

6 Month Progress Report to the Cystinosis Research Foundation:

Molecular and Pathogenesis Study of Cystinosis

(Period: Jul. 07- Dec. 07)

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Hypothesis:

We hypothesized that a cystine transporter defect in the lysosome of cystinotic patients results in a cysteine shortage in the cytosol, where the three major thio/disulfites, glutathione (GSH), thioredoxin and cysteine, are coupled. Cysteine is also one of the precursors for glutathione synthesis. Therefore, the glutathione and thioredoxin levels are decreased in the cytosol and mitochondria in patients with cystinosis. This affects mitochondrial function and other nuclear and cytoplasmic redox processes.

Strategy:

To study the redox status in cystinotic cells, we measured GSH and GSSG levels. To investigate the apoptosis rate and cell cycle properties, we performed APO-BrdUTM TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) Assay (Invitrogen) and generated cell growth curve for cystinotic fibroblasts versus normal cells. Total intracellular ATP concentrations were also determined to analyze mitochondrial functions in cystinotic cells. In addition, expression arrays were utilized to identify differentially expressed genes in cystinotic cells.

Preliminary Data:

Cell cultures and intracellular cystine levels:

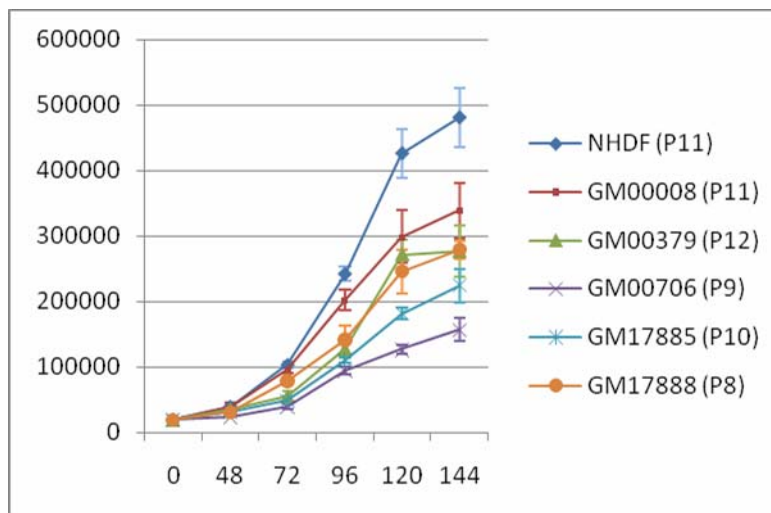
Cultures of normal and cystinotic human fibroblasts were obtained from the Coriell Cell Repositories and are maintained in the laboratory. The cystinotic lines represent different genotypes and total intracellular cystine levels of these fibroblasts were determined in Dr. Schneider's laboratory at UCSD.

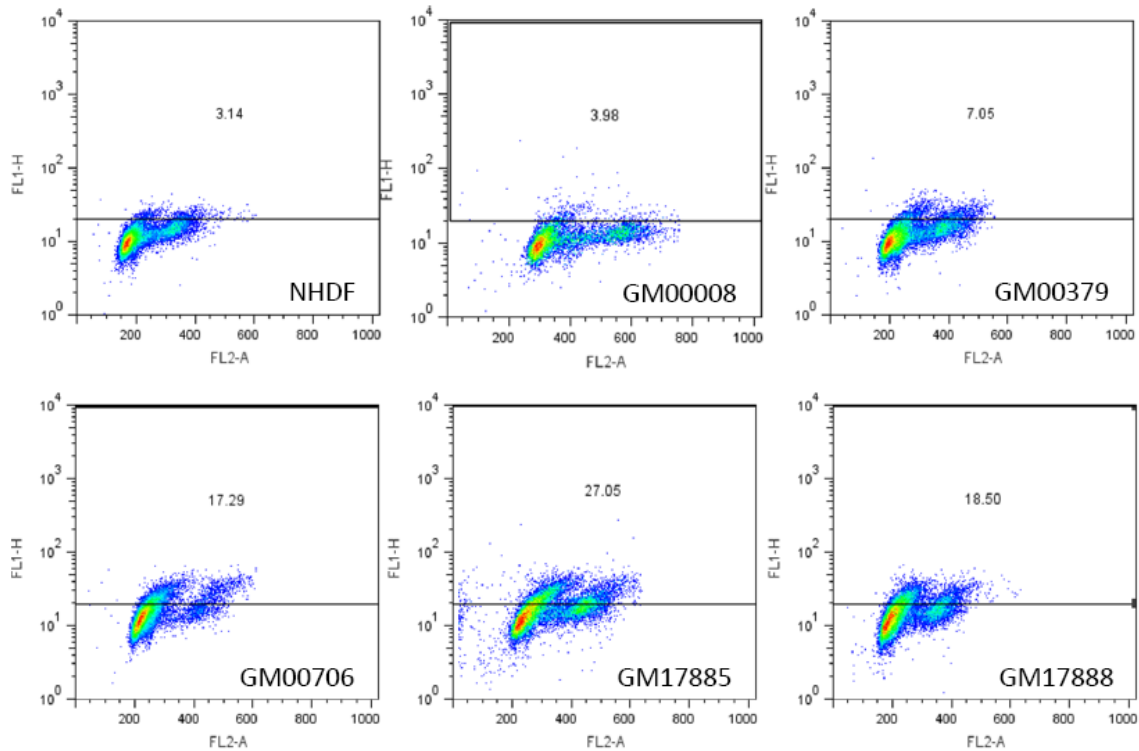
Cell line	Type of cystinosis	Age (Yrs)	Sex	Genotype	Mutation in Allele 1	Mutation in Allele 2	Cystine Levels (n mol/mg)
NHDF (control)	-----	0	M	-----	-----	-----	0.06
GM00008	nephropathic	5	F	homozygous	57 kb deletion	57 kb deletion	N/A

