5 Apr 05

Cystinosis Research Network Six Months Progress Report Jess Thoene, M.D.

In order to observe the sequence of events which occur among subcellular organelles after an apoptotic stimulus we studied apoptosis in cultured RPTE cells using antibody to cathepsin B and cytochrome C to observe intracellular permeablization of mitochondria and lysosomes:

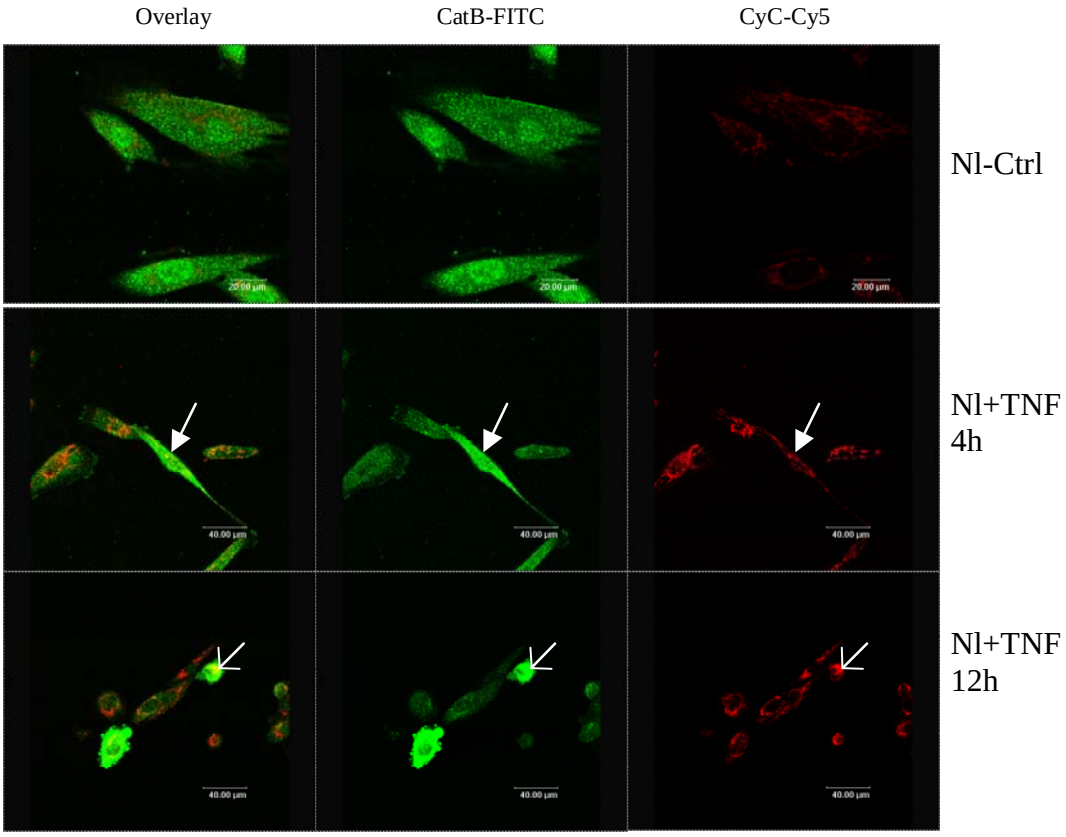

Fig 1: Loss of lysosomal and mitochondrial contents after treatment with TNFα.

At 4 hours after exposure of the cultured cells to TNFα(60ng/ml plus 2.0 µg/ml Actinomycin D), the discrete green granules representing lysosomal localization of cathepsin B have been lost, while the distribution of Cy5-antibody to cytochrome C (red) remains granular. At 12 hours of incubation, the red stain is also diffuse in the cytoplasm, and the cells show bleb formation, and thus are clearly apoptotic. At this point, both lysosomes and mitochondria have become permeablized. **Early lysosomal permeablization allows an opportunity for cells to potentially recover at this point, and for lysomal cystine to exert a pro-apoptotic effect.**

We performed preliminary studies on PKCδ because of the report from Chu, et al (1) that this key enzyme has greatly enhanced activity after cysteinylation in vitro. We demonstrated mixed disulfide formation in vitro by mixing pure PKCδ with ^{35}[S]-L cystine:

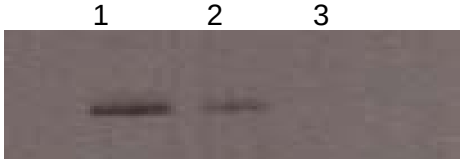

Fig 2: Autoradiogram of PKC δ Incubated in vitro with 35S-Cystine. PKCδ (Panvera) was incubated with ^{35}[S]-cystine (Amersham) (lane 1), plus MEA(2.0 mM, lane 2) or plus DTT (100 mM, lane 3). Samples were gel electrophoresed, blotted onto a nitrocellulose membrane and exposed to autoradiographic film for 24 h. Sensitivity of the autoradiography signal to added thiol demonstrates cystine is present in a disulfide bond.

