8-2016

Developing a more clinically-relevant mouse model of cisplatin-induced nephrotoxicity.

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DEVELOPING A MORE CLINICALLY-RELEVANT MOUSE MODEL OF CISPLATIN-INDUCED NEPHROTOXICITY

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A Thesis submitted to the faculty of the School of Medicine of the University of Louisville in partial fulfillment of the requirements for the degree of

Masters of Science in Pharmacology and Toxicology

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Louisville, Kentucky

August 2016

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DEDICATION

This thesis is dedicated to Bill Nye, who inspired me to be a scientist and to always be curious about the world around me from a very early age.

ACKNOWLEDGMENTS

I would like to thank my parents for fostering my curiosity and always answering my “why” and “how” questions. I would like to thank my friends for keeping me sane during this process. Finally, I would like to thank Drs. Siskind and Beverly for providing invaluable mentoring and shaping me into the scientist I am today.

ABSTRACT

DEVELOPING A MORE CLINICALLY-RELEVANT MOUSE MODEL OF CISPLATIN NEPHROTOXICITY

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Cisplatin is a nephrotoxic chemotherapeutic that causes acute kidney injury (AKI) in 30% of patients. Although recovery can occur after one episode of cisplatin-induced AKI, studies have indicated multiple episodes may lead to the development of chronic kidney disease (CKD), an irreversible disease with no current treatments. The standard mouse model of cisplatin-induced AKI consists of one, high dose of cisplatin (> 20 mg/kg) that is lethal to the animal three days later. This model doesn't accurately reflect the repeated dosing regimen patients receive, and doesn't allow for long-term outcome studies of pathologies associated with CKD. We have developed a repeated dosing model of cisplatin (7mg/kg once a week for four weeks). This model allows for the long-term survival of mice, and the associated pathology is fibrosis-the hallmark of CKD. Thus, data indicate that the repeated dosing model can be used to study AKI to CKD progression.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

INTRODUCTION

Acute kidney injury (AKI) is defined as a rapid loss in renal function accompanied by accumulation of waste products, such as urea and creatinine, in the blood. According to RIFLE criteria, which aims to standardize the definition of AKI, AKI is associated with a 50% loss in glomerular filtration rate (GFR), a doubling in serum creatinine (SCr) levels, and a significant decrease in total urine output (1, 2). Presently, the mortality rate of patients with AKI is 50%, and having AKI increases a hospital patient's risk of death 10-15fold (3, 4).

Cisplatin is a potent chemotherapeutic for the treatment of many solid tumor cancers, including ovarian, testicular, head and neck, and lung cancers (5, 6). While the potency of cisplatin makes it an excellent chemotherapeutic choice, there are very serious side effects associated with this drug. These include bone marrow suppression, ototoxicity, neurotoxicity, and nephrotoxicity (7). Of these, the risk of developing cisplatin-induced nephrotoxicity leading to AKI is high. After a single dose of cisplatin, 33% of patients will develop nephrotoxicity. Furthermore, cisplatin-induced AKI accounts for 19% of all cases of AKI, making it a prominent health problem (8).

The high relevance of cisplatin-induced nephrotoxicity can be explained by the ready uptake of cisplatin in the blood and kidney. The kidney contains glomeruli cells which can freely filter blood, and, as a result, cisplatin is also

freely filtered. Once cisplatin is filtered through glomeruli, it is taken up by Ctr1 and OCT2 transporters in an energy-dependent driven manner (9-11) . These transporters are responsible for secretion and absorption of organic molecules.

Once cisplatin is transported into proximal tubule cells, it is readily converted to a highly reactive metabolite and is conjugated to glutathione, proteins, RNA, and DNA (5, 12, 13). Cisplatin causes cross-linking of DNA strands, thereby inhibiting DNA replication and gene transcription (6, 12, 13). In the context of cancer, this is therapeutic as it leads to inhibition of growth, senescence, and cell death (6). However, in the proximal tubule cells, cisplatin accumulates to amounts 5 times higher than that in the serum, and thus these processes are extremely detrimental as they lead to severe proximal tubule cell death, ultimately resulting in kidney injury and subsequent loss of function (6, 14).

Cisplatin leads to two forms of cell death in proximal tubule cells- apoptosis and necrosis (7). It has been well documented that cisplatin activates the intrinsic apoptotic cell death pathway(15). This involves the accumulation of Bax, a BCL2 pro-apoptotic family member protein, in the mitochondria (16). This accumulation leads to the release of cytochrome c from the mitochondria and eventual activation of initiator and effector caspases. This apoptotic pathway plays a pivotal role in cisplatin-induced AKI pathophysiology, as $Bax^{-/-}$ mice are resistant to cisplatin nephrotoxicity (17).

Other BCL2 pro-apoptotic family members (such as PUMA-α) can be activated by p53,a tumor suppressor that is activated by DNA damage or

genotoxic stress (7, 18). Activation of p53 can result in the increased transcription of these pro-apoptotic proteins. Furthermore, p53 can act directly on BCL-2 proapoptotic family members to lead to the release of cytochrome c (18). Through these mechanisms, p53 plays a pivotal role in apoptosis, both in terms of killing cancer, and also as a mechanism of cisplatin-induced nephrotoxicity.

While apoptosis is predominately found in animal models of AKI, the main pathology associated with this human AKI is acute tubular necrosis (19, 20). Acute tubular necrosis is sudden death of the tubular epithelial cells that compromise the renal tubules, including both proximal and distal tubule cells(19). This pathology is seen in both animal and humans who have experienced AKI, and is often classified as acute tubular necrosis based on death to the distal tubules (which are affected to a greater extent than proximal tubules) and loss of brush borders (20). This pathology occurs to a greater extent in animal models of AKI than in humans. This is due to the fact that the level of injury patients endure is less than the level of injury achieved in animal models used to study AKI. Studies performed to examine AKI using nephrotoxins lead to very high levels of acute tubular necrosis due to that fact that the dose of the toxin being used is extremely high (20). It has also been suggested that this discrepancy in human and animal pathology may be due to the fact that human kidney biopsies are rarely performed, and when they are, they usually do not sample the outer medulla where the tubules are located (21). Furthermore, if biopsies are done, it

is usually during the recovery phase after AKI, and thus levels of necrosis do not accurately reflect levels that would be seen during injury phase (21).

The pathophysiology of cisplatin-induced AKI not only involves tubule cell death, but also several other processes. Of importance are the involvement of the cell cycle and immune cell infiltration/inflammatory pathways (7, 15). The cell cycle is a complex system of checks and balances to ensure that cell proliferation occurs without fault. Proliferation is a vital process in the kidney after injury, as remaining epithelial cells will dedifferentiate and proliferate to replace damaged tubular epithelial cells- an important step in adaptive recovery after AKI (21).

The ability of the cell cycle to proceed from G_1 to S, S to M, and M to G_2 phase in a subsequent order necessary for proper cell proliferation is controlled by cyclins (22). Cyclins are periodically synthesized and degraded during the cell cycle, and this leads to unidirectional progression of the cell cycle. Cyclins have been found to form heterodimers with Cdk subunits. Cdk subunits are serine/threonine protein kinases that are only active when bound to cyclins. Once cyclins binds, cdk subunits take on basal kinase activity. This kinase activity drives the progression of the cell cycle from one phase to another (22).

While cyclins and their heterodimerization with Cdks are the main drivers of cell cycle progression, there are also protein effectors that serve as checkpoints to ensure that each part of the cell cycle is completed successfully, further ensuring that the cell cycle proceeds without fault (22). These effectors monitor DNA damage, incomplete DNA synthesis, and whether or not mitosis is completed successfully. These effectors primarily control the entry of cells into

both S and M phases by inhibiting or stimulating kinase activity of Cdks (22). In humans, there are two families of protein effectors- the CDK interacting protein/ kinase inhibitory protein (CIP/KIP) family and the inhibitor of cyclin-dependent kinase 4 (INK4) family (23). Within the CIP/KIP family, p21 has been indicated as a potential therapeutic target for cisplatin-induced AKI.

p21 is expressed constitutively at low levels in the cell. Overexpression of p21, which can be induced by p53-dependent or independent mechanisms, leads to cyclin kinase inhibition and induction of senescence (24, 25). p21 has been found to play a major role in cisplatin-induced AKI, as it determines whether or not tubular cells undergo cell death or survive (24). Upon injury by cisplatin, many quiescent cells enter into the cell cycle as evidenced by increased PCNA staining and BrdU incorporation, and there is a marked induction of p21 (24). Higher levels of p21 are concentrated in the nuclei of distal and proximal tubule cells upon cisplatin injury (22).

While the mechanism of p21 protection is not fully understood, its role in regulation of the cell cycle may be key in repairing and regenerating injured cells (22, 24). Studies using p21 $^{-/-}$ mice have indicated that acute kidney injury-associated loss of function is exacerbated and worsened following cisplatin administration when compared to wild-type mice (26). Morphologically, cisplatin-induced AKI damage is restricted to the distal and proximal tubules. However, the damage in knockout mice was present throughout the entire kidney cortex, and apoptosis was also more widespread in these mice (22). Furthermore, there was an increase in nuclear size due to polyploid content of proximal tubule cells

as the result of an uncoordinated cell cycle, thus indicating the importance of p21 mediating AKI through cell cycle progression control. Furthermore, studies have also shown that when both p21 and 14-3-3σ (another cell cycle inhibitor protein) are expressed together, cell cycle progression is inhibited, resulting in repair of damaged cells and subsequent regeneration of healthy epithelial cells(27).

Animal knockout studies have been supplemented with *in vitro* studies. p21 overexpression in mouse proximal tubular cells instilled cytoprotective effects (27). Increasing levels of p21 expression as a potential renoprotective therapy has also been reported using prior administration of an adenovirus vector directing the overexpression of p21 (27). These studies indicate that p21 has renoprotective effects in cisplatin-induced AKI.

Taken together, these data suggests that upon initial cell stress, quiescent cells enter into the cell cycle and cell cycle inhibitors such as p21 and 14-3-3σ are induced to check the cycle at both G1 and G2 phases(27). Both p21 and 14-3-3σ are needed to successfully coordinate the cell cycle so that injury to the cell can be properly repaired, and this in turn leads to repair and regeneration of renal cells after acute injury (27).

Most research on cyclin-dependent kinase inhibitors has focused mainly on the CIP/KIP family, especially p21. Little is known about the INK4 family, but recent studies have shown that p18 is important. p18 interacts with CDK4/6 and inhibits the cell cycle in early G1 phase (28). Studies using p18 $^{-/-}$ mice have shown, over a course of 28 days, p18 $^{-/-}$ mice have significantly worse survival (28). Furthermore, these mice showed increased markers of endoplasmic

reticulum stress (ER stress), and worse injury morphology. Taken together these data indicate that cisplatin-induced AKI was aggravated in p18 $^{-/-}$ mice, and that p18 may play a role in protecting the kidney by interaction with ER stress cell death pathways (28). This was confirmed in studies with p18 overexpressing LLC-PK1 renal tubular epithelial cells. Overexpression of p18 in these cells inhibited the cell cycle in the G1 phase, and this was associated with a decrease in apoptosis levels (29). Commonly, the ER stress pathway promotes cell survival, but in times of prolonged stress (as associated with AKI), the ERS pathway favors apoptosis. Thus, it was concluded that p18 protects from cisplatin-induced AKI by inhibiting the ER stress pathway and therefore apoptosis (29).

