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REVIEW

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Potential role of apoptosis in development of the cystinotic phenotype

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Abstract Much still remains unclear about the proximal biochemical effects of mutations on development of the phenotype in inborn errors of metabolism. Cystinosis is an example of this phenomenon. We have recently shown that cystinotic cells undergo apoptosis at a two- to fourfold higher rate than controls. Cystinotic cells pre-treated with cysteamine, normalizing cystine content, display a four- to fivefold decrease in apoptosis, while normal cells pre-treated with cystine dimethylester, increasing lysosomal cystine, exhibit a fivefold increase in apoptosis. We speculate that cystine exits the lysosomal compartment during early apoptosis and affects apoptotic proteins in the cytosol, causing an inappropriate commitment to proceed to cell death. The resulting chronic hypocellularity could account for all the characteristics of the nephropathic cystinotic phenotype. The milder variants of cystinosis may result from modifying mutations within an apoptotic protein, ablating the proapoptotic effects of cystine. Failure of the mouse knockout for cystinosis to show renal involvement may be the result of differences in apoptotic processes between man and mouse. Apoptosis is a major final common pathway for many disease states. Therefore, a better understanding of the effect of lysosomal cystine on apoptosis may help to clarify development of other diseases.

Keywords Lysosomes · Lysosomal storage disorders · Thiol · Disulfide · Protein kinase C-delta · Cystine

Cystinosis

Cystinosis is an autosomal recessive disorder due to defects in cystinosin, the lysosomal membrane transporter for the disulfide amino acid cystine. Cystinosin is a 367-amino acid lysosomal membrane protein. It contains seven transmembrane motifs, and is the only well-characterized lysosomal amino acid transport protein.

Patients with the nephropathic form of the disorder are of normal length and weight at birth, lack dysmorphic features, but have onset of short stature and failure to thrive in the 1st year of life. Patients display ocular abnormalities, including corneal clouding due to cystine crystals, photophobia, peripheral retinopathy, and recurrent corneal erosions in later life. Renal abnormalities include the swan neck deformity of the renal proximal tubule, the renal Fanconi syndrome, and end-stage renal failure by 10 years of age if untreated [1, 2]. They display polyuria and polydipsia in the first years of life and may have recurring episodes of dehydration. Primary hypothyroidism occurs, delayed puberty is frequent, and ricketts, myopathy, difficulty swallowing, and diabetes mellitus also develop at later ages [2]. The most-common mutation in nephropathic cystinosis is a 57-kilobase deletion found mostly in the people of northern European descent [3], which ablates most of the gene, leaving no residual lysosomal cystine transport activity.

There are two variant forms of cystinosis. Intermediate cystinosis patients acquire symptoms similar to nephropathic patients, but the onset of symptoms is about a decade later [4]. Ocular (previously called benign) cystinosis patients display only eye symptoms without involvement of any other system [5]. Mutations in the gene for cystinosis (*CTNS*) in some variants display some residual transport activity, but fibroblasts from patients with each of the three forms of cystinosis have overlapping cystine content [2], thus, the cause of these rare variants remains unexplained.

Apoptosis

Apoptosis (programmed cell death, PCD) is a set of characteristic morphological and physiological changes that a cell undergoes to commit suicide without provoking