We then measured the activity of native PKCδ derived from TNFα -treated normal and cystinotic fibroblasts after purification by immunoprecipitation:

Cell Line/ Stimulus	*Relative PKCδ Activity (n=3)*	*p-Value*
Norm/Ctrl	*1.0 ± 0.0*	
Norm+TNF	*7.2 ± 6.5*	
Cys/Ctrl	*6.0 ± 4.0*	*0.05*
Cys+TNF	*28.2 ± 15.3*	

Table 1: PKCδ Activity in Normal and Cystinotic Fibroblasts : PKCδ was assayed in normal and cystinotic fibroblasts after exposure to TNFα (30 ng/mL, 4 h). The cytosolic proteins were immunoprecipitated using anti-PKC δ, and PKC δ activity assessed using a commercially available non-radioactive fluorescent peptide (Promega). The amount of PKCδ protein present was assessed by western blot. The enzyme activity values were divided by the densitometry units derived from the western blots, and then normalized to the normal untreated samples to control for differences in the intensity of the western blots. This experiment was done in triplicate. P= 0.05 for PKCδ activity between TNF α treated normal fibroblasts versus TNF α treated cystinotic fibroblasts .

The enzyme recovered from the cystinotic cells has significantly more activity (p=0.05) after treatment with TNFα than that recovered from the normal fibroblasts, as predicted from the in vitro data by Chu, et al (1).

We designed 22-mers for PKCδ that ablate its RNA, leading to >90% diminution of protein on Western blot of normal and cystinotic fibroblasts as shown below for fibroblasts in Fig 3

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Fig 3: The effect of siRNA against PKCδ on PKCδ protein expression in normal and cystinotic fibroblasts.

siRNA against PKCδ reduces the increased apoptosis seen in cystinotic fibroblasts compared to that seen in normal fibroblasts by about 30% (79 to 54%, $p<.05$, table 6). This suggests that PKC δ may account for a significant amount of the enhanced apoptosis due to lysosomal cystine storage.

	% Apoptosis	
Cell line/Inducer	Control siRNA	PKCdelta siRNA
Norm/Control	5.5±1.8	6.1±1.8
Norm/TNF-alpha	40.2±12.5	32.7±2.9
Cys/Control	5.1±2.4	8.0±0.9
Cys/TNF-alpha	78.2±2.5	51.5±2.9

Table 2: The Effect of siRNA against PKCδ on the augmented apoptosis seen in cystinotic fibroblasts. Fibroblasts were pre-treated with siRNA to PKCδ (0.625 µl/mL) for 6 h, then incubated 72 h. Fibroblasts were then treated with TNFα as previously described for 16 h, and apoptosis was assessed using CaspACE followed by FACS analysis. $p<0.05$ for cystinotic cells treated with TNF-alpha and control siRNA versus cystinotic cells treated with TNFα and PKCδ siRNA.

Cystinosis patients display a typical swan neck deformity of the proximal tubule (4,5), concomitant with development of the Fanconi syndrome, and consistent with hypocellularity of that structure. We have recently studied a cystinotic kidney removed during renal transplantation for ESRD due to cystinosis. Serial sections (4 µ) were prepared at Tulane and studied by Ramesh Nair MD and Patrick D Walker MD of Nephropathology Associates, Little Rock, AR. They quantified the occurrence of atubular glomeruli in the cystinotic sample and in three control samples. Of 50 cystinotic glomeruli so studied, 49 had no associated tubule, compared to only 1 atubular glomerulus of 30 normal glomeruli so studied (Chi^2 <0.001). We believe this finding is consistent with progression of the renal tubule portion of this disease ultimately resulting in the swan neck deformity. It may also explain the delay in glomerular failure in cystinosis, as tubule destruction slowly occurs with progressive hypocellularity from increased apoptosis. Atubular glomeruli are implicated in the chronic ESRD of diabetic nephropathy (36).Representative photomicrographs of a normal and an atubular cystinotic glomerulus are as follows:

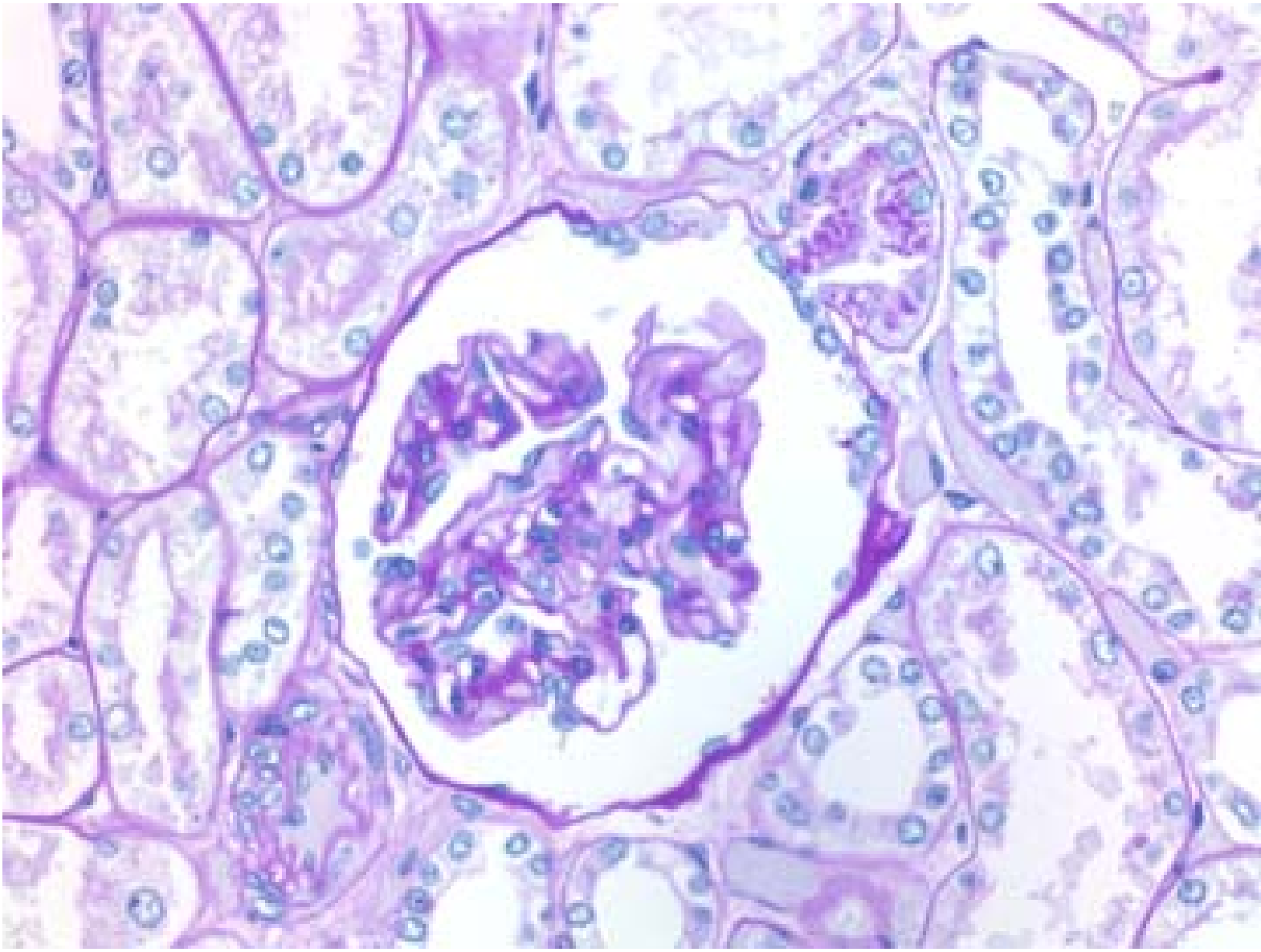

Figure 4: A 4 µ section through a normal glomerulus, showing the attached renal tubule at the upper right