Activation of inflammatory pathways and immune cell infiltration are also involved in cisplatin-induced AKI. After an acute injury, injured resident kidney cells release tumor necrosis factor α (TNFα) which serves as a chemoattractant to recruit immune cell types, mainly neutrophils to the site of injury in the kidney (30). Once recruited, neutrophils can initiate the activation of the downstream immune system. Once this activation occurs, M1 subtype macrophages eat dead proximal tubule cells and neutrophils (31). It has been shown that recruitment of this type of macrophages amplifies initial injury in the kidney (21).

While neutrophils and macrophages are involved in the initial inflammatory response after injury, many other immune cell types have been reported as mediators of cisplatin-induced AKI. Of importance are CD4+ cytotoxic T cells, CD4+CD25+ T regulatory cells, and dendritic cells(15). Of these cells types, the

role of CD4+ cytotoxic T cells has been well characterized. Mice depleted of CD4+ cytotoxic T cells had better renal function and less injury when treated with cisplatin and when these mice had their CD4+ cytotoxic T cell levels restored, renal function decreased and injury worsened(32). This may have been due to the fact that these cells produce FasL and Tim-1 which are important for T-cell mediated cytotoxicity, leading to the death of proximal tubule cells (32).

Whereas CD4+ cytotoxic T cells are detrimental in AKI, CD4+ CD25+ T regulatory cells are protective. These cells play a role in innate immune suppression as well as suppression of CD4+ T cell –mediated cytotoxicity (33). In animal studies where T regulatory cells were depleted, AKI was worsened (34).

Dendritic cells are anti-inflammatory as evidenced by their production of IL-10, an anti-inflammatory cytokine (35). Furthermore, dendritic cells can induce T regulatory cells, suggesting that they are protective in cisplatin-induced AKI (36). This is in fact the case- depletion of dendritic cells resulted in decreased renal function, increased tubular injury, and higher mortality rates when mice were treated with cisplatin (37).

Taken together, previous studies suggest that AKI is marked by a very complex mechanism of injury. In addition to cell death, cell cycle arrest, and immune cell infiltration, AKI pathophysiology also includes oxidative stress as mediated by mitochondrial dysfunction, glutathione depletion and inactivation, and endothelial cell dysfunction (7, 15).

Most of what is known about cisplatin-induced AKI pathology exists because we are able to study these processes in mouse models of AKI. As a result, studies for the development of therapies for protection against AKI and reversing injury as the result of AKI have also been performed in animal models. Unfortunately, while there have been many experimental therapies developed for the treatment of cisplatin-induced AKI, none have made it past clinical trials (7, 15). Currently, only palliative care therapies exist for severe, sustained AKI (1, 3, 4). In order to prevent cisplatin-induced AKI in patients, several precautions are taken. When possible, lower doses of cisplatin are used in conjunction with newer, less toxic chemotherapeutics to minimize cisplatin nephrotoxicity while maintaining its therapeutic efficacy(38). However, this type of dosing regimen is usually only used for palliative care. In instances where cisplatin can be used to cure cancer, particularly in the case of germ cell line cancers, a high dose of cisplatin administered over a short period of time is still used, putting these patients at the highest risk for developing AKI (38). Currently, the standard preventative measure for cisplatin nephrotoxicity and subsequent AKI is intravaneous saline infusions given before cisplatin administration. Saline induces diuresis, and by doing so, lowers overall nephrotoxicity, but the mechanism by which saline is preventative is not well-defined (38).

Alternatively, the administration of less nephrotoxic platinum drugs may be given to patients who run a high risk of developing AKI. Of the platinum alternatives, carboplatin is commonly used. Carboplatin was designed specifically to reduce the side effects of cisplatin (39, 40). Unfortunately,

carboplatin can still have nephrotoxic side effects, especially in patients who have previously received cisplatin and have increased nephrotoxicity sensitivity (41). Furthermore, cisplatin is still the better of drug of choice for most cancers due to its higher potency (39).

An alternative to creating a new, less nephrotoxic platinum chemotherapeutic would be to design a drug that could be used to protect the kidney from injury during the course of cisplatin treatment. This would not only avoid the development of AKI, but also allow for higher doses of cisplatin to be administered, which would make for a more efficacious cancer treatment option. Unfortunately, there is only one renoprotective agent available for cisplatin-induced AKI, and it is no longer used clinically for this purpose (42). In the early 2000's, amifostine was FDA-approved to be used as a renoprotective agent with cisplatin treatment of ovarian cancer (43). In a Phase III clinical trial of women with ovarian cancer, amifostine lowered the risk of nephrotoxicity from 33% to 10% overall, and the percentage of patients that required a delay in the next treatment or discontinuation of treatment altogether due to nephrotoxicity decreased from 36% to 10% (43, 44).

High doses can be given due to amifostine's mechanism of action, thereby increasing its efficacy as a renoprotective agent. Amifostine is rapidly converted to an active thiol metabolite via dephosphorylation by alkaline phosphatase. This thiol metabolite is preferentially taken up by normal tissue (42). Once uptake occurs, this active thiol metabolite scavenges for free radicals and depletes them. At the same time, it can also bind directly to cytotoxic drug molecules to

inactivate them (42). Furthermore, amifostine has few side effects, most of which are reversible and manageable. The most serious side effects include transient hypotension, allergic skin reactions, and severe nausea(42). Most importantly, amifostine doesn't attenuate the efficacy of chemotherapeutics. In fact, pre-clinical studies have suggested that amifostine may protect against the development of secondary malignancies associated with chemotherapeutic treatment (42)

While extensive pharmacokinetics have been studied in mice, little is known about the pharmacokinetics of amifostine in humans. Most clinical trials done with amifostine and cisplatin use a single, high dose of amifostine given before a single dose of cisplatin (38). Furthermore, the dose of cisplatin used is usually very high. Currently, most cisplatin regimens consist of lower doses of the drug given over an extended period of time, or in multiple doses to curtail nephrotoxic side effects. Thus the efficacy of amifostine with these newer low dosing regimens has yet to be elucidated. Case studies have reported that amifostine given before treatment of ovarian cancer with cisplatin and etoposide can lead to severe nephrotoxicity, ototoxicity, and neurotoxicity(45). Due to lack of renoprotective and injury ameliorating therapeutics available, nephrotoxicity of cisplatin must be monitored closely. This is done by extensively measuring changes in kidney function. Clinically, the golden standard is measurements of GFR and changes in serum creatinine (46-49). Serum creatinine is used as an indirect measure of GFR, as a doubling in serum creatinine equates a 50% decrease in GFR (loss of kidney function). Unfortunately, measuring serum

creatinine levels is an insensitive and faulty assay(49). Changes in serum creatinine usually are not detectable until there is 50% nephron loss. At this point, the level of AKI sustained is very high and may not be reversible. Furthermore, serum creatinine levels can be altered by levels of protein uptake and changes in protein in catabolism (47-49). This is due to the fact that creatinine is the byproduct of creatine breakdown from muscle catabolism. Thus, basal serum creatinine levels differ based on age and weight (47-49). These factors may falsely represent a full recovery from AKI, but rather suggest that there is injury/ change in kidney function, but that it is undetectable by this method.

This may be key in explaining why, even in patients with one instance of AKI in their lifetime, there is increased susceptibility to developing chronic kidney disease later in life (50-52). There are approximately 600,000 new cases of AKI each year, and it has been suggested that 20% of these cases go on to develop CKD (51, 53). This 20% accounts for patients who sustain severe AKI requiring dialysis and are able to recover. Patients who receive an acute tubular necrosis diagnosis go on to develop CKD 18-24 months later (50). This is surprising as it was formerly believed that patients who recovered from AKI had generally good renal outcomes. However, studies on AKI recovery used the development of end-stage renal disease to define a poor renal outcome. Studies including the progression of AKI to CKD indicate that the poor prognosis rate of patients who "recover" from AKI is actually greater than expected (50). Furthermore, the likelihood of developing CKD is also associated with the number of times a

person sustains AKI in their life (54). Thus, there is a need to monitor kidney function even after recovery.

Chronic disease kidney refers to a kidney disorder of varying severity, and thus affects many people (55). Chronic kidney disease is a progressive disease, and the early stages are often asymptomatic and reversible. However, the problem with chronic kidney disease arises from the worsening of the disease over the period of many decades (55). As a result, it is obvious that chronic kidney disease is often associated with aging (56). This is in part due to the fact that in older populations, there is an increased prevalence of diabetes and hypertension (56). These comorbidities worsen chronic kidney disease. Ultimately, a patient who has chronic kidney disease and one or more comorbidities are more likely to progress from chronic kidney disease to end-stage renal disease (55). Much like AKI, there are currently no therapies to ameliorate chronic kidney disease or halt progression to end stage renal disease. As a result, most patients will be placed on dialysis, which is costly and is often not that useful. Eventually, the kidney failure worsens, leading to high rates of mortality. There are approximately 400 per million people deaths in the U.S. from chronic kidney disease alone (55).

Clinically, CKD is defined as the presence of kidney damage, particularly albuminuria and proteinuria, and decreased kidney function as measured by serum creatinine levels for a period of three months or more (55). The severity of this disease is classified based on the total loss of GFR, as is the case for AKI as well. The pathology of chronic kidney disease differs from that of AKI. Whereas

proximal tubule cells are the source of most damage in AKI, glomeruloclerosis and nephrosclerosis are commonly associated with chronic kidney disease (55). Furthermore, chronic kidney disease is marked by an increase in interstitial kidney fibrosis. Fibrosis is the hallmark of CKD, and is characterized by the pathological deposition of excessive extracellular matrix (57). This matrix is often rich in collagens type I and III, as well as fibronectin (58, 59). In the presence of fibrosis, tubule cells cannot secrete toxins or receive nutrients from circulation due to the separation from peritubular capillaries by fibrosis (57). The processes that underlie fibrosis are similar to those that occur in normal wound healing. Whereas in wound healing myofibroblasts play a beneficial role, the persistence of this cell type results in detrimental processes indicative of fibrogenesis (57).

Myofibroblasts are the cell type most responsible for the production of cytokines and chemokines leading to increase deposition of extracellular matrix proteins, particularly collagens (57). Under normal conditions, myofibroblasts are not detectable in the kidney. During pathogenic processes, these myofibroblasts are found to increase greatly in number and are found usually in the interstitium and around injured glomeruli, where fibrosis most commonly occurs (57). The source of these myofibroblasts is still a matter of debate. Intricate fate-mapping studies have shown that a small percentage of myofibroblasts arise from resident fibroblasts in the kidney, while most are derived from pericytes (57) .

