Cystinosis Research Foundation Progress Report

Title: *Cysteamine effects on extracellular matrix accumulation in chronic kidney disease.* Grant number: 412560090101 Investigators: Allison Eddy M.D. and Daryl Okamura M.D. Funding Period: July 1, 2008 to June 30, 2011 Progress Report: January 1, 2010 to June 30, 2010

OVERVIEW

The project is progressing according to the original research plan. The three mouse lines are all actively breeding (*Ctns-/-*, *vanin-/-* and *Ctns-/-vanin-/-* double knockouts). The animal work for the first cysteamine *in vivo* study has been completed and tissue analyses continue. We now have a cohort of 10 *Ctns-/-vanin-/-* double knockout mice who are nearly 12 months of age. Although data are still preliminary, it appears that the males are polyuric with mild renal insufficiency compared to the *Ctns+/-vanin+/-* double heterozygous sibling controls.

1) <u>Aim #1:</u> To perform preliminary studies to develop an optimal model and drug delivery strategy to investigate the effect of cysteamine in a mouse chronic kidney disease model.

Searching for a better mouse model of cystinosis-associated nephropathy:

We hypothesize that the rate of progression of the renal phenotype in *Ctns-/-* mice may be attenuated by the endogenous expression of cysteamine synthesized via an enzymatic pathway

that is encoded by the *vanin* gene. We have generated a colony of *Ctns-/- Vanin-1-/*double knock-out mice. Similar to the study published by Dr. Antignac and colleagues (*Nephrol Dial Transplant. 25;* 2010), we found significantly increased rates of polyuria in our double knock-out mice at 6

months, however, the difference diminished at 9 months and there was no difference in weight (Table 1). There was a significant increase in BUN at 10 months in the double knock-out mice compared to heterozygous controls (Heterozygous controls vs. Double *knock-out*, =8/group: 17.6 ± 1.0 vs. 22.7 ± 1.4 mg/dL, *P* =0.01). Masson trichrome staining did suggest an increase in glomerulosclerosis and mild interstitial fibrosis at 12 months in the double knock-out mice (See Figure 1). Serum creatinine levels will be measured at 12 and 18 months. We will also examine the histology of the double knock-out,

	Ctns+/- Vnn1+/- 6 months	Ctns-/- Vnn1-/- 6 months	Ctns+/- Vnn1+/- 9 months	Ctns-/- Vnn1-/- 9 months
Body weight (BW) (g)	24.4 ± 1.1	28.2 ± 2.4	29.3 ± 1.8	31.4 ± 2.9
Urine volume (ml/24h)	0.64 ± 0.12	1.12 ± 0.16*	0.70 ± 0.07	0.90 ± 0.18
Diuresis (μl/min/g BW)	0.016 ± 0.002	0.026 ± 0.003*	0.017 ± 0.002	0.018 ± 0.002

Table 1. Weight and urine values in 6- and 9- month *Ctns-/- Vnn1-/-* and heterozygous controls. * *P* <0.05, *n*=9-10/group



Figure 1. Histologic lesions in Ctns-/- Vanin-1-/- mouse. Representative staining with Masson trichrome and hematoxylin in 12 month old mouse. (A) Arrow indicates focal glomerulosclerosis and intense interstitial mononuclear infiltrate. (B) Arrows indicates area of tubular atrophy and (*) global glomerulosclerosis.

heterozygous littermate controls, and CTNS-/- mice at 18 months for proximal tubules (lotus lectin), picrosirius red, in addition to PAS and trichrome stains.

Findings from a study of the unilateral ureteral obstruction (UUO) model in 8-week old male mice, comparing the degree of fibrosis between littermate controls (*Vanin+/+*) and *Vanin-/-* mice, suggest that vanin-deficiency alone does not alter the degree of kidney fibrosis. However, as reported in our last progress report there was a non-significant trend toward more renal fibrosis in Vanin-/- at day 21 after UUO (Vanin+/+ vs. Vanin-/-;). We did note that values for total collagen were much lower than typically seen in C57BL/6 mice and we are investigating the strain of the Vanin-/- mice to determine if backcrossing to a pure C57BL/6 background will accentuate the differences observed.

2) <u>Aim #2</u>: To investigate the efficacy of cysteamine therapy for interstitial renal matrix protein reduction in chronic kidney disease and to determine its mechanism of anti-fibrotic action.

Cystagon® added to the drinking water that was freshly made every 24h) on the degree of renal fibrosis was investigated using two doses: 400 and 600 mg/kg/day. Groups of mice (n = 8 at each time-point) were studied 3, 7 and 14 days after the onset of chronic injury induced by UUO. Both doses were shown to significantly reduce kidney collagen levels by day 14 by 21% compared to the untreated group (shown on previous progress report). Studies designed to extend these observations to a 21-day UUO study and glomerular model of chronic kidney disease are currently underway. In collaboration with Dr. Jon Gangoiti and Dr. Bruce Barshop at UCSD, serum cysteamine levels in mice at the time of sacrifice were measured by mass spectrometry. Interestingly, the plasma levels of cysteamine were low in the Cystagon® treated mice at day 14 after UUO (400mg/kg - 0.81 ± 0.09umole/L; and 600mg/kg - 1.04 ± 0.15umole/L; levels were undetectable in the vehicle alone group). This may be attributable to the short half-life of cysteamine and the nocturnal feeding habits of the mice.