GM00379	late-onset	4	M	heterozygous	753G>A		IVS11+2T>C	18.52
GM00706	nephropathic	1	M	homozygous	57	kb	57 kb deletion	12.06
GM02894	nephropathic	9	F	homozygous	57	kb	57 kb deletion	3.38
GM17885	nephropathic	9	F	heterozygous	57	kb	Gly308Arg (G308R) deletion	12.29
GM17888	non-nephropathic	26	M	heterozygous	IVS10-3C>G		545delTCCT	3.46

Apoptosis /cell cycle analysis:

We noticed that cystinotic cells did not grow as fast as the NHDF cells, as shown in the cell growth curve. Depletion of intracellular cystine by cysteamine (incubation for 20 hr) did not enhance cell growth (data not shown). Retarded cell growth may indicate slow proliferation, elevated cell death, or both. Increased apoptosis had been reported in cystinotic cells and proposed to be an important factor in the pathogenesis of cystinosis. We used TUNEL assay to study the apoptosis rate and cell cycle properties of cystinotic cells.





Cell Line	Apoptosis Rate (%)	G1 (%)	S (%)	G2 (%)	UV only	CySH+UV
NHDF	3.14	53.79	36.56	7.51	8.74%	23.69%
GM00008	3.98	47.16	41.84	8.11	2.78%	4.25%
GM00379	7.05	55.87	33.21	6.28	9.80%	10.74%
GM00706	17.29	62.83	33.21	3.39	N/A	N/A
GM17885	27.05	48.21	44.52	5.22	34.41%	33.75%
GM17888	18.50	49.92	41.71	5.64	45.95%	16.06%

From our data, even under basal conditions (without apoptotic stimuli) we saw enhanced apoptosis rates of cystinotic cells, some of which were unreasonably high. The milder forms of cystinosis could display higher basal cell death rate than the more severe nephropathic types. We also used UV (60 mj) radiation to trigger apoptosis and 1 hr incubation of 1 mM cysteamine-HCl to remove cystine before UV treatment. However, we did not see decreases in apoptosis rate due to lysosomal cystine depletion (except for GM17888) as previously reported.

Gene expression analysis:

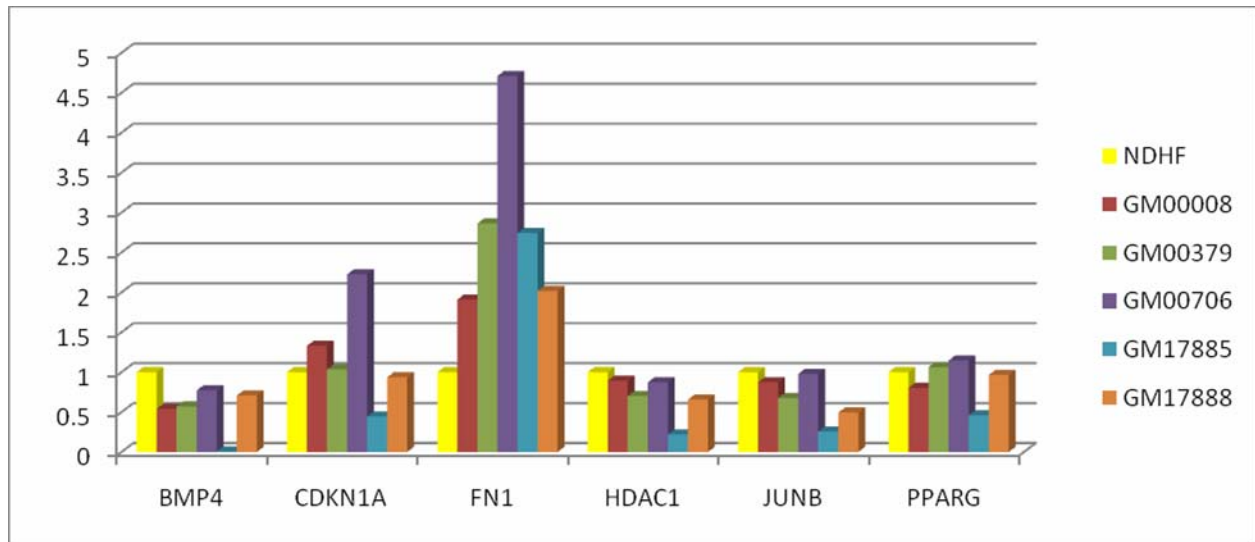
To investigate if cystine accumulation in the lysosomes affects gene expression levels in cystinosis, we used Affymetrix Human Genome U133 Plus 2.0 Array to identify differentially expressed genes in cystinotic cell. We used cystinotic GM00706 (homozygous for 57 kb deletion) and NHDF as control. These two cell lines were sex, age,