Immune cells, particularly macrophages, play a pivotal role in resolving fibrogenesis. This is due to the fact that macrophages are able to internalize

collagen and degrade it with MMPs (60) . Once degraded, collagen can then be further destroyed by cathepsins and lysosomes (60). In doing so, these macrophages can ameliorate low levels of fibrosis. Currently, available animal models to study chronic kidney disease focus on this fibrotic aspect and developing therapies to ameliorate fibrosis. These models include the UUO, IR, and folic acid models (61).

As previously discussed, AKI is associated with an increased likelihood of developing chronic kidney disease based on severity and the number of AKI occurrences (50). However, recent studies have suggested that AKI can progress to CKD, and that AKI and CKD are interconnected syndromes (62). The process by which this AKI to CKD transition occurs is very complex, but focuses on the underlying mechanism of maladaptive repair. To understand the processes that occur during maladaptive repair, it is first important to understand the processes involved in adaptive repair.

After AKI, neutrophils are responsible for the activation of the immune system and recruit M1 type macrophages to the site of injury (15, 21). While M1 macrophages have been associated with worsening of injury, M1 macrophages quickly take on a M2 phenotype, which is essential for adaptive repair (63, 64). These M2 macrophages remove cellular debris and secrete TGFβ, fibronectin, and other growth factors that are essential for supporting the growth of new epithelial cells to replace those that have undergone apoptosis or necrosis (63, 64). The source of these new epithelial cells has been extensively studied, and

data suggest that these cells arise from existing epithelial cells which are able to dedifferentiate and proliferate to replace lost cells (21, 57).

There are several overlapping processes that define adaptive and maladaptive repair. The true difference between these forms of repair depends on whether or not the injury is singular (as is often the case with AKI), or if it is recurrent. Grgic *et al*. developed a Six2-Cre-LoxP technology to selectively express the diphtheria toxin receptor in proximal tubule cells (65). Using this model, they showed that a tubule cells are able to repair injury from a single dose of diphtheria toxin (65). However, after three doses of the toxin, persistent inflammation ensued, leading to increased levels of TGFβ1, collagen Type 1a1 (col1a1), and fibronectin (65). This ultimately resulted in progressive fibrogenesis and secondary glomerulosclerosis, indicative of CKD (65).

To better understand the processes occurring with recurrent injury, maladaptive repair in terms of both the cell cycle of tubular epithelial cells and the role of immune cells, particularly macrophages, has been extensively studied in the context of CKD. In several models of CKD, it has been shown that cells often become senescent, and Grgic *et al.* have hypothesized that senescence may play a pivotal role in maladaptive repair associated with recurrent injury (65). Senescence is a detrimental process for two reasons. For one, senescent cells will remain present in the kidney and cannot be cleared by recruited macrophages. Thus, they cannot be replaced with new epithelial cells. Ultimately, this results in a decline in kidney function that is permanent. Secondly, even in this senescent state, epithelial cells continue to produce pro-

fibrotic factors, thus increasing fibrogenesis (21, 66). Yang *et al.* demonstrated that this senescent state was the result of G2/M cell cycle arrest in these epithelial cells (66).

Furthermore, it has been shown that recruited M1 macrophages are unable to undergo the switch to M2 type macrophages, thereby leading to maladaptive repair and worsened injury (67). While M2 subtype macrophages are regarded as protective against maladaptive repair, recent studies have suggested that M2 macrophages can also be key in fibrogenesis (68). When present at high levels in a unilateral ureteral obstruction model, M2 macrophages produce large amounts of TGFβ, which is an inducer of fibrotic pathways (68). When M2 macrophages were depleted in this model, fibrosis was ameliorated (68). Ultimately, maladaptive repair mechanisms are associated with recurrent, persistent injury and models of CKD. Of maladaptive repair mechanisms, cell cycle arrest and the role of both M1 and M2 macrophages have been shown to play a role in kidney fibrosis.

With the increasing prevalence of CKD and more studies are emerging on the importance of the AKI to CKD transition/ recurrent injury, it is important to have animal models that can be used to recapitulate the process by which this happens. This is especially true for cisplatin-induced AKI, as approximately 10-20% of all cancer patients will receive cisplatin alone or in combination therapy, and 33% of these run the risk of developing AKI. Furthermore, most of what is known about maladaptive repair involved in AKI to CKD transition has been studied in ischemia/reperfusion models of AKI. Unfortunately, no such models exist for

cisplatin-induced AKI leading to CKD. Furthermore, the standard model of cisplatin-induced AKI does not recapitulate the type of treatment regimen patients receive and does not allow for long-term studies. With the standard model of AKI, mice are administered a single, high dose of cisplatin (>20 mg/kg). This dose is lethal to the mouse beyond 72 hours, and thus examining the AKI to CKD transition, a process that may take place over several decades in humans, is not possible. Furthermore, humans receive multiple low doses of cisplatin. This is done to decrease the likelihood of AKI while maintaining a dose that is efficacious for the treatment of the cancer.

Thus, we have developed a low dose, repeated dosing model of cisplatin induced kidney injury that both recapitulates the type of dosing regimen patients receive, as well as allows for long-term analyses of what happens to the kidneys post-AKI. We have found that with this model, processes indicative of AKI such as high levels of cell death do not occur, but that the ultimate pathological outcome is fibrosis. Thus, this model can be used to examine the transition from AKI to CKD in a mechanistic way. In doing so, this will provide insight into when maladaptive repair occurs temporally and if processes that occur in this model are similar to those seen in models of CKD. By understanding more about what happens in this transition mechanistically, this will provide insight into possible therapeutic targets for preventing injury or ameliorating fibrosis.

MATERIALS AND METHODS

Reagents and Antibodies

The following antibodies were purchased from Cell Signaling (Beverly, MA) unless otherwise noted: Cleaved Caspase 3 (#9664), C/EBP homologous protein (CHOP; #2895), c-Jun N terminal kinases (JNK; #9258), p-JNK (#4668), transforming growth factor β (TGF-β; #3712S), fibronectin (F3648, Sigma-Aldrich, St. Louis, MO), phospho- Mothers against decapentaplegic homolog 3 (pSMAD3; #12747), α-tubulin (Santa Cruz, Dallas, TX, SC-23948) and β-actin (Santa Cruz, SC-47778). Cisplatin (P4394, Sigma Aldrich, St. Louis, MO) was used for experiments comparing the effects of a high, single dose model (euthanized three days later) with the new repeated dosing model. Pharmaceutical grade cisplatin (purchased directly from the University of Louisville hospital pharmacy) was used for experiments comparing the effects of single versus repeated injury from cisplatin. Similar effects were observed for both sources of cisplatin.

Animals

FVB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12-hour light/12-hour dark cycle and provided food and water *ad libitum*. Animals were maintained under standard laboratory conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville and followed the guidelines of the

American Veterinary Medical Association. Cisplatin at 25 mg/kg in PBS (200 µL/animal) was administered by i.p. injection. Seventy-two hours after cisplatin injection, these mice were euthanized. Another cohort of mice received either 7 or 9 mg/kg cisplatin in PBS administered by i.p. injection once a week for four weeks. For these survival studies, mice were monitored for weight loss and signs of discomfort/distress on a daily basis. Mice exhibiting a weight loss of 20% or more total body weight or high levels of discomfort and stress were euthanized. Mice that survived the course of treatment were sacrificed 72 h after the fourth injection of cisplatin. Serum was prepared and stored at -80°C. The kidneys were flash frozen in liquid nitrogen or fixed in 10 % neutral buffered formalin for histological purposes.

Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr) Determination

BUN (DIUR-500) and SCr (C7548-120) were determined using kits from Bioassay Systems (Hayward, CA) and Point Scientific Inc. (Canton, MI), respectively following the manufacturers' instructions. For SCr, this specific assay kit employs a two reagent enzymatic assay system to eliminate interference by endogenous creatine and ascorbic acid.

Protein Quantification, Western Blot Analysis, and ELISAs

Homogenates were made from kidney cortex by homogenization in Cell Extraction Buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl,1 mM imidazole, 1 mM magnesium acetate, 20 mM EGTA, 10 mM β-mercaptoethanol) containing a Complete Protease Inhibitor Cocktail Tablet and Phosphatase Inhibitor Cocktail Tablet (Roche, Indianapolis, IN). Homogenates were centrifuged at 15,000 *X g* for 10

minutes at 4° C. Supernatants were removed, vortexed, aliquoted, and stored at -80°C until use. Protein concentrations were determined using Bradford Reagent (Bio-Rad, Hercules CA). Kidney homogenate (40 µg) was separated on 4–12% gradient tris-glycine-SDS polyacrylamide gels and transferred to PVDF membranes that were blocked in 5% (w/v) dried milk in tris-buffered saline containing 0.1% Tween-20 (TBST) for 15 minutes. Membranes were incubated with 1:5000 dilutions of primary antibody overnight at 4°C. The next morning, membranes were washed 3 times for 5 minutes each with TBST containing 5 % (w/v) dried milk. After incubation for 1 hour at room temperature with secondary antibodies conjugated with horseradish peroxidase (1:40,000 in TBST containing 1.25% (w/v) dried milk), membrane proteins were detected by chemiluminiscence substrate. ELISAs for KIM-1 (DY1817, R&D systems, Minneapolis, MN) and NGAL (DY1857 R&D Systems) were performed on the urine as directed by the manufacturer.

Gene Expression

Total RNA was isolated using RNA-STAT 60 (TEL-TEST Inc., Friendswood, TX) combined with mini bead-beater glass beads and a Mini Bead Beater machine (Cole-Palmer, Vernon Hills, IL). Other than using the bead beater glass beads to disrupt the tissue, the protocol provided by TEL-TEST was used. cDNA was made from 1 µg of total RNA using High Capacity Reverse Transcriptase (Life Technologies, Grand Island NY) following manufacturers' instructions. Gene specific cDNAs were quantitated using real-time PCR using predesigned TaqMan assays. Tumor necrosis factor-alpha (TNF-α, Mm00443258_m1), interleukin-6

(IL6; Mm00446190_m1), interleukin 1-beta (IL1β; Mm0043228_m1), chemokine ligand (c-x-c motif) 1 (CXCL1; Mm04207460_m1), monocyte chemotactic protein-1 (MCP-1; Mm00441242_m1), plasminogen activator inhibitor-1 (PAI-1; Mm00435860_m1), alpha- smooth muscle actin (α-SMA ; Mm1546133_m1), bone morphogenetic protein-7 (BMP-7; Mm00432102_m1), collagen type 4a1; (COL4a1; Mm01210125_m1), cyclin dependent kinase inhibitor 2a (Cdkn2a; Mm00491449-m1), connective tissuse growth factor (CTGF; Mm01192932_g1), collagen type 1a1 (Col1a1; Mm00801666_g1), and the normalization genes beta 2 microglobulin (B2M; Mm00437762_m1) and β-actin (Mm01205647-g1) were purchased from Life Technologies (Grand Island NY) and used in combination with 2X Gene expression Master Mix (Life Technologies).