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an inflammatory response. The requirement for ATP and new protein synthesis distinguishes this process from necrosis, a process that harms adjacent cells. There are overlapping characteristics between the two processes. Apoptosis is characterized morphologically by cell shrinkage, membrane blebbing, and nuclear disintegration. Molecularly, this process is characterized in some cell types and after some stimuli, by the appearance of phosphatidylserine moieties on the outer surface of the plasma membrane, caspase activation, mitochondrial transition pore formation, and double-stranded DNA breaks [6]. Apoptotic pathways can be activated by two classes of triggers. Intrinsic triggers (for example UV light) induce the apoptotic response from within the cell, while extrinsic triggers [for example TNFR1 stimulation by tumor necrosis factor (TNF)-alpha or Fas/CD95 stimulation by anti-Fas antibodies] cause apoptosis by trimerization of receptors on the plasma membrane. Many stimuli can cause cells to become senescent, necrotic, or apoptotic, depending on environmental conditions, cell type, and stage in the cell cycle [7]. PCD is a complex process involving hundreds of proteins and many pathways. TNF-alpha and anti-CD95 (or anti-Fas) antibodies activate apoptosis by trimerizing TNF receptors or CD95 receptors, respectively. UV light induces apoptosis by introducing double-stranded DNA breaks, in addition to trimerizing surface receptors and perturbing oxidative stress pathways. After initiation of apoptosis, initiator procaspases are recruited and cleaved to active forms. Activation of upstream caspases is followed by activation of Bcl-2 family member proteins. Proapoptotic Bcl-2 family member proteins (such as Bid and Bax) participate in the formation of the mitochondrial permeability transition (MPT) pore, which allows depletion of the mitochondrial membrane gradient and exodus of proapoptotic proteins such as cytochrome C [8]. Executioner procaspase zymogens are then activated, followed by chromatin condensation, DNA fragmentation, and nuclear disintegration. DNA repair proteins such as PARP are inactivated to further accelerate DNA degradation. Finally, cells undergoing apoptosis divide into caspase-positive membrane-bound fragments known as apoptotic bodies, which are then phagocytosed by neighboring cells [6, 9]. There are other caspase-independent pathways leading to apoptosis, involving different systems from that summarized here [10, 11].

Lysosomes and apoptosis

Involvement of lysosomes in cell death has been observed since 1997 [12]. During the apoptotic response, the lysosomal membrane becomes permeabilized with concomitant translocation of cathepsins B and D (lysosomal cysteine and serine proteases) to the cytosol. The lysosomotropic agent MSDH (*O*-methyl-serine dodecylamide hydrochloride) has been shown to cause TUNEL positivity, a marker of DNA damage, in J-774 cells, in a dose-dependent manner [13].

A number of studies of lysosomal cysteine proteases (cathepsins) and their role in apoptosis show that lysosomes are permeabilized early in the pathway. It has been shown by Ishisaka et al. [14] that lysosomal proteases may be indirectly involved in the caspase-3 cleavage pathway, independent of the mitochondrial cytochrome C cascade. Cathepsins leaked from lysosomes during apoptosis cleave Bid, a protein involved in mediating the mitochondrial permeability transition pore formation, at Arg65. Caspase-8 cleaves this molecule at Arg59 and granzyme B at Arg75 [15, 16]. Cathepsin B translocates from lysosomes to the cytosol after treatment of mouse hepatocytes with TNF-alpha. In vitro release of cytochrome C from mitochondria occurs after incubation with cathepsin B, and this release is inhibited by caspase-8 inhibitor CrmA [17]. Thus, lysosomal permeabilization most likely occurs upstream from the mitochondrial cytochrome C release. The precise sequence of events in lysosome and mitochondrial permeabilization during apoptosis is still being determined. The point at which the process becomes irrevocable is an important, but unanswered question.

The protein kinase C (PKC) family of proteins includes 11 isozymes. These fall into three distinct categories: classical PKCs (α, βI, βII, and γ) require Ca^{2+}, and are activated by DAG (1,2-dioleoyl-sn-glycerol); novel PKCs (δ, ϵ, η, and θ) are activated by DAG but do not require Ca^{2+}; and atypical PKCs (ζ, μ, and ι/λ) are not activated by DAG and do not require Ca^{2+}. Typically, the classical and atypical subcategories, and also PKCϵ, are associated with promoting cell survival, whereas the novel subcategory is associated with promoting apoptosis [18].

PKCδ is the most abundant and well-characterized of the PKCs. Studies have shown an association between PKCδ loss and tumor growth. Also, PKCδ overexpression leads to growth arrest during the G2/M phase of the cell cycle, and PKCδ is a target for caspase-3 cleavage during UV-induced apoptosis. Cleavage of PKCδ by caspase-3 results in a 40-kDa kinase-active fragment, which alone can induce apoptosis [18, 19]. GSSG (oxidized glutathione), cystine, and $(Cys\text{-}Gly)_2$ increase PKCδ activity in vitro. These compounds also inactivate PKCs α, βI, γ, ϵ, and ζ, all of which are either anti-apoptotic or pro cell survival [20].