Based on the findings from a pilot study, the effect of cysteamine (administered as



Figure 2. Interstitial macrophage infiltration was abrogated by cysteamine treatment at advanced time-points. (A) Graph summarizes semi-quantitative image analysis of F4/80 staining. (B-D) Representative F4/80 (red) confocal images demonstrates reduced interstitial macrophages at day 14 in cysteamine treated mice. † P < 0.01. NS – not significant.

Future studies are planned to determine the area under the curve for cysteamine treated mice. Kidney tissue studies are currently focusing on elucidating the mechanism by which
Cystagon® reduces kidney fibrosis. One potential pathway may be its ability to reduce collagen synthesis rates. Extracellular matrix (ECM) gene mRNA levels have now been measured by real-time qPCR in all mice 3, 7 and 14 days after UUO (See last progress report). ECM gene transcription levels were significantly down-regulated in UUO kidneys of cysteamine-treated mice: procollagen I mRNA levels were 56% lower in the mice treated with 600 mg/kg at day 14; and at day 7, despite no difference in total collagen, there was a nearly 40% reduction in kidney

fibronectin and procollagen III mRNA levels in mice treated with 400mg/kg and a nearly 60% reduction in fibronectin, procollagen I and procollagen III at higher doses of cysteamine (600mg/kg). The peak effect of Cystagon® was observed at 7 days at both treatment doses. Additional mechanisms must also contribute to the anti-fibrotic

	Cystagon 400mg/kg Day 7	Cystagon 600mg/kg Day 7	Cystagon 400mg/kg Day 14	Cystagon 600mg/kg Day 14
TGF-β	0.52 ± 0.13	0.74 ± 0.19**	0.61 ± 0.21	0.66 ± 0.22
TGF- β receptor	0.72 ± 0.30	1.38 ± 0.36**	0.56 ± 0.22	0.42 ± 0.13**

Table 2. Relative profibrotic cytokine gene expression in total UUO kidneys from cysteamine treated mice compared to control mice at day 7 and 14 after UUO. Normalized to two housekeeping genes: 18S and GAPDH. n=7-8/group; ** P < 0.01.

actions of Cystagon®. Computed-assisted image analysis of immunohistochemically stained kidney sections at day 14 UUO show significantly less interstitial inflammation as measured by the number of F4/80+ interstitial macrophages and significantly fewer interstitial myofibroblasts. The profibrotic cytokine TGF- β was significantly down-regulated by 26% at day 7 at high doses of Cystagon® compared to control mice (See Table 2). Studies are currently underway to investigate the mechanisms in which cysteamine modulates TGF- β and TGF- β receptor expression during fibrosis.

There was a significant reduction in α -SMA positive myofibroblasts by nearly 30% in both doses at day 14 in cysteamine-treated mice. There was a significant reduction in interstitial macrophage infiltration by 34% in mice treated with 600mg/kg/day (no difference was seen at day 7, see Figure 2). Total kidney thiol content, a measure of antioxidant status, was significantly increased by 36% in high dose cysteamine-treated mice (600mg/kg) compared



Figure 3. Total kidney thiol content is augmented in cysteamine treated mice at day 14 after UUO. Total kidney thiol was quantified using the Measure-iT Thiol Assay (Invitrogen).

to controls at day 14 after UUO (See Figure 3, no difference was seen in day 7). *In vitro* studies investigating the effect of cysteamine on myofibroblast activation are currently underway.

3) <u>Aim #3:</u> To investigate the effect of cysteamine on apoptosis of renal tubular epithelial cells, oxidant stress, and other novel target pathways of chronic kidney disease. As originally proposed, the effects on apoptosis, glutathione activity and novel cysteamine targets will be the primary focus of these studies. These aspects will be investigated as part of the *in vitro* studies discussed above.

In summary, early data from the first Cystagon® treatment study establishes its significant anti-fibrotic effects. Ongoing studies are planned to identify how this effect is achieved and to determine if it is sustained when chronic kidney damage persists for longer periods of time. Over the next 6 months we will be able to confirm the advanced renal phenotype of the *Ctns-/-vanin-/-* double knock-out mice compared to Ctns-/- mice and published results from Dr. Antignac.

BUDGET EXPENDITURES

Category	Award *	Prior expenditures **	Expenditures this period ***	Anticipated expenditures ****	Total expenditures this period	Total expenditure s to date	Remaining balance
Personnel	\$118,989	\$71,603	\$23,601	\$ 2,600	\$26,201	\$97,805	\$21,184
Animals/ Maintenance	\$11,165	\$10,455	\$2,845	\$655	\$3,500	\$13,955	\$(2,790)
Supplies	\$26,390	\$24,885	\$3,023	\$296	\$3,318	\$28,204	\$(1,814)
Travel	\$3,045	-	\$34	-	\$34	\$34	\$3,011
Subtotal	\$159,589	\$106,943	\$29,503	\$3,551	\$33,054	\$139,997	\$19,592
IDC	\$15,959	\$10,695	\$ 2,950	\$355	\$3,305	\$14,000	\$1,959
Total	\$175,548	\$117,637	\$32,453	\$3,906	\$36,359	\$153,997	\$21,551

January 1, 2010 to June 30, 2010

* Award amount for 07/01/08-06/30/10

** Prior expenditures: 07/01/08-12/31/09

*** Expenditures this period: 01/01/10-06/28/10

*** Anticipated expenditures: 06/28/10-06/30/10

12% budget remaining