Histology

Kidney sections (5 microns thick) from cisplatin-treated and untreated animals were stained with H&E and PAS, and the degree of morphologic changes was determined by light microscopy in a blinded fashion by a renal pathologist. The following measures were chosen as an indication of morphologic damage to the kidney after treatment with vehicle or cisplatin: proximal tubular necrosis, loss of brush border, proximal tubule degradation, tubular casts, presence of inflammatory cells, and interstitial fibrosis These measures were evaluated on a scale from 0 to 4, which ranged from not present (0), mild (1), moderate (2), severe (3), and very severe (4).

Immunohistochemistry

Kidney sections (5 microns thick) were rehydrated in Histoclear followed by an ethanol gradient. Antigen retrieval was performed in citric acid buffer pH 6.0 at 95°C in a steamer for 30 min. Endogenous peroxidases were inhibited with 3% hydrogen peroxide and dual endogenous enzyme blocker (Dako, S2003) for 10 minutes, followed by two 5 minute PBS washes. Slides were then blocked with avidin for 10 minutes followed by a PBS wash, and then biotin for 10 minutes followed by another wash in PBS (Dako, X0590). Slides were further blocked with 5% normal goat serum in 0.1% TBST for 1 hour at room temperature. α-SMA primary rabbit antibody (Abcam, ab5694) was added to slides at a concentration of 0.5 µg/mL, and allowed to incubate at 4°C overnight. Biotinylated goat anti-rabbit IgG antibody (1:25000) (Vector laboratories, BA-1000) was added to each section and incubated for 30 minutes at room temperature. Slides were rinsed twice with PBS for 5 minutes each. Vector ABC reagent (Vector laboratories, PK-7100) was added to each section and incubated for 30 minutes at room temperature. Slides were rinsed 2 times with PBS for 5 minutes each, followed by the addition of NovaRed Substrate to detect HRP (Vector Laboratories, SK-4800) to each section for 5-7 minutes. Slides were rinsed in distilled water for 5 minutes, counterstained with modified Mayer's hematoxylin (Thermo-scientific, 72804), then dehydrated in an ethanol gradient to Histoclear, followed by mounting with Permount (Fisher Scientific, SP15). Positive staining for α-SMA was quantified using Metamorph Image Analysis software, and percent positive pixels were calculated as follows: (threshold area/(total area-acellular area)).

Sirius Red/ Fast Green Staining

Kidney sections (5 microns thick) were rehydrated in Histoclear followed by an ethanol gradient. Slides were then dipped into a Coplin jar containing PBS-T (PBS+0.1% tween-20) and incubated for 5 minutes. Slides were washed with distilled water twice for 5 minutes each and then incubated in saturated picric acid (1.2% w/v, Ricca Chemicals; 5860-32) containing 0.1% Sirius Red (Sigma, 365548) and 0.1% Fast Green (Sigma, F7258). Slides were washed with 5% glacial acetic water until water ran clear. Tissue samples were then dehydrated and fixed using Permount (Fisher, F-SP15-100). Positive staining for Sirius Red was quantified using Metamorph Image Analysis software, and percent positive pixels were calculated as follows: (threshold area/(total area-acellular area)).

Statistical Analysis Data

Data are expressed as means ± SEM for all experiments. Multiple comparisons of normally distributed data were analyzed by one-way ANOVA, as appropriate, and group means were compared using Tukey post-tests. Single comparisons were analyzed by Student's t-test where appropriate. The criterion for statistical differences was $p < 0.05$*, $p<0.01$** and $p<0.001$***. For statistical analysis of the survival curve, Log-Rank (Mantel-Cox) test was used.

RESULTS

Effects of dosing regimens on mouse survival. The current, standard model used to study cisplatin-induced AKI does not allow for the analysis of long-term effects on kidney function and physiology, nor does it recapitulate the repeated dosing regimen of cisplatin patients receive in the clinic. We hypothesized that administration of a low dose of cisplatin once a week for several weeks, which better recapitulates the human dosing regimen, would improve mouse lifespan, thus allowing for analysis of long-term effects on kidney function and physiology. We compared survival of mice given a single high dose of cisplatin (standard dosing model; 25 mg/kg) to mice given a dose of cisplatin (7 or 9 mg/kg) once a week for 4 weeks. All mice subjected to the standard dosing regimen were sacrificed three days after cisplatin injection due to moribund status. Mice treated with the 7 mg/kg repeated dosing regimen survived the course of treatment and were sacrificed 3 days after the fourth treatment (day 24). Ninety percent of mice treated with a 9 mg/kg repeated dosing regimen survived until day 24 (Fig1). These data indicate that the repeated dosing model with 7 mg/kg enables longer-term survival of mice, and thus can be used for long-term studies of kidney function and physiology.

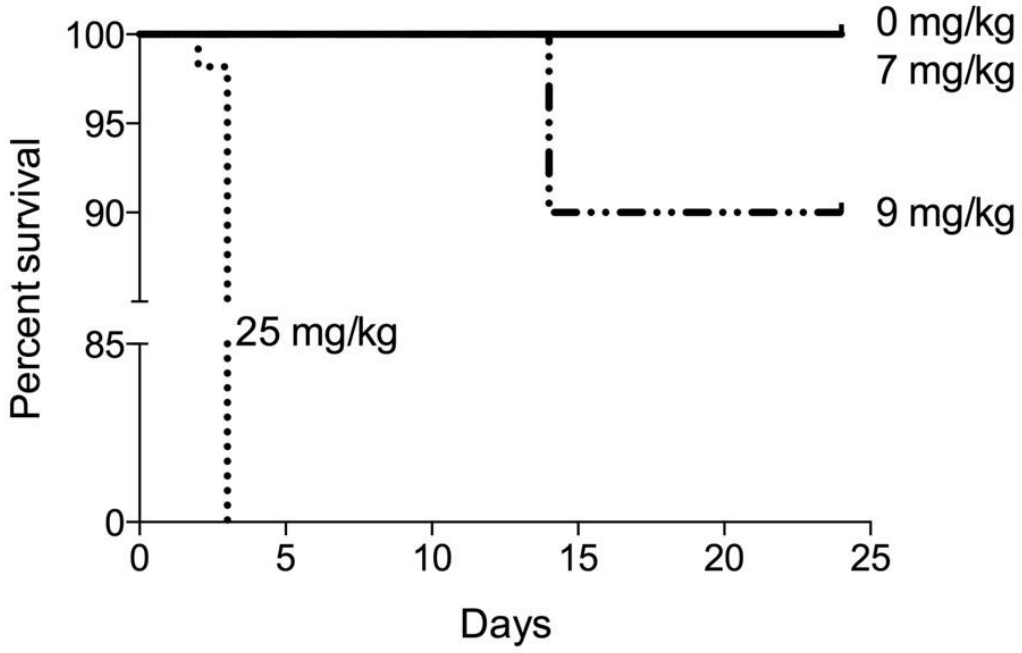

Figure 1. Survival curve of animals treated with the standard dosing regimen and repeated dosing regimen of cisplatin. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard model). Mice were monitored daily for weight loss and changes in overall well-being and were sacrificed when moribund in accordance to IACUC guidelines. At day 24, surviving mice were sacrificed and analyzed.

Effects of dosing regimens on kidney injury and function. In order to assess the impact of repeated, low dosing of cisplatin on the kidney, we measured markers of kidney function and injury in the serum and urine of mice, respectively. Blood urea nitrogen (BUN) and serum creatinine (SCr) are standard measures of kidney function as their levels increase in the blood when the filtering capacity of the kidney is significantly reduced (69). BUN levels of mice treated with the standard dosing model were significantly increased at 72 hours post-treatment (Fig2A). In the repeated dosing model, BUN also increased, but not significantly (Fig2A). SCr levels were significantly increased for both the repeated and standard dosing models, but SCr levels were higher in the standard dosing model (Fig2A). Kidney injury was evaluated by levels of urinary KIM-1 and NGAL, sensitive biomarkers of AKI (70, 71). Urinary KIM-1 and NGAL levels were significantly increased in the standard dosing model, but only NGAL levels were significantly increased in the repeated dosing model, albeit to a lesser extent than in the standard model (Fig 2B). These data indicate that the repeated dosing model of cisplatin causes less loss of kidney function and less kidney injury than the standard dosing model.

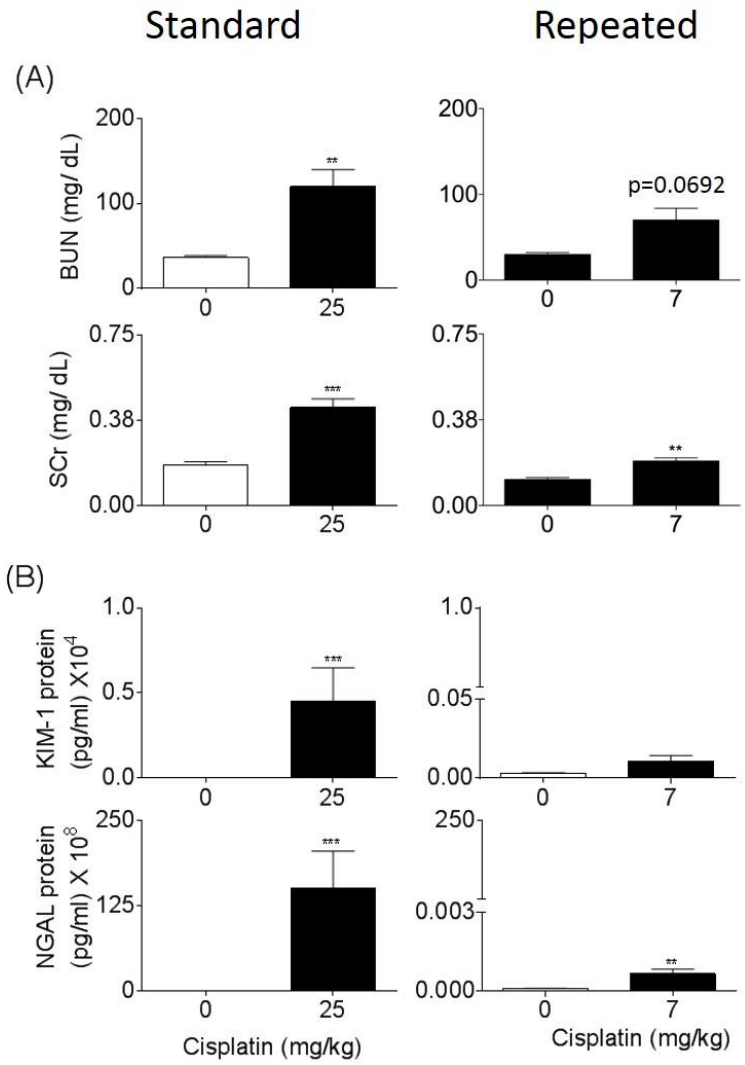

Figure 2. Comparison of kidney function and injury between standard dosing model and repeated dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard model). Animals were sacrificed 72 h after last injection. Levels of (A) BUN and SCr measured in the serum. (B) KIM-1 and NGAL measured in the urine. Data expressed as mean ± SEM, n=10. Statistical significance was determined by Student's t-test ** indicates $p<0.01$ and ***indicates $p<0.001$.