Apoptosis is increased in cultured cystinotic cells

Cystinosis causes renal death by the age of 10 years if untreated. This phenomenon is unexplained on the cellular level, since lysosomal cystine is isolated from the cytosol. Lysosomal membranes become permeable in the early stages of apoptosis [15, 17], and cystine could therefore exit into the cytosol during this process. We have investigated apoptosis in normal and cystinotic fibroblasts, and have demonstrated that cell lines from cystinotic patients undergo apoptosis in tissue culture at a higher rate than control cell lines from normal individuals [21]. Table 1 shows that normal fibroblasts exhibit about 5%–7% apoptosis after three typical inducers (TNF-alpha,

Table 1 The apoptosis rate in nephropathic cystinotic, variant cystinotic, and normal fibroblasts. Cells were treated with apoptosis inducers tumor necrosis factor (TNF)-alpha (30 ng/ml) plus actinomycin D (2 μg/ml), anti-Fas antibodies (500 ng/ml) plus actinomycin D (2 μg/ml), or UV light (60 mJ). Cells were then stained with CaspACE (Promega), a fluorescein isothiocyanate-conjugated form of the pan-caspase inhibitor VAD-Fmk that fluoresces upon binding to caspases, and analyzed by fluorescence microscopy. A minimum of 250 cells were scored per condition, and all analyses were performed in triplicate; thus, at least 750 cells were scored per condition

Cell line	Phenotype	% Apoptosis			
		TNF-alpha	Anti-Fas	UV	Control
GM008	Nephropathic	14.9±0.6	17.7±2.9	12.8±4.8	2.2±0.2
GM760	Nephropathic	13.5±4.3	14.4±2.7	26.3±5.4	3.1±0.7
GM046	Nephropathic	16.1±4.1	22.3±2.9	13.1±2.0	2.2±0.6
Average		14.8	18.1	17.4	2.5
GM08761	Ocular	8.2±0.98	5.9±1.9	6.4±2.0	2.3±0.3
GM00379	Intermediate	11.4±2.0	7.7±1.0	8.4±0.94	3.1±0.2
GM010	Normal	9.2±1.9	6.5±2.3	7.0±0.6	2.8±0.2
GM05399	Normal	6.3±1.9	4.9±0.8	7.2±1.0	1.9±0.1
Average		7.8	5.2	7.1	2.4

T-statistics: $P<0.001$ for average nephropathic vs. average normal, $P<0.05$ for ocular vs. nephropathic, and $P>0.05$ for ocular vs. normal, intermediate vs. nephropathic, and intermediate vs. normal. This experiment was performed a total of three times