race, and passage-matched and the microarray expression analysis were implemented in triplicates for each. Combined analysis of two algorithms (LIMMA and Cyber-T) as well as straight fold change yielded 990 protein IDs that were significantly differentially expressed in GM00706 versus NHDF. Functional annotation of the 990 genes found some enriched biological themes that may be involved in the pathogenesis of cystinosis.

Category	Gene number
Apoptosis	39
Cell Cycle	64
Cell Proliferation	52
Transcription regulator	100
Development	204
Signal Transduction	187
Cell-Cell signaling	50
Ion Transport	18
Glutathione Metabolism	7
Oxidoreductase	44
Carboxylic Acid Transport	11

Next, Pathway Studio was used to identify the genes that played central roles in the differentially expressed functional groups for cell proliferation and apoptosis, transcriptional regulation, development and signal transduction (listed in Table). Quantitative RT-PCR was used to verify the expression profiles of these genes generated by Affymetrix arrays. Then, the expression levels of these six genes were studied in the other four cystinotic cell lines.

Tag	Gene	Function	Expression in GM00706
BMP4	bone morphogenetic protein 4	development, differentiation	Down
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	suppress cell growth, senescence marker	Up
FN1	fibronectin 1	cell adhesion, morphology, senescence marker	Up
HDAC1	histone deacetylase 1	transcriptional regulation	Down
JUNB	jun B proto-oncogene	cell proliferation	Down
PPARG	peroxisome proliferative activated receptor, gamma	anti-inflammatory, repress tumor	Down



When compared to normal control, expression of FN1 was up-regulated while BMP4, HDAC1 and JUNB were down-regulated in all the five cystinotic fibroblasts investigated.

Redox status and energy metabolism analysis:

Total GSH and oxidized GSH levels of cystinotic cells were determined by enzymatic cycling assays using both a commercial kit (Cayman Chemical) and the method developed in Dr. Luderer's laboratory at UCI. Total intracellular ATP contents were measured with the ATP Bioluminescence Assay Kit HS II (Roche).

	cystine	apoptosis rate	ATP	GSH	GSSG	GSSG/GSH
NHDF	0.06	3.14	24.06	103.5	2.1	4.00%
GM00008	N/A	3.98	21.59	N/A	N/A	N/A
GM00379	18.51	7.05	22.17	120.6	10.4	8.60%
GM00706	12.11	17.29	25.61	30.9	0.9	6.11%
GM17885	12.31	27.05	31.22	146.9	2.6	3.50%
GM17888	3.52	18.5	19.45	86.27	1.88	4.37%

Generally, cystinotic cells had reduced ATP content and total GSH level, and increased oxidized GSH ratio when compared to normal cells. Nevertheless, there were always exceptions (see GM17885). There was no clear correlation between severity of the disease and level of the biochemical indicators characterized here.

Summary and Future Directions:

We observed a slower growth rate of cystinotic cells when compared to normal cells. Accordingly, augmented programmed cell death in cystinotic cells was recorded even without apoptotic stimuli, suggesting that apoptosis does play an important role in

pathogenesis of cystinosis. Cystinotic cells generally displayed reduced ATP content and total GSH level, as well as an increase in GSSG/total GSH ratio, indicating perturbed redox balance due to cystine trap in lysosomes and resultant defective energy production capability of the mitochondria. However, the differences in GSH or ATP contents between cystinotic and normal cells were not definite; this further demonstrated the complexity of the disease and could partially explain the inconsistencies of similar data in previous reports. Meanwhile, we performed the first comprehensive gene expression analysis of human cystinotic cells and had identified four differentially expressed genes in cystinotic cells that are involved in cell proliferation and development.

We are now analyzing the expression data in more details. We will obtain more control cell lines in the near future and will use quantitative RT-PCR to validate the differentially expressed genes. Apoptosis rate, GSH and ATP levels of additional control cell lines will also be investigated to further understand the differences in redox status and energy metabolism of cystinotic versus normal cells. Based on our preliminary data, mitochondrial malfunction could be a major factor in cystinosis. We will analyze mitochondrial respiration rate and complex I-IV activities to see if mitochondrial respiratory chain activities are compromised in cystinotic cells. ROS production will also be studied by measuring either aconitase activity or MitoSox fluorescence. We expect to see elevated ROS levels in cystinotic cells, since ROS can damage the mitochondria, reduce ATP production and initiate apoptosis. In addition, the effects of antiapoptotic reagents (broad-spectrum caspase inhibitors) on cystinotic fibroblasts will be investigated to see if they can reverse the increased apoptotic rates in cystinotic cells we observed.