Effects of dosing regimen on inflammatory cytokine and chemokine levels. One of the components of the standard dosing model of AKI is a large inflammatory response. We compared the levels of inflammatory cytokines and chemokines between the standard and repeated dosing models. TNFα is a potent cytokine that mediates inflammatory tissue damage in the kidney, and activates downstream cytokines and chemokines, particularly IL1-β, MCP-1, and IL-6 (4, 72). CXCL1 plays a role in neutrophil recruitment to sites of tissue inflammation (73). mRNA levels of *Tnfα, Il1-β, Mcp-1, and Cxcl1* were increased significantly and approximately to the same extent in both the standard and repeated dosing models (Fig3A, C, D, E). *Il-6* mRNA was significantly increased in the standard dosing model, but only increased 5.51±2.01-fold in the repeated dosing model (Fig3B). These data suggest similar effects of dosing on activation of inflammatory cytokines and chemokines.

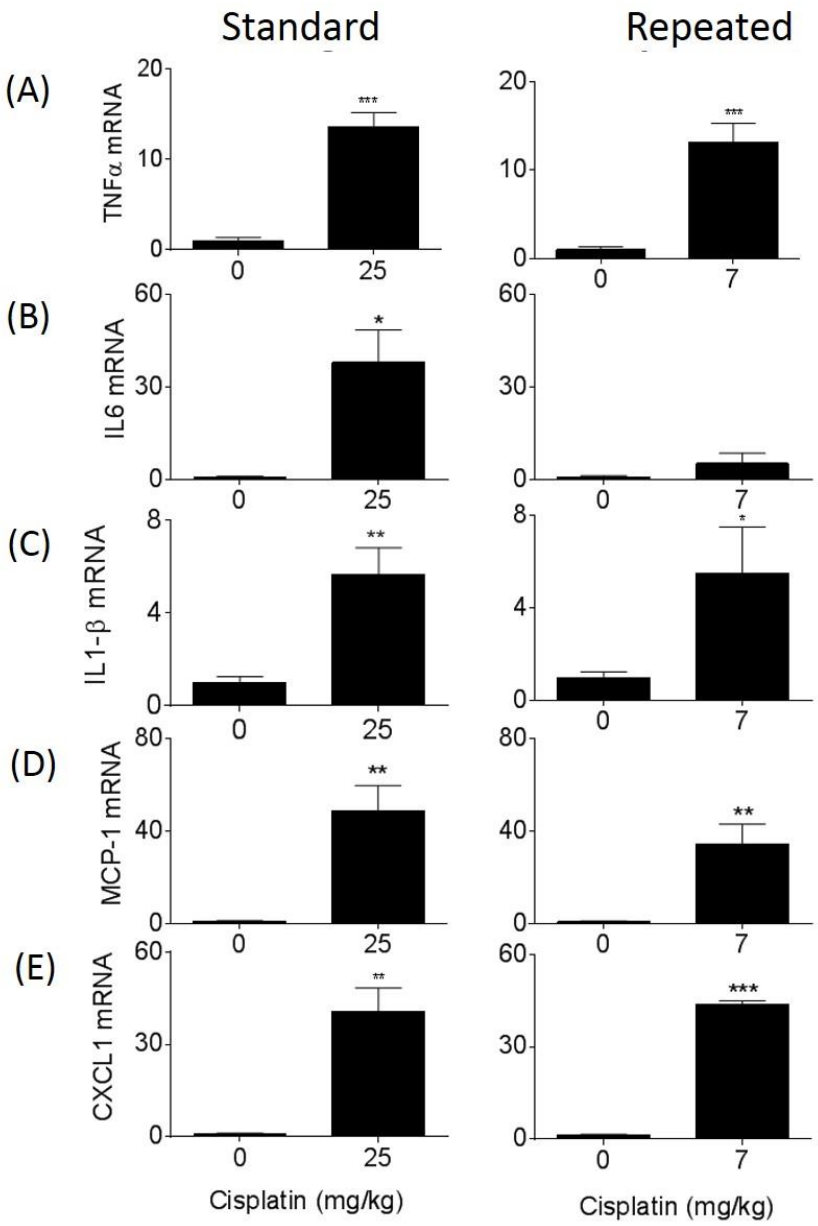

Figure 3. Comparison of inflammatory markers between standard dosing model and repeated dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard model). Animals were sacrificed 72 h after last injection. mRNA levels of (A) TNFα, (B) IL6, (C) IL1-*β*,(D) MCP-1, and (E) CXCL1 were assessed in kidney cortex via real-time qRT-PCR and were normalized to their vehicle control. Data expressed as mean ± SEM, n=10. Statistical significance was determined by Student's t-test *indicates $p<0.05$, ** indicates $p<0.01$ and ***indicates $p<0.001$.

Effect of dosing regimen on activation of ER stress and cell death proteins. Cell death and ER stress are characteristic of cisplatin-induced AKI and inhibiting/deleting key players in apoptosis protects the kidney from cisplatin in the standard dosing model (7, 74, 75). Therefore, we assessed cellular markers of ER stress and cell death proteins in both models. JNK phosphorylation and activation is associated with ER stress-induced apoptosis and G2/M cell cycle arrest (15, 66). We found that JNK was phosphorylated and activated in both models (Fig4). CHOP is also associated with ER stress, and was also activated in both models (Fig4) (76). However, cleaved caspase 3 (CC3), a marker of apoptosis, didn't show an increase in the repeated dosing model. (Fig4). These data suggest that while both models show similar trends in activation of ER stress proteins, there is less cell death activation in the repeated dosing model.

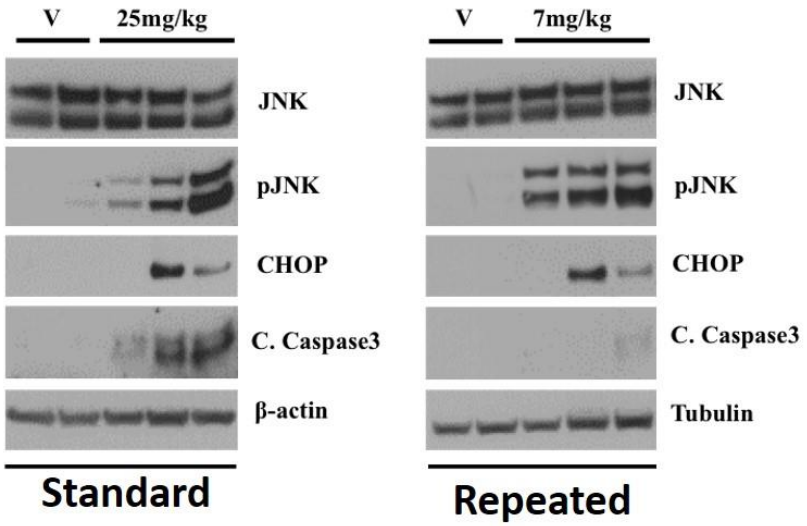

Figure 4. Repeated dosing model shows similar increases in ER stress and cell death markers as compared to standard dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard model). Animals were sacrificed 72 h after last injection. Markers of ER stress and cell death were assessed in kidney cortex homogenates via western blot analysis.

Effect of dosing regimen evident in tissue pathology. The standard dosing model of cisplatin-induced AKI is associated with changes in kidney pathology, particularly with increased levels of tubular necrosis and loss of brush borders (21, 77). We compared kidney pathology of the standard and repeated dosing models and examined tubular necrosis, loss of brush borders, tubule dilation, cast formation, presence of inflammatory cells and interstitial fibrosis, all of which are indicative of kidney injury and damage. Blinded analysis by a certified pathologist indicated tubular necrosis was significantly higher in the standard dosing model, compared to the repeated dosing model (Fig5A). In contrast, there was a significant loss of brush borders (Fig5B), an increase in tubular dilation (Fig5C), and an increase in cast formation (Fig5D) in both models. Interestingly, only the repeated dosing model displayed a significant increase in the presence of inflammatory cells and interstitial fibrosis (Fig5E,F). These data demonstrate that there are key differences in kidney pathology between the standard and repeated dosing models.

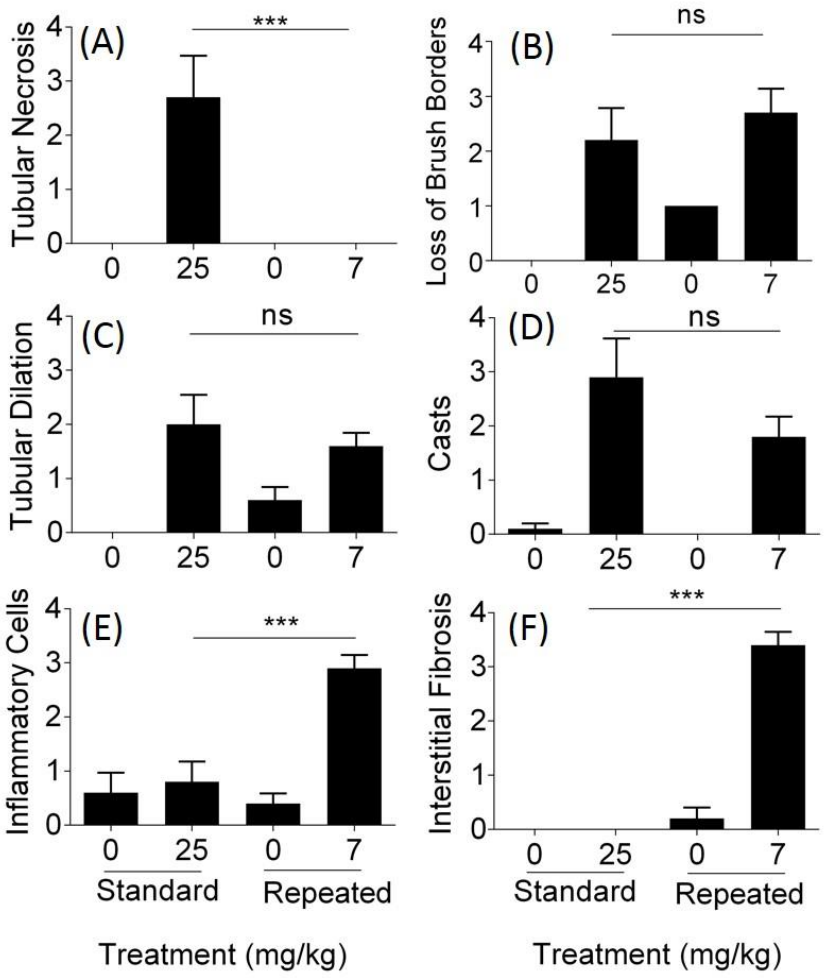

Figure 5. Quantification of various kidney tissue pathology indices. Renal histological changes were assessed on H&E and PAS stained sections 5 microns thick. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard model). Animals were sacrificed 72 h after last injection. (A) Tubular necrosis, (B) loss of proximal tubule brush borders, (C) proximal tubule dilation, (D) proximal tubule cast formation, (E) presence of inflammatory cells and (F) interstitial fibrosis were scored in a blinded manner by renal pathologist Dr. Megyesi using a scale of 0-4 (0=not present, 1=mild, 2=moderate, 3=severe, and 4=very severe renal histological changes in the proximal tubules). n = 5-10; data are the mean ± SEM. Statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test, ***indicates $p<0.001$.