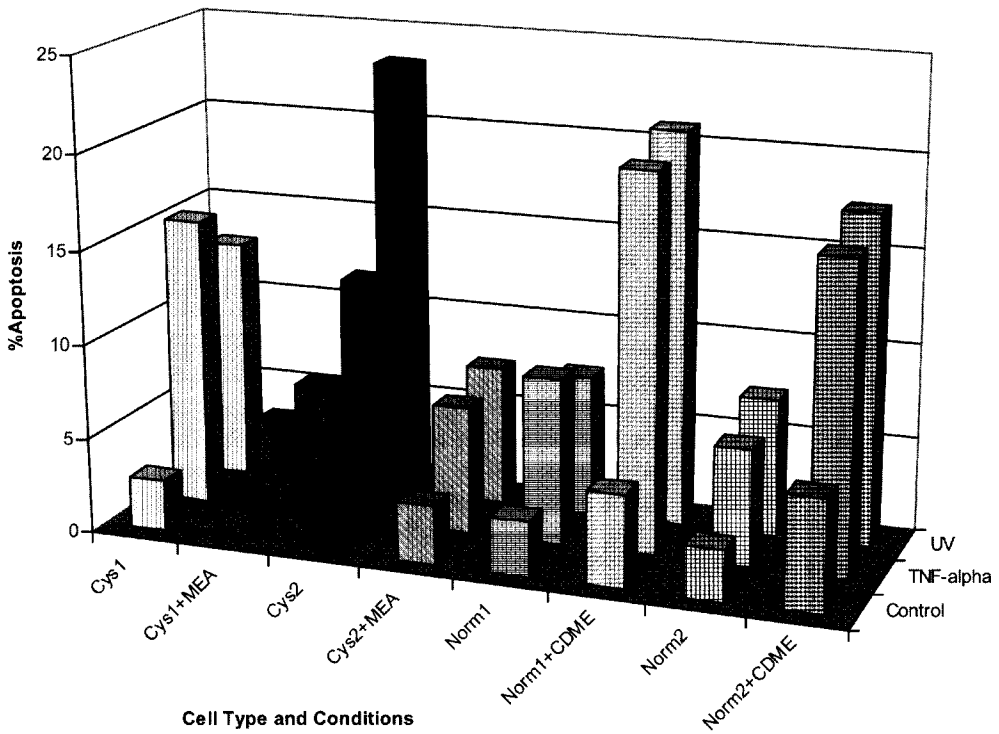

Fig. 1 The effect of cysteamine (*MEA*) or cystine dimethylester (*CDME*) on apoptosis in cystinotic or normal fibroblasts. Cystinotic cells [GM00008 (cys1) and GM00760 (cys2), passage 7–13] were pre-treated with MEA (1.0 mM, 1 h) prior to exposure to apoptotic triggers, thus depleting them to a normal lysosomal cystine content (0.01–0.13 nmol cystine per mg protein). Normal cells [GM05399 (norm1) and GM00010 (norm2), passage 7–13] were pre-treated with 0.5 mM CDME for 1 h prior to apoptotic triggers, thus loading their lysosomes with cystine (0.47–1.95 nmol cystine per mg protein). The cells were then treated with apoptotic stimuli [tumor necrosis factor (*TNF*)-alpha, 30 ng/ml or UV light, 60 mJ], and incubated for 16 h in cystine-free or CDME-containing medium. All cell lines were purchased from Coriell Cell Repositories and are untransformed. Apoptosis was assayed by CaspACE, and scored using fluorescence microscopy (250 cells per condition in triplicate). The apoptosis rates for the individual cystinotic cell lines were significantly different compared with both individual normal cell lines ($P<0.05$)

anti-Fas antibodies, and UV light). Cystinotic fibroblasts, however, display 14%–18% apoptosis after exposure to the same inducers ($P<0.05$, Table 1). Moreover, when normal fibroblasts are pre-treated with cystine dimethylester (CDME), causing lysosomal cystine loading, apoptosis increases from an average of 7.6% to an average of 23.6%. When cystinotic fibroblasts are pre-treated with cysteamine, causing depletion of lysosomal cystine to a normal concentration, the rate of apoptosis decreases from an average of 13.7% to an average of 6.9% (Fig. 1).

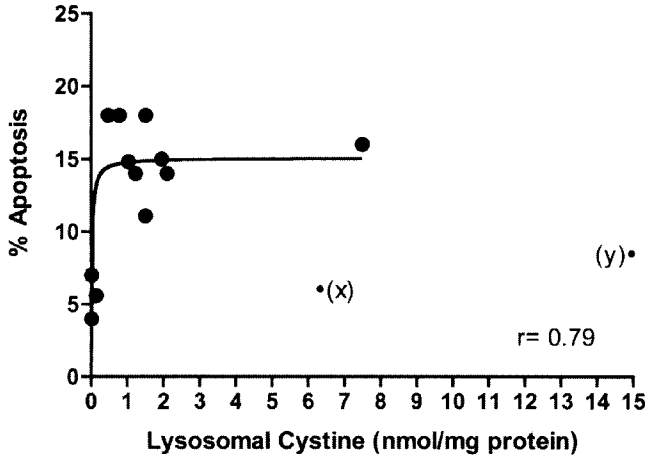

Fig. 2 Lysosomal cystine content versus apoptosis rate. The lysosomal cystine content was measured by CBP at the time the cells were harvested [21] and plotted against the apoptosis rate displayed 16 h after exposure to TNF-alpha (30 ng/ml). Nephropathic cystinotic and normal cells are represented by *filled circles*. The benign cell line is represented by x and the intermediate cystinotic cell line is represented by y. The correlation coefficient r applies to the *filled circles* only

There are two variant forms of cystinosis: intermediate (juvenile) cystinosis and ocular cystinosis. These forms have a less severe phenotype with later onset (juvenile) or no renal involvement (ocular) than the more common nephropathic form. The lysosomal cystine content of these forms overlaps that of the nephropathic. When apoptosis rates were measured in ocular and juvenile cystinotic untransformed fibroblasts, they were not significantly different from normal cell apoptosis rates ($P>0.05$, Table 1). A plot of lysosomal cystine measured at the time the cells were harvested versus the apoptosis rate in normal and nephropathic fibroblasts forms a rectangular hyperbola with a K_m of about 0.2 nmol cystine/mg protein, close to the normal cystine content. Apoptosis rates determined in fibroblasts from the benign (x) and intermediate (y) variant lines do not fall on the curve (Fig. 2). Thus, they displayed no increase in apoptosis induction despite elevated cystine content.

