

CRF 12-mo detailed progress report

Title of proposal: Gene transfer studies for cystinosis

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Initial specific aims

The causative gene of cystinosis, *CTNS*, encodes a novel protein, named cystinosin. Corinne Antignac's laboratory, of which I was previously a member, generated a knock-out mouse (*Ctns*^{-/-}) model for the disease. Our research project is to use viral-mediated gene transfer to reduce lysosomal cystine levels *in vivo*. The efficiency of cystine clearance will be compared to that obtained with cysteamine, the drug currently administered to patients. Initially in this proposal, we will primarily target the ocular and CNS anomalies associated with this disease, which can be incapacitating or potentially life-threatening.

Our project is divided into 3 main subjects:

i) Validate preliminary *in vitro* gene transfer studies on primary murine hepatocytes by *in vivo* gene transfer to the liver.

ii) Generate viral vectors (helper-dependent canine adenovirus serotype 2 and adeno-associated virus serotype 8) expressing *CTNS* and perform eye-targeted gene transfer studies to correct the corneal anomalies of cystinosis.

iii) Finish characterising the CNS anomalies in *Ctns*^{-/-} mice, and begin CNS-targeted gene transfer to correct these anomalies.

Results obtained over the last 12 months

I) Validating *in vitro* gene transfer studies by *in vivo* gene transfer

BACKGROUND:

We previously 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. This study is now almost complete and the results are summarised as follows:

RESULTS:

Short-term (1 wk) gene transfer experiments: We targeted the liver of young (2 and 3 mo-old) and older (6 and 9 mo-old) mice with first generation (deleted only in the E1 & E3 viral genes) adenovirus vectors expressing green fluorescent protein (GFP; AdGFP), cystinosin (AdCTNS) or cystinosin fused to GFP (AdCTNSGFP). We evaluated transduction efficiency by IF studies of GFP fluorescence from AdGFP or AdCTNSGFP. Regardless of age, the transduction efficiency of both vectors was on average ~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 on average ($p < 0.05$) 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, we show that it is feasible to reduce lysosomal cystine levels *in vivo* by viral-mediated gene transfer. Furthermore, our *in vivo* observations confirm our *in vitro* data that the efficiency of cystine clearance over a short transduction period is age-dependent.

Long-term (1 mo) gene transfer experiments: 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 adenovirus vectors 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, a mild immunosuppression protocol (using cyclosporin A (CsA) delivered via a subcutaneously implanted osmotic pump) was begun one day prior to transduction and used to extend the post-transduction period 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 in cystine levels with the AdGFP vector that was not significant and that was lower (1.2-fold) than that observed in the short-term experiments (2-fold). 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. As was the case for AdGFP, the observed 2.5-fold decrease was lower than that obtained with AdCTNS or AdCTNSGFP in the short-term transduction experiments. As a negative control, in the absence of an immunosuppressive protocol, cystine levels were not reduced in 3 mo-old mice 1-mo post-transduction with AdGFP, AdCTNS or AdCTNSGFP. One-month post-transduction of 5 mo-old immunosuppressed *Ctns*^{-/-} mice with AdCTNS or AdCTNSGFP, we did not detect 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.

Kupffer cell regeneration post-transduction non-specifically affects cystine levels: Contrary to our expectations, cystine levels were more efficiently reduced in short-term as opposed to long-term transduction experiments. Furthermore, the non-specific effect of AdGFP on cystine reduction was more pronounced 1-wk versus 1-mo post-transduction. These observations suggest that cystine levels are partly non-specifically increasing over time. Intravenous adenovirus vector entry destroys Kupffer cells, which could account for the decrease in cystine levels observed after short-term AdGFP transduction experiments. In turn, we hypothesised that a long post-transduction period may result in the regeneration of Kupffer cells and hence cause a rise in cystine levels.