Fibrotic markers and fibrosis in the repeated dosing model. Since pathology of kidney sections revealed tubulointerstitial fibrosis in the repeated dosing model, we wanted to examine known markers of maladaptive repair involved in the induction of fibrosis. TGF-β is released by immune cells in response to kidney injury as a way to make conditions suitable for new epithelial cells to grow (22). However, during maladaptive repair, dedifferentiated epithelial cells in a senescent state can also release TGF- β, leading to fibrosis (21). TGF-β can signal through its receptor to lead to phosphorylation of SMAD3, thereby activating pathways that increase extracellular matrix protein deposition, particularly fibronectin (78, 79). BMP-7 is also a member of the TGF-β superfamily and works to counteract the profibrotic activity of TGF-β (80). TGF-β, p-SMAD3, and fibronectin were all increased at the protein level in the repeated dosing model (Fig6A). *Bmp-7* mRNA expression was significantly decreased (Fig6B). *Col1a1*, which encodes for collagen type I protein and is a transcript marker of fibrosis, also significantly increased at the message level in the repeated dosing model (81)(Fig6B). PAI-1 is associated with maladaptive repair and is produced by resident and intrarenal inflammatory cells and inhibits fibrinolysis, leading to the accumulation of scar tissue in the kidney (82). *Pai-1* mRNA expression was increased in the repeated dosing model (Fig6B). *Cdkn2a* encodes for p16, and increased expression of *Cdkn2a* is associated with maladaptive repair and cell cycle arrest at the G2/M phase (66); *Cdkn2a* expression was significantly increased following repeated dosing (Fig6B). Myofibroblast numbers increase during kidney fibrosis and are responsible for synthesis of collagen (83, 84). These cells can be identified by

their expression of α-SMA. α-SMA IHC indicated increased numbers of myofibroblasts following repeated dosing of cisplatin (Fig6D). Collagen deposition as the result of extracellular matrix production can be quantified with Sirius red, fast green staining (SR/FG) which stains collagen red. We performed SR/FG staining and found that collagen levels increased in the kidneys following repeated dosing of cisplatin (Fig6C). Taken together, these data indicate that there are alterations in key mediators of kidney fibrosis in the repeated dosing model, indicative of maladaptive repair. Furthermore, since fibrosis is the hallmark of CKD, these data suggest that the ultimate outcome of our repeated, low dosing cisplatin model is CKD.

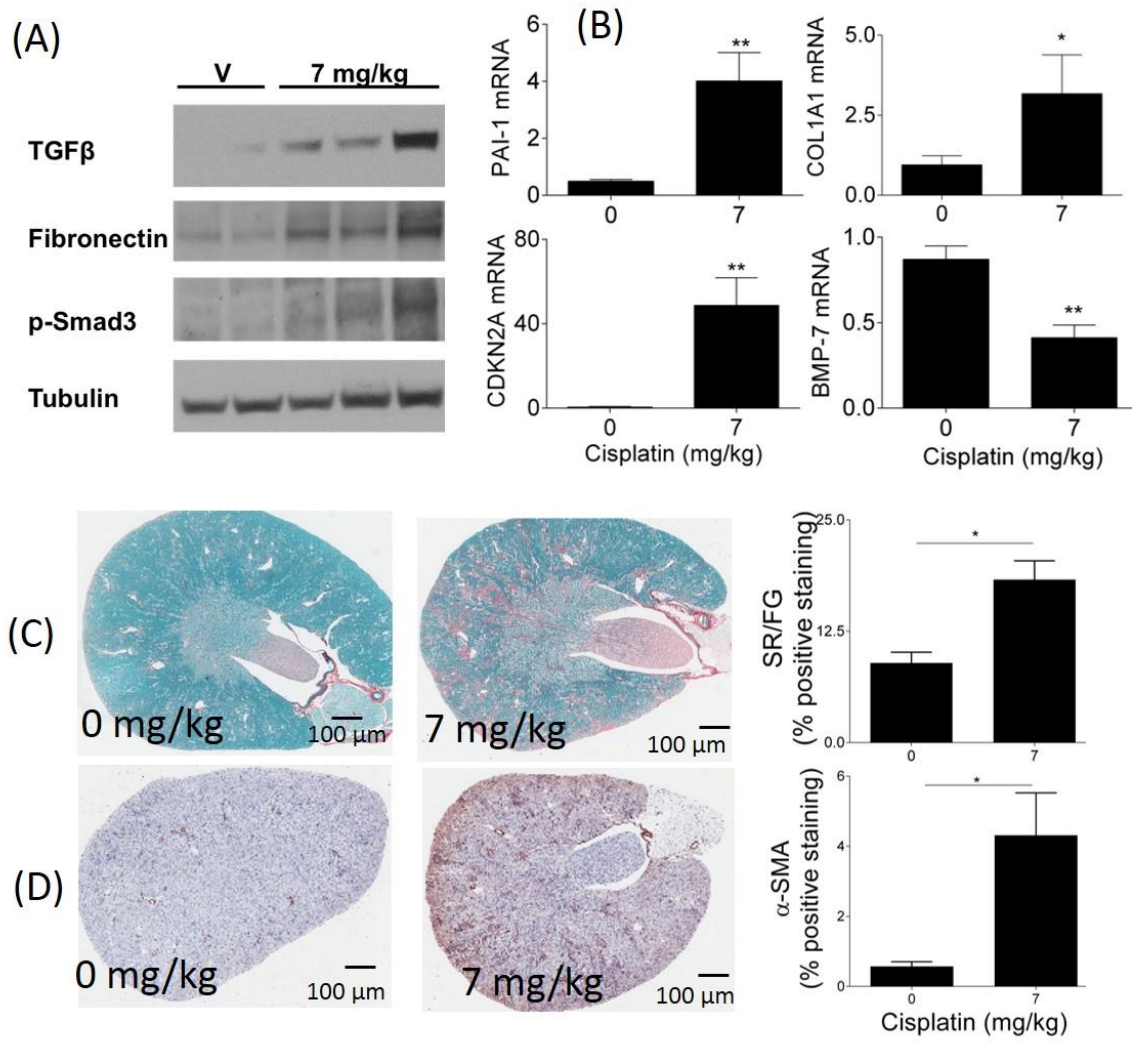

Figure 6. Assessment of fibrosis and pro-fibrotic markers. Eight-week old FVB mice were injected (i.p.) with saline vehicle, or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), Animals were sacrificed 72 h after last injection. (A) Markers of fibrosis in the kidney cortex assessed via Western blot. (B) Measurement of mRNA levels of fibrotic marker in the kidney cortex assessed via real-time qRT-PCR. (C) SR/FG staining of kidney sections and quantification of staining (D) α-SMA IHC staining in the kidney cortex and quantification of staining. Data expressed as mean ± SEM, n=5-10. Statistical significance was determined by Student's t-test, *indicates $p<0.05$** indicates $p<0.01$.

Comparison of a single, low dose of cisplatin (7VVV) to repeated dosing model. To determine if fibrosis is a result of a single, low dose of cisplatin, or rather repeated injury from several low doses, mice were administered a low dose of cisplatin (7 mg/kg) followed by three weekly vehicle injections (7VVV) and compared to mice receiving 4 weekly cisplatin injections (7777). BUN and SCr both increased significantly in the repeated dosing model, but not following a single low dose (Fig7A). Likewise, levels of *Tnfα* and *Il6* mRNA expression increased with repeated dosing (7777) of cisplatin, but not following a single dose (7VVV; Fig7C). Western blot analysis of TGF-β and fibronectin in the kidney indicated that a single dose was not sufficient to cause an increase in these fibrotic markers (Fig7B). mRNA expression of *Pai-1, Cdk2na*, and *Col1a1* did not increase, and *Bmp-7* mRNA levels did not decrease in the single, low dose regimen (Fig7C). Quantification of SR/FG staining for collagen deposition and IHC staining for α-SMA for myofibroblasts also indicated that a single low dose was insufficient to elicit changes (Fig7D,E). These data suggest that fibrosis is a result of repeated injury to the kidney by several low doses of cisplatin.