The mouse knockout model for *CTNS* described by Cherqui et al. [22] does not demonstrate any of the typical characteristics of the human disease, even though the tissue cystine concentration is elevated to levels found in the human disease. Studies of apoptosis in cultured mouse, human, and rat fibroblasts, and mouse and human renal proximal tubule epithelial (RPTE) cells, show that the mouse cells do not display the significant increase in apoptosis when pre-treated with CDME that is seen in human fibroblasts or RPTE cells or rat fibroblasts (Table 2 and Fig. 3). A human transformed fibroblast line (GM00637 from Coriell cell repositories), when loaded with cystine, displays 52.5% apoptosis compared with 4.9% when not loaded with cystine ($P<0.05$). The mouse CRL-6475 transformed fibroblast line from the ATCC, however, only demonstrates 27.5% apoptosis when cystine loaded, compared with a control of 5.8%. In contrast, the rat transformed fibroblast cell line 1764 displays an apoptosis rate of 83.8% when cystine loaded, compared with 5.1% at baseline ($P<0.05$). After TNF-alpha exposure, the mouse line displayed a decrease in apoptosis after CDME loading from 70.3% to 55.1%, but the human line showed an increase in apoptosis induced by combined CDME and TNF-alpha exposure. The mouse cells display a smaller increase in lysosomal cystine after CDME loading than do the human and rat cell lines (human cells increased from 0.6 to 2.0 nmol cystine/10^6 cells, while mouse cells increased from 0.4 to 0.7 nmol cystine/10^6 cells). As shown in Fig. 3, cultured mouse and human RPTE cells also differ in their apoptotic response. Human RPTE cells display a large increase in apoptosis after CDME exposure, whereas mouse RPTE cells show little response. These differences are seen when CDME is used alone, or with TNF-alpha or anti-Fas exposure.

Discussion

The apoptotic response is constituted to recognize a number of adverse changes in the cell's environment, including an increase in reactive oxygen species that could threaten DNA integrity. The lysosomal cystine accumulation in cystinotic patients can be released during the early permeabilization phase of lysosomes in apoptosis, possibly perturbing redox balance. Cystine could also act to enhance the production of reactive oxygen species or enhance formation of the MTP complex by interacting with thiols on the mitochondrial surface. We further hypothesize that lysosomal cystine released in the early stages of apoptosis increases the activity of one or more proapoptotic proteins by cysteinylation. PKCδ has been shown to display an increase in its proapoptotic

Table 2 The effect of cystine dimethylester (CDME) on apoptosis in mouse, rat, and human fibroblasts. CRL1764, a Fischer rat-derived cell line purchased from the ATCC, CRL6475, a C57/Bl6 mouse-derived cell line also purchased from the ATCC, and GM00637, a transformed normal human cell line purchased from Coriell Cell Repositories, were used in these experiments. Cells were treated with the apoptotic triggers described in Table 1 with or without prior exposure to 0.5 mM CDME for 16 h, then stained with caspACE, and analyzed via fluorescence activated cell sorting (FACS). P values were calculated for non-CDME-treated versus CDME-treated cells and are in *bold* if less than 0.05. In total three separate experiments were performed

Treatment	Rat % positive	P	Mouse % positive	P	Hum % positive	P
Control	5.1±2.4	**0.04**	5.8±4.6	0.18	4.9±2.1	**0.04**
CDME	83.8±4.1		27.5±20.6		52.5±15.4	
TNF	8.6±3.1	0.07	70.3±14.8	0.07	63.9±15.3	0.14
TNF+CDME	64.6±39.2		55.1±10.8		85.2±5.1	

