

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Therefore, accumulation of p62-positive and ubiquitin-positive aggregates in autophagy-deficient proximal tubular cells suggests that autophagy could exert a cytoprotective function by degrading otherwise toxic unfolded protein (aggregates) during cisplatin-induced AKI.

These proposed mechanisms by which autophagy guards against cisplatin-induced AKI (ie, DNA damage, mitochondrial injury and ROS, and protein aggregates) are depicted schematically in Supplemental Figure S3 (available at http://ajp.amjpathol.org).

The renoprotective role of autophagy prompts us to assume that activation of autophagy can be beneficial in preventing AKI. To date, several molecules are proposed as clinically applicable regulators of autophagy. In contrast, it has been demonstrated that some types of cancer cells use autophagy to survive against lack of nutrients and hypoxia in their microenvironments. In line with this notion, compounds that can reverse autophagy (eg, chloroquine) have been applied in clinical settings as a sensitizer for conventional chemotherapeutic compounds, which may in turn deteriorate cisplatin-induced AKI injury. A better understanding of the effect of autophagy on the relationship between cancer and cisplatin nephrotoxicity would ultimately enable us to harness autophagic pathways as clinically applicable interventions.

In conclusion, we have demonstrated that autophagy guards proximal tubular cells against AKI by modulating several deteriorative pathways that lead to tubular cell death.

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**Figure 5.** Accumulation of p62-positive and ubiquitin-positive aggregates in the autophagy-deficient kidney proximal tubules at 3 days after cisplatin treatment (15 mg/kg). Immunohistochemical staining demonstrated accumulation of p62-positive (A) and ubiquitin-positive (B) aggregates in proximal tubules of cisplatin-treated Atg5F/F:KAP mice (n = 4 to 7). Megalin was co-stained in red as a marker of proximal tubules. The number of p62-positive or ubiquitin-positive dots was counted in at least 10 high-power fields (×352). Original magnification, ×1058. Data are given as mean ± SE. Statistically significant differences (*P < 0.05) are indicated. F/F, Atg5F/F mice; F/F:KAP, Atg5F/F:KAP mice. Images are representative of multiple experiments.
References


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