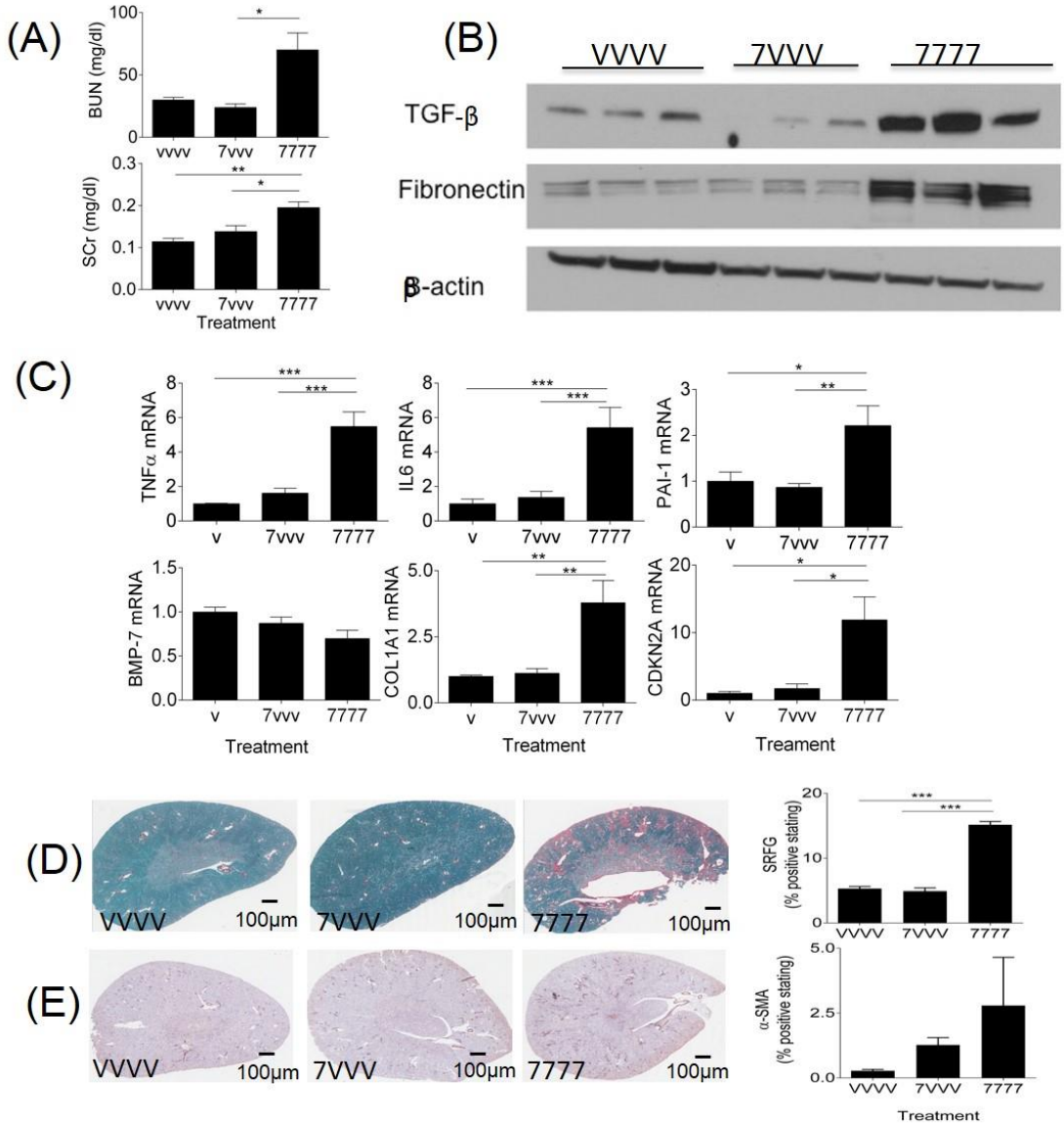

Figure 7. Comparison of single, low dose and repeated dosing regimens. Eight-week old FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once, or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), Animals were sacrificed 72 h after last injection.(A) Levels of BUN and SCr assessed in the serum .(B) Markers of kidney fibrosis assessed via western blot. (C) mRNA levels of inflammatory cytokines and fibrotic marker in the kidney cortex measured by real-time PCR. (D) SR/FG staining of kidney sections and quantitation of staining. (E) α-SMA IHC staining in kidney and quantitation of staining. Statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test, *indicates $p<0.05$** indicates $p<0.01$ ***indicates $p<0.001$.

DISCUSSION

The dosing regimen of cisplatin varies by cancer type as well as the ability of the patient to handle prescribed doses. In lung cancer, for example, patients receive 60-100 mg/m^2 of cisplatin intravaneously every 21 days in conjunction with other antineoplastic drugs, while patients with cervical cancer receive 40 mg/m^2 of cisplatin intravaneously once a week for six weeks in conjunction with radiation therapy (85). Regardless, treatment of human cancers with cisplatin often leads to a nephrotoxic effect that is cumulative and dependent on the dose of cisplatin being administered. AKI occurs in some individuals even after one low dose of cisplatin, and when AKI occurs, oncologists will dramatically lower the dose of cisplatin used- usually decreasing the dose by 50-75% (85). As a result, this leads to a less efficacious cancer treatment. Thus, the development of renoprotective agents would greatly aid in allowing patients to receive the full dose of cisplatin for a more efficacious treatment, while at the same time decreasing the likelihood of an AKI event. This area of research has been ongoing for thirty or more years. Unfortunately with the exception of amifostine, which is no longer used as a renoprotective agent in the clinic, there are no renoprotective agents available and none have succeeded in clinical trials. This is in opposition to the fact that many renoprotective agents have been tested in rats and mice and have shown a significant protective effect.

Furthermore, it is established that multiple episodes of AKI can lead to CKD (51). This is of importance as, until recently, it was widely held that patients who suffer from an episode of AKI usually undergo full recovery. This is a biased view as the standard measure of kidney function in the clinic is SCr, which is highly unreliable and can only detect changes in function when there is fifty percent nephron loss.

In an ideal situation, researchers would be able to study the long-term effects of cisplatin-induced AKI in a model that recapitulates a dosing regimen similar to what is used in humans- mainly the fact that patients receive multiple doses of cisplatin over an extended period of time. Unfortunately, there are no models to study this. Currently, the standard dosing model of cisplatin-induced AKI has limitations that cannot be overcome for studying AKI to CKD progression. For one, a single, high dose regimen is not clinically relevant. Patients are administered multiple low doses of cisplatin over an extended period of time. Secondly, with the standard dosing model, mice cannot be aged out to study long-term effects associated with repeated AKI, namely fibrosis—the underlying pathology of CKD (86). Furthermore, models of CKD, which often lead to fibrosis, often are short term models that lead to this pathology within a week (i.e. UUO/ IR models), and there no models of cisplatin-induced CKD. Here, we report a new model for studying the nephrotoxic effects of cisplatin that mimics the repeated administration of cisplatin given clinically, and allows for analysis of long-term effects on kidney function. Data obtained from this model indicate that repeated cisplatin injury induces interstitial fibrosis which is indicative of CKD, and suggests

that targeting fibrotic mediators may prevent both short- and long-term renal side-effects of cisplatin.

The standard dosing model of cisplatin induces high levels of kidney injury and cell death through apoptosis and necrosis, which are processes known to be involved in AKI (7). This in turn results in a rapid loss of kidney function. With the repeated dosing model, cleaved caspase 3 as a measure of apoptosis is low, and pathology reveals a low level of tubular necrosis. This coincides with lower injury levels and a smaller decline in overall kidney function. These lower levels of injury and the maintenance of kidney function with the repeated dosing model may be key to explaining how mice treated with multiple low doses of cisplatin are able to survive for 24 days, and beyond that.

As opposed to cell death, the repeated dosing model of cisplatin induces interstitial fibrosis. Fibrosis is regarded as the result of maladaptive repair, which is marked by prominent inflammation, G2/M cell cycle arrest leading to cellular senescence, and the continual release of growth factors by epithelial cells that ultimately culminate in fibrosis (21, 65, 66). While inflammation is also indicated in AKI, it is associated with adaptive repair, and is usually short-lived. In maladaptive repair, however, inflammation persists and is marked by the inability of M1 macrophages to become M2-type (36). M1 macrophages are highly associated with worsening of kidney injury, and thus suggests that the persistence of this cell type is something that needs to be further examined within our model. As far as inflammatory cytokines and chemokines, both models show a similar activation of TNFα and its downstream targets, with the exception of IL6. In the

repeated dosing model, IL6 was not significantly elevated. Although the role of IL6 in kidney injury is not well understood, it is correlated with onset and severity of AKI, and has been indicated as a potential urinary and plasma biomarker of AKI (87-89). The differential of IL6 elevation between a high injury model (standard AKI model), and a low injury model (repeated dosing model) provides further validity of the sensitivity of IL6.

In AKI, p21 and p53 play major roles in the adaptive processes after injury (18, 22, 26, 90). p21 is responsible for cell cycle arrest before S phase, so that damage to injured proximal tubule cells can be repaired before proceeding through the cell cycle, while p53 mediates apoptosis (15, 22). These processes are pivotal in removing injured epithelial and tubule cells, and also allowing for the growth of new, fully functioning ones to restore kidney function. In maladaptive repair, p16 mediation leads to cellular senescence as opposed to apoptosis. This is mediated by G2/M cell cycle arrest (65). Grgic *et al*. have shown in a model of diphtheria toxin receptor expression on proximal tubule cells leading to PTC only injury, that kidney function can be recovered after a single round of injury induced by diphtheria toxin (65). In contrast, recurrent injury resulted in persistent inflammation, cellular senescence, and G2/M cell cycle arrest of the tubule epithelial cells, which led to maladaptive repair and interstitial fibrosis (65). G2/M arrest and cellular senescence leading to maladaptive repair and fibrosis have also been indicated in the ischemia-reperfusion and unilateral ureteral obstruction mouse models (66). G2/M arrest is also associated with an increase in JNK phosphorylation (66). However, JNK has also been indicated as a mediator of cell

death as well. The increase in activated JNK (pJNK) observed in the repeated dosing model did not correlate to cell death. Instead, this increase in pJNK suggests G2/M cell cycle arrest may be occurring. This is supported by the increased expression of *Cdk2na*, a gene responsible for p16 (66). However, we do not know if cellular senescence is occurring in epithelial cells in this model, although there are increased levels of TGF-β and other pro-fibrotic cytokines which are released by dedifferentiated epithelial cells in the senescent state (21). To better determine if senescence is occurring, an assay for senescence-associated beta-galactosidase needs to be performed.

In conclusion, we have found that fibrosis, not cell death, is the main pathological outcome of our more clinically relevant repeated, low dosing cisplatin model. Previously developed renoprotective agents that have fared well in animal studies have focused on targeting cellular death pathways as a way to mediate renal injury. These drugs include caspase inhibitors to block the initiation and execution of apoptosis (91). Mice with caspase-1 deficiency are protected against cisplatin-induced AKI due to blocking activation of proinflammatory cytokines IL-1β, IL-18, and IL-6, suggesting that targeting caspases in humans may be worthwhile (74). Furthermore, pifithrin-a, a p53 inhibitor, also decreases apoptosis to preserve renal function during AKI (92). Since cell death is relatively low in our model, it is likely that these therapies would not have a renoprotective effect in the repeated dosing model of cisplatin. Our new model does provide several new target pathways for the development of renoprotective agents. Our data suggest that maladaptive repair processes are taking place in our model. Thus, potential

therapeutics include targeting G2/M cell cycle arrest to stop the process of cellular senescence and subsequent extended release of profibrotic cytokines.

Furthermore, studies on the role of the immune system need to be performed. While the role of different immune cell types have been somewhat defined in AKI models, very few studies have examined the role of these immune cells in fibrotic processes, other than the role of macrophages (15, 31, 57). Thus, understanding the mechanistic role of immune cells may provide better insight into what types of cells to target to ameliorate fibrosis or protect from the development of fibrosis, and lead to the potential development of immunotherapies for cisplatin-induced kidney injury.

Furthermore, maladaptive repair processes are very similar to those involved in normal aging. It is well documented that during aging, the kidney loses function naturally and kidney structure changes (93-95). Namely, aging is associated with increased profibrotic markers, and age is a contributing factor to number of deaths from CKD (95, 96). This suggests that aging plays an important role in kidney injury and subsequent pathologies. In addition, cancer is regarded as a disease associated with age, as there are approximately 4,750 per 100,000 patients who are 50 or older when diagnosed with cancer (97). This is especially true for lung cancer patients as median age of diagnosis and of death is 70 and 72 respectively (97). Since lung cancer is the leading cause of cancer deaths in Kentucky, this implies that improving on our current model by adding in factors such as advanced age and lung cancer would provide a more comprehensive view of cisplatin-induced nephrotoxicity, and potential interplay between kidney injury

and lung cancer. This is of utmost importance as fibrosis and inflammation are altered during aging and play a role not only in renal fibrosis, but also in cancer development and metastasis (98).