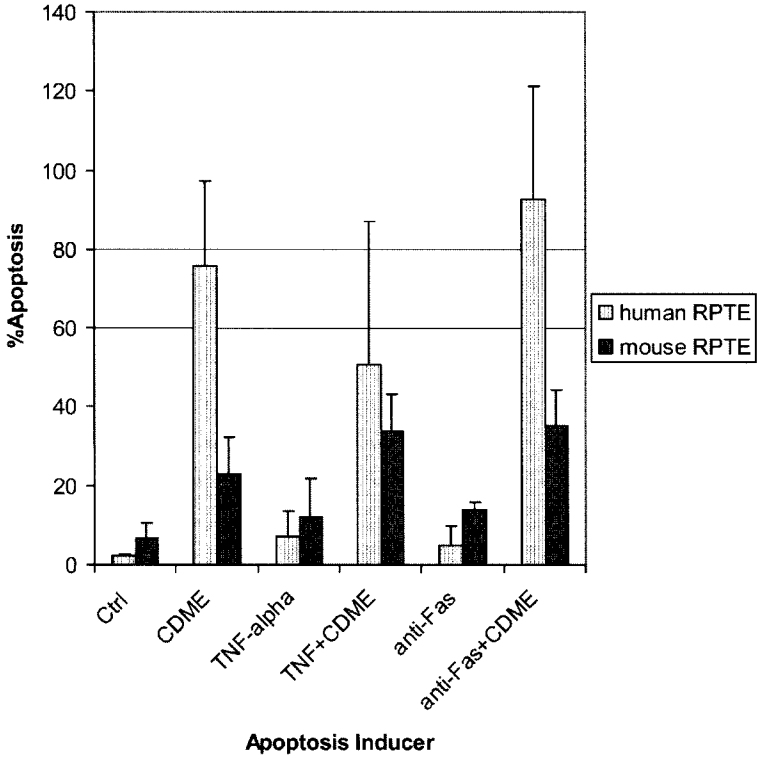

Fig. 3 Apoptosis in normal human and mouse RPTE cells. Human RPTE cells, purchased from Biowhittaker and mouse RPTE cells harvested and cultured from kidneys of the strain C57/Bl6, were exposed to TNF-alpha or anti-Fas antibodies for 16 h. Apoptosis was analyzed by CaspACE staining followed by flow cytometry with a Beckman Coulter FACS. *P* values were significant for all CDME-treated versus non-CDME-treated conditions in human cells ($P<0.05$). However, the apoptosis rate in mouse cells was not significantly increased by treatment with CDME except when treated with anti-Fas antibodies ($P<0.05$)

activity in vitro upon cysteinylation [20]. Inhibition of binding of anti-apoptotic proteins to their activator counterparts (e.g., TRX inhibition of ASK1) could also result from cysteinylation of thiols at the active sites of these proteins and thereby increase the apoptotic signal. The net result would be enhanced apoptosis in cells whose lysosomes are loaded with cystine as shown by Park et al. [21] and Figs. 1, 2, and 3 and Tables 1 and 2.

The milder phenotypes in the variant forms of cystinosis may be explained by hypothesizing mutations that ablate the enhanced apoptotic response resulting from lysosomal cystine release. This mutation could take the form of a bulky amino acid that precludes access to a critical thiol by cystine, or could be the result of other mutations that eliminate the thiol stimulation function of a proapoptotic protein. Since intermediate and ocular cystinosis breeds true in families, and since there is a significant difference in phenotype, we predict that different mutations altering the effect of lysosomal cystine on different proteins will be found in pedigrees of the two diseases.

Failure of the Ctns -/- mouse to develop the human cystinosis phenotype may be the result of a difference between man and mouse in which a key thiol group is missing in a critical proapoptotic protein in the mouse. There are significant differences in the structure of PKCδ in man, mouse, and rat. Man and rat share a binding domain critical for activity of PKCδ and the kinase domain of man and rat possess five cysteine residues compared with mouse, which contains six [23, 24, 25]. To the extent that augmentation of PKCδ phosphorylation activity occurs upon cysteinylation, failure of this activity in the mouse could explain why the mouse model of cystinosis does not display the human disease characteristics.

Aberrant apoptosis plays a central role in disease processes such as Huntington disease, systemic lupus erythematosus, and cancer [26, 27, 28]. Cystinosis possibly offers another example of abnormally increased apoptosis. All the findings in the nephropathic phenotype are explicable by a progressive generalized hypocellularity. The swan neck deformity, poor growth, retinopathy, myopathy, and other features clearly could be the result of inadequate cell numbers due to inappropriate apoptosis. A sudden increase in disulfide content of the cytosol following a normally dampened apoptotic stimulus may perturb the critical homeostatic balance necessary for continued cell survival. Cystinotic cells may be useful in understanding the importance of local concentrations of small molecules in apoptosis. Future research to identify the different proteins affected by cystine is needed.

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