To test this hypothesis, we artificially depleted Kupffer cells from 2 and 5 mo-old *Ctns*^{-/-} mice by treating with liposomal clodronate (Clodrolip), a potent antimacrophage agent. Mice were sacrificed 1-wk (at which time there should be > 90% depletion) or 1-mo post-treatment. Regardless of age, hepatic cystine levels were ~25% of control levels 1 wk after Clodrolip administration. In contrast 1 mo later, cystine levels had increased to ~70% of control levels. In parallel, we performed immunostaining studies with an antibody to a macrophage marker to demonstrate the absence and presence of Kupffer cells 1-wk and 1-mo post-treatment, respectively.

Taken together, these data suggest that Kupffer cell regeneration causes a non-specific increase in cystine levels. This correlates with the observation that cystine levels rose to 85% of control levels (from 50% at 1 wk) 1-mo post-transduction with AdGFP. In contrast, cystine levels rose to only 40% of control levels (from ~20% at 1-wk) 1-mo post-transduction with AdCTNS, indicating a continued specific role of *CTNS* in cystine clearance. Finally, our observations suggest that the cystine content of the Kupffer cells, which represent only 10% of the liver cell population, accounts for 75% of hepatic cystine levels in the mouse model.

ONGOING WORK:

We are currently complementing this study with some additional experiments:

- 1) We have not been able to successfully use the anti-cystinosis antibody to detect cystinosis expression on liver sections by immunofluorescence studies (hence the use of AdCTNSGFP). We are currently performing immunostaining studies, a more sensitive technique, with the anti-cystinosis antibody to try to detect cystinosis expression in liver sections.
- 2) As mentioned above we did not detect a reduction in cystine levels 1-mo post-transduction of non-immunosuppressed mice with AdGFP, AdCTNS or AdCTNSGFP. Conversely, we did detect GFP expression from AdGFP (~50% of cells transduced) but not from AdCTNSGFP. These observations suggest that cystinosis or cystinosis-GFP may be more immunogenic than GFP. We are currently performing assays to verify this hypothesis.
- 3) In parallel, we are preparing a manuscript detailing the first *in vitro* and *in vivo* gene transfer studies for cystinosis.

II) Ocular gene transfer studies

BACKGROUND:

Our article detailing the ocular anomalies in the cystinosis animal model was accepted in March and published in August 2007:

Kalatzis, V., Serratrice, N., **Hippert, C.**, Payet, O., Arndt, C., Cazevielle, C., Maurice, T., Hamel, C., Malecaze, F., Antignac, C., Müller, A. & Kremer, E.J. (2007) A temporospatial guide to the ocular anomalies in a cystinosis mouse model. *Pediatr. Res.* 62: 156-162.

The next step of this project is to perform gene transfer studies to the cornea, one of the first tissues affected in cystinosis patients and in the *Ctns*^{-/-} mice.

RESULTS & ONGOING WORK:

To date, we have used first generation vectors for the *in vitro* and *in vivo* liver studies. However, if we hope to eventually use gene therapy in the clinic, we need to perform *in vivo* eye studies with more clinically relevant *CTNS*-expressing vectors. We chose two such vectors that are devoid of all viral genes and that can readily transduce the cornea: an adeno-associated vector (AAV8) and a state-of-the-art, helper-dependent canine adenovirus vector (HD CAV-2). To facilitate vector production and subsequent analysis we needed to include a marker gene, such as GFP, in our expression cassette in addition to *CTNS*. Thus we opted for the use of an IRES sequence as opposed to fusing GFP to *CTNS*. In this way we will avoid possible hindering cystinosin function, or rendering cystinosin more immunogenic, with a GFP tag.

