CRN 24-mo progress report

(Funding begun September 2005)

Development, and *in vivo* testing, of novel therapies for cystinosis

Our 2 yr research project was a joint proposal between:

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Progress report Project 1 – Cysteamine trial

The first aim of the project was to generate antibodies against cystinosin, since the antibodies raised against a C-terminal peptide of cystinosin (published by Haq et al., 2002) had run out . As several attempts of making polyclonal antibodies against the same peptide have been unsuccessful, we planned to generate a polyclonal antibody against the amino-terminal part of the protein, which corresponds to the intra-lysosomal part of cystinosin. Given that this part of the protein is highly glycosylated, we choose to produce the protein using the baculovirus expression system, which provides correct folding of the recombinant protein by allowing post-translational modifications. Thus a 300 bp fragment of the human *CTNS* gene corresponding to aa 24-123 of cystinosin with a 6His tag at the N terminus was cloned in the pFastBac plasmid (Invitrogen) and recombinant baculovirus was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen) and used to infect Sf9 cells. The recombinant protein was purified in denaturing conditions using the 6HIS tag and injected to one rabbit (5 injections at Day 1, 7, 28, 56 and 84). Sera were obtained at 35, 42, 49, 63, 77 and 105 days after the injections, and were tested by western blot. All of them were able to detect human cystinosin overexpressed in various cell lines, but not endogenous cystinosin (probably too low level of expression). Unfortunately, given the fact that the antibodies were raised against a non-native protein, they do not work for immunofluorescence experiments on cell lines and for immuno-precipitation experiments. We are now testing them on tissue sections. These antibodies are freely available for the scientific community.

We postponed the second aim of the project (cysteamine trial) to better characterize the renal phenotype of the *Ctns-/-* mice on 2 genetic backgrounds (C57Bl/6 and FVB/N) throughout the lifespan of the mice. Interestingly, we showed that, although both strains accumulated cystine, only the C57BL/6 *Ctns*$^{-/-}$ mice

presented renal anomalies. Clinically, the C57BL/6 *Ctns*$^{-/-}$ mice developed renal failure from 15 months of age. From 9 months, we observed histological lesions with focal tubular atrophy that worsened with age, resulting in wide areas of fibrosis and cellular infiltration without tubules at 18 months. Immunolabelings with various markers of tubules clearly indicated that proximal tubules are the initial and most affected structures. A manuscript reporting these results is in preparation and will be submitted shortly.

Progress report Project 2 – Development of gene transfer studies

Our initial proposal in 2005 comprised 2 major sections:

i) *In vitro* gene transfer studies

ii) *In vivo* gene transfer studies targeting the eye, CNS and kidney

i) *In vitro* gene transfer studies

Previous work:

Prior to our proposal in 2005, we showed that viral-mediated gene transfer is feasible for reducing cystine levels *in vitro* in *CTNS*$^{-/-}$ human fibroblasts and *Ctns*$^{-/-}$ murine hepatocytes. Furthermore, our data from the murine hepatocyte studies suggested that the efficiency of cystine reduction is age-dependent. The next step was to validate these observations *in vivo* by directly targeting the liver in *Ctns*$^{-/-}$ mice. As mentioned in our 18-mo update, we targeted the liver of young (2 and 3 mo-old) and older (6 and 9 mo-old) mice with adenovirus vectors expressing green fluorescent protein (GFP; AdGFP), cystinosin (AdCTNS) or cystinosin fused to GFP (AdCTNSGFP). One week post-transduction, we sacrificed the mice and evaluated transduction efficiency by immunofluorescence studies of GFP fluorescence from AdGFP or AdCTNSGFP. Regardless of age, the transduction efficiency of both vectors was ~50%. With respect to hepatic cystine levels, significant differences were observed between the experimental groups of the young mice but not between those of older mice. Regardless of age, we observed a ~2-fold reduction in cystine levels one-week post-transduction with the control AdGFP vector, which was not significant ($p > 0.05$). In contrast, transduction of young *Ctns*$^{-/-}$ mice with AdCTNS or AdCTNSGFP resulted in a significant ~5-fold decrease ($p < 0.05$) in cystine levels as compared to cystine levels in nontransduced mice. Following transduction of older *Ctns*$^{-/-}$ mice with AdCTNS or AdCTNSGFP, we did not significantly reduce cystine levels beyond that observed with AdGFP.

Taken together, this work showed that it is feasible to reduce lysosomal cystine levels *in vivo* by viral vector-mediated gene transfer. Furthermore, our *in vivo* observations confirmed our *in vitro* data that the efficiency of cystine clearance over a short transduction period could be age-dependent.

Recent work:

A possible explanation for the age-dependent efficiency of cystine clearance is that a longer duration of cystinosin expression is required to reduce the higher cystine levels in older mice (2- to 7-fold higher than those of younger mice). The first generation, E1-deleted, adenovirus vectors used in this study are prone to short-term expression in most immunocompetent rodents, especially in the liver. This is due to T-cell mediated destruction of transduced cells, which normally occurs from day 7. Therefore to inhibit the T-cell response, an immunosuppression protocol (using cyclosporin A (CsA) delivered via a subcutaneously implanted osmotic pump) was begun one day prior to injection and used to extend transgene expression from 7 to 28 days. A whole blood immunoassay demonstrated that CsA was continually administered over the 28-day period.