In fact, recent studies have suggested the existence of a pulmonary-renal syndrome in which inflammatory cytokines released by the lung during forms of acute lung injury (ALI) may lead to the onset of AKI (99). Furthermore, the kidneys may play a role in production and elimination of inflammatory mediators of ALI (99). In animal models of kidney ischemia-reperfusion, Rabb *et al.* determined that there were changes in gene expression of genes involved in pulmonary regulation of salt and water handling in alveolar epithelial cells (100). Furthermore, ischemia-reperfusion models have also been associated with altered cytokine production in the lung, showing increased levels of IL-1β, IL-6, and IL-12 in the lung (101). While these studies were done in the context of ALI, it is known that cancer is a largely inflammatory-mediated process (98). Thus, it is feasible that inflammatory changes occurring in the lungs may influence AKI and subsequent kidney disease in humans, and that inflammatory processes associated with cisplatin-induced AKI may have an effect on lung cancer.

Finally, the biggest advantage of our repeated low dosing model is that comprehensive, temporal studies can be performed by looking at changes that occur after each dose of cisplatin. We have shown that one dose at 7 mg/kg does not have a long-term effect on renal outcome, but that four doses does. However, we can now look at what happens at doses 2 and 3 as well to determine when fibrosis starts occurring, and correlate this to when changes in kidney function and

injury also start to change. Furthermore, we can perform immune cell studies at each of these time points to determine when certain immune cell types are most important.

Overall, our repeated, low dose cisplatin model of nephrotoxicity indicates that fibrosis may be a more physiologically relevant process to target in humans for the development of renoprotective or injury reversing agents. Not only are we able to examine long-term outcomes of multiple doses of cisplatin, but our model affords us the ability to go back and examine changes occurring temporally to provide better mechanistic insight into when maladaptive repair processes are taking place. Now that this model has been established, it is important to better characterize the processes that occur during the injury phase. This is of importance as most nephrotoxic therapeutic agents have focused on amelioration of injury, rather than protection against injury. By better understanding when and how injury occurs, we can better target nephrotoxicity leading to AKI or CKD before it happens.

SUMMARY

Cisplatin is a chemotherapeutic used for the treatment of many cancers but has nephrotoxic side effects leading to acute kidney injury (AKI). Thirty percent of patients administered cisplatin will develop kidney injury, requiring the oncologist to withhold or reduce the next dose, leading to a less effective therapeutic regimen. Although recovery can occur after one episode of cisplatin-induced AKI, longitudinal studies have indicated multiple episodes may lead to the development of chronic kidney disease (CKD). Furthermore, the severity of AKI sustained is also a predictor of CKD. CKD is an irreversible disease with no current treatments that can progress to end-stage renal disease (ESRD), and eventual mortality.

Most of what is known about the complex pathophysiology of AKI has been studied in animal models. The standard mouse model of cisplatin-induced AKI consists of one, high dose of cisplatin (> 20 mg/kg) that is lethal to the animal three days later. This model doesn't accurately reflect the dosing regimen patients receive (multiple doses over weeks or months), and doesn't allow for long-term studies to examine outcomes associated with CKD, particularly the development of fibrosis. We have developed a repeated dosing model with administration of cisplatin once a week for four weeks in mice. Comparison of this repeated dosing model to the standard dosing model demonstrated that inflammatory cytokines and chemokines were induced in the repeated dosing model, but levels of cell death

were lower in the repeated dosing model. The repeated dosing model had increased levels of fibrotic markers (fibronectin, TGFβ, and α-SMA), and pathology revealed that interstitial fibrosis was occurring. As fibrosis is the hallmark of CKD, these data demonstrate that the repeated dosing model can be used to study AKI to CKD progression.

With this newly established model, we hope to identify potential targets of the fibrotic pathway for the development of renoprotective agents. In order to do so, we need to better understand the mechanism by which AKI can progress to CKD. Planned future studies include examining immune cell populations at various time points to determine the temporal role of immune cells during this transitional process. Furthermore, we can also use this model to measure temporal changes in both kidney injury and function to better determine key points in the injury phase that are important to target for the development of renoprotective agents.

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LIST OF ABBREVIATIONS

AKI: acute kidney injury

CKD: chronic kidney disease

RIFLE: risk injury failure loss end-stage kidney disease

GFR: glomerular filtration rate

SCr: serum creatinine

Ctr1: copper transporter 1

OCT2: organic cation transporter 2

BCL2: B cell CLL/ lymphoma 2

BAX: BCL2-associated X protein

PUMA-α: p53 upregulated modulator of apoptosis

ATN: acute tubular necrosis

Cdks: cyclin dependent kinases

CIP/KIP: Cdk interacting protein/ kinase inhibitory protein

INK4: inhibitor of cyclin-dependent kinase 4

PCNA: proliferating cell nuclear antigen

BrdU: bromodeoxyuridine

ER (Stress): endoplasmic reticulum (stress)

MMPs: matrix metalloproteinases

UUO: unilateral ureteral obstruction

IR: ischemia reperfusion

LLC-PK1: Lilly laboratories porcine kidney 1 cells

IL-10: interleukin-10

TNFα: tumor necrosis factor alpha

FasL: Fas ligand

Tim-1: T-cell immunoglobulin and mucin domain-1

(p)SMAD3: (phospho) mothers against decapentaplegic 3

TGFβ-(1): transforming growth factor beta- (1)

Col1a1: collagen type 1a1

CHOP: CCAAT/enhancer-binding protein homologous protein

(p)JNK: c-JUN N-terminal kinase

BUN: blood urea nitrogen

IL6: interleukin 6

IL1-β: interleukin 1-beta

CXCL1: chemokine (C-X-C motif) ligand 1

MCP1: monocyte chemotactic protein 1

PAI-1: plasminogen activator inhibitor-

α-SMA: alpha-smooth muscle actin

BMP-7: bone morphogenetic protein-7

Cdkn2a: cyclin-dependent kinase inhibitor 2a

CTGF: connective tissue growth factor

B2M: beta-2 microglobulin

SR/FG: sirius red/ fast green

KIM-1: kidney injury molecule-1 (see also Tim-1)

NGAL: neutrophil-gelatinase associated protein

CC3: cleaved caspase 3

ALI: acute lung injury

CURRICULUM VITAE

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EDUCATION

2009-2013 Transylvania University
B.A. Biology
Minors in Chemistry and Psychology
GPA: 3.74

2014-present University of Louisville
Ph.D. Pharmacology and Toxicology
GPA: 3.9
Expected graduation: May 2018

HONORS

2015 1st place poster presentation Master's category- Research!Louisville
2015 Graduate Student Council Travel Award- University of Louisville
2014 Graduate Student Council Travel Award- University of Louisville
2014 IPIBS Fellowship covering tuition, benefits and stipend- University of Louisville
2013 Graduated *cum laude*- Transylvania University
2013 Graduated with Biology honors- Transylvania University
2013 Who's Who Among American College and University Students
2011 Alpha Lambda Delta Honorary Society Service Chair- Transylvania University
2009-2013 Dean's List (>3.5 GPA, 6 semesters)-Transylvania University

Related Experience

May 2012-August 2012 College Intern- Marathon Petroleum

Summer intern whose duties included running analytical tests on gasoline and jet fuel samples as part of quality control; initiation of a safety training program for common workplace hazards that was used throughout the entire refinery.

Research

2014-present IPIBS Graduate Fellowship- University of Louisville
Working in the lab of Dr. Leah Siskind, my research focuses on developing a more clinically relevant model of cisplatin nephrotoxicity and using this model to elucidate the mechanism by which acute kidney injury progresses to chronic kidney disease.
2013-2014 Research Technician- University of Louisville
Working in the lab of Dr. Uma Sankar, my project focused on developing an enzymatic assay to screen compounds that were chosen as potential CAMKII inhibitors. In addition to this, I performed mouse survival surgeries.
2012 Student Research- Transylvania University
Spent 3 months working with Dr. Belinda Sly breeding betta fish and examining and detailing the embryonic development of offspring. This was done in an effort to determine how betta fish and zebrafish development differed, in hopes of establishing the betta fish as an alternative developmental biology model.
2012-2013 Research Assistant- University of Kentucky
Working in the lab of Dr. Charles Waechter, my project focused on determining activity of mutated flippases involved in the glycosylation of newly synthesized proteins.

Peer-Reviewed Publications

Sharp CN, Doll MA, Dupre TV, Shah PP, Subathra M, Siow D, Arteel GE, Megyesi J, Beverly LJ, Siskind LJ. Repeated Administration of Low-Dose Cisplatin in Mice Induces Fibrosis. American journal of physiology Renal physiology. 2016:ajprenal 00512 2015. doi: 10.1152/ajprenal.00512.2015. PubMed PMID: 26739893.

Abstracts

Sharp CN, Doll MA, Dupre TV, Siow D, Marimuthu S, Shah P, Beverly LJ, and Siskind LJ. Developing a clinically relevant model of cisplatin induced nephrotoxicity.
Dupre, TV**,** Doll, MA, Shah, PP, **Sharp, CN,** Scherzer, MT, Casson, L, Megyesi, J, Beverly, LJ, Schnellmann, RG, Siskind, LJ. Suramin Protects from cisplatin-induced acute kidney injury.

Lang, AL, Kaelin, BR, Yeo, H, Hudson, SV, Poole, LG, McKenzie, CM, **Sharp, CN**, Arteel, GE, Beier, JI. Critical role of mammalian target of rapamycin (mTOR) in liver damage caused by VC metabolites in mice.

Provisional Patent:

U.S. Application Number: 62/234,427
Filing Date: 09/29/2015
Applicant: University of Louisville research Foundation, Inc., Louisville, KY
Title of Invention: Methods for Treating Chemotherapy-Induced Kidney Injury
Inventors: Siskind, Beverly, Schnellmann, Dupre, Doll, **Sharp**

Presentations

Developing a clinically relevant model of cisplatin induced nephrotoxicity. Southeastern Regional Lipid Conference, November, 2015 (Poster)
Developing a clinically relevant model of cisplatin induced nephrotoxicity. American Society of Nephrology Kidney Week, November, 2015 (Poster)
Developing a clinically relevant model of cisplatin induced nephrotoxicity. Research!Louisville, October, 2015 (Poster)

Grant/Fellowship Support

Submitted

NIH (F31) F31DK111127 Sharp (PI)
Project Period: 07/01/2016-TBD
Title: Characterization and Mechanistic Studies of a Repeated Cisplatin Dosing Kidney Injury Mouse Model
Goal: To elucidate the mechanism by which acute kidney injury progresses to chronic kidney disease using a repeated, low dose cisplatin model. The goal is to determine potential targets for the development of renoprotective and kidney injury-reversing therapeutic

Memberships

Alpha Lambda Delta Honorary Society
American Society of Nephrology
Beta Beta Beta National Biology Honors Society

Extracurricular Activities

Louisville Regional Science and Engineering 2016 Judge- Translational Medical Science