Firstly, we generated the AAV8 vector containing the *CTNS*-IRES-GFP expression cassette. We performed the initial subcloning step of inserting the expression cassette into an AAV shuttle plasmid. We then tested the plasmid for correct cystinosin expression, localisation and function. Subsequently, we subcontracted the production of the AAV8 vector to the Vector Production Platform at the Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain). Claire Hippert, the recipient of the CRF Ph.D. fellowship, spent a week in Barcelona learning the AAV production steps. We received a first batch of the AAV-*CTNS*-IRES-GFP vector in July 2007, however due to a problem during the purification steps, the initial stocks were not sufficient for *in vivo* experiments production. Thus we re-ordered a second batch, which we received at the end of December 2007. The next step is to test the functionality of the transgenes. Last week, we injected AAV-*CTNS*-IRES-GFP and AAV-GFP in the tail vein of 2 mo-old *Ctns*^{-/-} mice. We will sacrifice these mice 1-wk post-injection and will assay the liver, kidney, lung, spleen, heart and brain for a reduction in cystine levels by cystinosin and for GFP expression. Once we have verified that the vector is functional, we will begin our cornea transduction experiments.

Secondly, concerning HD CAV-2 vector production, our progress in optimising the transfection of canine cells to begin production has advanced slower than we had hoped. However, in December 2007, Sandy Ibanes, a technician, joined our team and is working on HD CAV-2 vector production fulltime so we hope to advance significantly this year.

III) Characterisation of the CNS anomalies in cystinosis mice

BACKGROUND:

We previously performed behavioural studies of 3 (young) and 13 (middle-aged) mo-old *Ctns*^{-/-} mice and showed that the middle-aged mice have marked spatial and working memory defects, reminiscent of those seen in some patients, which were most likely hippocampal in origin.

RESULTS:

As the memory defects we detected in the *Ctns*^{-/-} mice suggested a hippocampal defect, we wanted to assay the cystine levels in the hippocampus with regards to other brain structures. Thus 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 work suggests 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

ONGOING WORK:

Having identified the brain regions affected in the *Ctns*^{-/-} mice, the next step is to identify the cell type. Clearly neurons are affected, but we do not know if this due to a direct cystine accumulation within this cell type or due to the indirect destruction of supporting cells. We will set up primary neuron, microglial, astrocyte, and oligodendrocyte cultures from the brains of *Ctns*^{+/+} and *Ctns*^{-/-} mice, and assay their respective cystine levels to determine which cell type(s) is predominately involved. Primary cultures can only be obtained from newborn mice that are younger than 7 days. Therefore, it is not certain that the cystine levels will be high enough to detect a difference. However, these cells can be kept in culture for 2 weeks during which time we hypothesise that the *Ctns*^{-/-} cells will accumulate cystine at a faster rate than wild-type cells. However, the caveat of culturing cells is that the *in vitro* culture conditions poorly mimic the redox environment *in vivo*, therefore the results we obtain may not mirror the *in vivo* situation. To palliate this problem, we will, in parallel, attempt to isolate individual cell types from the brains of different-aged mice and directly assay cystine levels. We will homogenise different brain regions and isolate single cell populations using

fluorescent-labelled cell markers (NGF receptor for neurons, EAAT1 for astrocytes, myelin oligodendrocyte glycoprotein for oligodendrocytes, and CD11b for microglial cells) and fluorescence-activated cell sorting. We will assay the cystine levels in each cell population and compare the data with those obtained by assaying the primary *in vitro* cultures. These complementary approaches will give us a better grasp of the *in vivo* situation.

If we are able to culture neurons and micro- and macro-glial cells from young *Ctns*^{-/-} mice as detailed above and detect differences in cystine levels as compared to wild-type cells, we will initially attempt to reduce cystine levels *in vitro* in these cultures using the AAV-CTNS-IRES-GFP vector. These studies will be a first step to our long-term goal of targeted gene transfer to the brain *in vivo* using stereotactic injection.

CONCLUSION:

We have made significant progress in the characterisation of the anomalies of the *Ctns*^{-/-} mice, resulting in **2 published papers**, and in providing the proof-in-principal that gene transfer can be used for reducing lysosomal cystine levels due to defective cystine efflux (**manuscript in preparation**). We have now laid the necessary foundations for the more challenging eye and brain-targeted gene transfer studies that we will undertake in the second year of our two-year grant.