We transduced two groups of immunosuppressed *Ctns*$^{-/-}$ mice, aged 3 and 5 mo, with AdGFP, AdCTNS or AdCTNSGFP. Interestingly, and consistent with our short-term *in vivo* transduction data, significant differences were observed in the hepatic cystine levels following sacrifice between the experimental groups aged 3 mo but not between those aged 5 mo, regardless of a similar transduction efficiency for both ages (~30%; as estimated by GFP fluorescence from AdGFP). For both age groups, we observed a reduction (1.2-fold) in cystine levels with the AdGFP vector that was not significant. In contrast, long-term transduction of 3 mo-old *Ctns*$^{-/-}$ mice with AdCTNS or AdCTNSGFP resulted in a significant 2.5-fold decrease ($p < 0.05$) in cystine levels as compared to cystine levels in nontransduced mice. One-month post-transduction of 5 mo-old immunosuppressed *Ctns*$^{-/-}$ mice with AdCTNS or AdCTNSGFP did not result in a significant reduction in cystine levels beyond that observed with AdGFP.

Thus our long-term transduction data continue to show an age-dependent reduction of cystine clearance and suggest that this phenomenon is due to a factor other than duration of cystinosin expression.

ii) *In vivo* gene transfer studies

Previous work:

We used the liver as the proof-of-principle organ for determining the feasibility of gene transfer for cystinosis because of its accessibility. However, the organs we initially proposed to target were the eye, brain and kidney. The age-dependent efficiency of cystine reduction via gene transfer that we demonstrated in our liver study indicated that a spatial and temporal guide of the appearance of anomalies in *Ctns*$^{-/-}$ mice was a necessary prelude to *in vivo* gene transfer studies. We performed a thorough characterisation of the ocular and CNS anomalies in C57Bl/6 *Ctns*$^{-/-}$ mice. The results of our ocular study and the corresponding paper were summarised in our 12-mo progress report and won't be repeated here. The results of our CNS study are summarised below. The proposed gene transfer studies to the kidney were beyond the scope of our 2-year funding period.

Recent work:

We performed a panel of behavioural tests (open field, rotarod, Morris water maze (both visible and invisible platforms), passive avoidance tasks, spontaneous alternation) on 3 and 13 mo-old mice to assay their behaviour as well as learning and memory abilities, as a measure of CNS defects. Our data on 3 mo-

old mice showed that they are i) hyperactive in an open field environment, suggesting difficulty in spatial adaptation (linked with cerebral defects), and ii) unable to stay on a rotarod for longer than 96 seconds (s) (167 s for the wild-type mice), indicative of cerebellum and/or basal ganglia defects. These 3 mo-old mice performed well in Morris water maze (both visible and invisible platforms), passive avoidance tasks, and spontaneous alternation. We then used the same panel of tests with 13 mo-old *Ctns*$^{-/-}$ mice. In addition to more pronounced difficulties in the open field and rotarod tests, the older mice showed significant difficulties in the passive avoidance tasks, spontaneous alternation and, most markedly, in the Morris water maze. Concerning the latter, the *Ctns*$^{-/-}$ mice performed well with a visible platform (indicating correct vision), however the difficulties arose when the platform was invisible or moved. These data showed that the 13 mo-old *Ctns*$^{-/-}$ mice have severe spatial working and reference memory defects (controlled by the hippocampus).

Because the memory defects we detected in the *Ctns*$^{-/-}$ mice suggested a hippocampal defect, we wanted to assay the cystine levels in the hippocampus with respect to other brain structures. We dissected the brains of the young and middle-aged mice used in the behavioural studies, as well as additional age-matched controls (92 mice in total), and assayed the cystine levels in the hippocampus, residual forebrain, cerebellum and brainstem. Consistent with the age-related defects we identified, we observed higher cystine levels in all brain structures of middle-aged as compared to young *Ctns*$^{-/-}$ mice (cystine levels were already elevated in all tissues of young *Ctns*$^{-/-}$ mice as compared to age-matched controls). Furthermore, consistent with the memory impairments, the hippocampus, a structure playing a prominent role in spatial and contextual memory encoding, showed the highest cystine levels. This was followed by the cerebellum and brainstem, which are structures involved in particular, motor skilled memories.

In parallel, we performed a histological study to detect the presence of cystine crystals. The cystine levels in the hippocampus were not elevated enough to result in the formation of detectable cystine crystals. However, we did detect crystals in the choroid plexus and clustered around capillaries in the parenchyma. The ensemble of our observations are similar to those reported for cystinosis patients.

Taken together, our results strongly suggest that the cystinosis-associated CNS anomalies are due to progressive cystine accumulation. The article describing this study was accepted in September and published on-line in November 2007:

Maurice, T., **Hippert, C.**, Serratrice, N., Dubois, G., Jacquet, C., Antignac, C., Kremer, E.J. & **Kalatzis, V. (2007)** Progressive cystine accumulation in the CNS of a cystinosis animal model results in severe age-related memory deficits. ***Neurobiol. Aging*** Epub ahead of print: 10.1016/j.neurobiolaging.2007.09.006