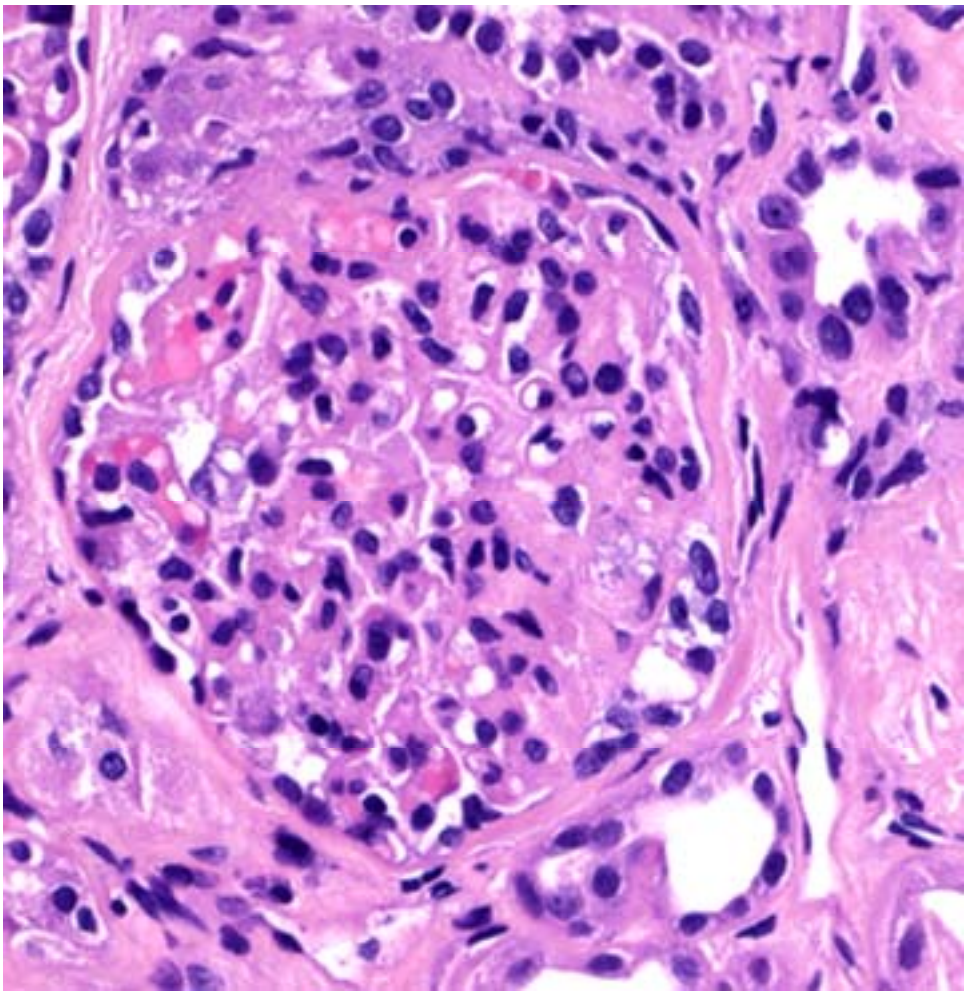

Figure 5: A 4µ section through a cystinotic glomerulus showing no connection to the tubule. These findings were confirmed by analyzing 50 reconstructed glomeruli using 4µ serial sections .

Based on the literature, our preceding data above, and that already published by us (3,7), we hypothesize that lysosomal cystine alters the rate of apoptosis, at least in part by cysteinylation or inappropriate mixed-disulfide formation of key apoptotic thiols, such as PKC δ, p53, Bcl-2, or other thiol-containing elements of the apoptotic cascade This effect is not due to altered gene expression, because the lysosomal cystine effect upon apoptosis is observed in the presence of 2µM Actinomycin D, which inhibits transcription by greater than 90% at the interval and concentration we used (3,6). We hypothesize that, as renal injury develops in cystinosis from inappropriate apoptosis, the tubules degenerate, passing through the swan neck deformity stage, and then become disconnected from the glomeruli, as shown in Fig 5 above, thus impeding glomerular filtration, and ultimately causing glomerular failure. We will continue examination of cysteinylation of key proteins in the known apoptotic pathways in human cells for the duration of this award, as we originally proposed. Study of the pathway(s) by which lysosomal cystine results in augmentation of the apoptotic cascade will lead to a clearer

understanding of the regulation of apoptosis, and a better understanding of the development of the cystinotic phenotype.

We have one publication in this interval citing CRN support: M Park, J Thoene, Increased Apoptosis Produces the Phenotype in Nephropathic Cystinosis " Pediatric Nephrology 20:441-446, 2005. Reprints have not been received yet, as this is the April 2005 issue. We will forward them when available.

References:

1)Chu F, Chen LH, O'Brian C. Cellular protein kinase C isozyme regulation by exogenously delivered physiologic disulfides- implications of oxidative protein kinase C regulation to cancer prevention. Carcinogenesis 25, 585-596, 2004.

2)Najafian B, Kim Y, Crosson JT, Mauer M. Atubular glomeruli and glomerulotubular junction abnormalities in diabetic nephropathy. J Am Soc Nephrol. 2003 14 :908-17.

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4)Jackson JD, Smith FG, Litman NN, Yuile CL, Latta H. The Fanconi syndrome with cystinosis: Electron microscopy of renal biopsy specimens from five patients. Am Journ Med 33, 893-910, 1962.

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6) . Sawicki SG, Godman , GC On the recovery of transcription after inhibition by actinomycin D J Cell Biol 1972 55, 299 -309

7) M Park, J Thoene, Increased Apoptosis Produces the Phenotype in Nephropathic Cystinosis " Pediatric Nephrology 20:441-446, 2005.