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Genetics

Lack of complementation in somatic cell hybrids between fibroblasts from patients with different forms of cystinosis

(somatic cell genetics/complementation analysis)

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ABSTRACT **Cystinosis is an autosomal recessive disease in which three clinical forms are recognized: infantile nephropathic, with renal tubular damage by 1 year of age and progressive glomerular insufficiency; intermediate, with tubular and glomerular insufficiency beginning at a later age; benign, with no kidney damage. Skin fibroblasts cultured from patients with all types of cystinosis show increased intralysosomal free (nonprotein) cystine; however, fibroblasts from heterozygotes have normal free-cystine values. To determine whether genetic complementation occurs between the different forms, somatic cell hybrids were constructed between cells from a patient with infantile nephropathic cystinosis and cells from patients with other types of cystinosis. If complementation occurred, the hybrids would be expected to have normal cystine levels. To construct hybrid cells, a "universal parent" cell type (TG1-neo), which was hypoxanthine/aminopterin/thymidine (HAT) sensitive and G418 resistant was constructed from an infantile nephropathic cystinosis fibroblast strain. Polyethylene glycol fusion of TG1-neo with other cells that are not HAT sensitive or G418 resistant allowed for selection of hybrid cells in a medium containing HAT and the aminoglycoside G418. As indicated by elevated cystine levels, complementation did not occur between TG1-neo and two different benign cystinosis strains, an intermediate cystinosis strain, or another nephropathic cystinosis cell strain. When a normal fibroblast strain was fused with TG1-neo, all 15 hybrid clones studied contained normal amounts of intracellular free cystine.**

Cystinosis is an autosomal recessive disease characterized biochemically by the accumulation of free (nonprotein) cystine within lysosomes (1). The cystine accumulates in this location because of a defect in the normal egress of this amino acid from the lysosome to the cytosol (2–7). Three forms of cystinosis are recognized clinically: infantile nephropathic, with proximal tubular damage before 1 year of age and progressive glomerular insufficiency; intermediate (late onset), with tubular damage and glomerular insufficiency beginning at a later age; benign, with no kidney damage (1). Further heterogeneity occurs within the intermediate form with an age of onset of ≈4 years in some families (8, 9), 10–17 years of age in other families (10–13), and 25 years in one family (1). Peripheral leukocytes and fibroblasts cultured from patients with all types of cystinosis show increased amounts of intralysosomal free (nonprotein) cystine. Although polymorphonuclear leukocytes from cystinotic heterozygotes contain increased amounts of free cystine (14) cultured fibroblasts from heterozygotes contain normal amounts of cystine (1). The purpose of this study was to see whether somatic cell hybrids formed between fibroblasts from a patient with infantile nephropathic cystinosis and fibroblasts from patients with other forms of cystinosis would demonstrate correction of the defect—i.e., genetic complementation.

To search for genetic complementation between different forms of cystinosis, we required hybrid cells capable of prolonged growth so that enough cells could be obtained for study and a system that could select for the hybrid cells. We constructed such a cell in a stepwise process. First, a fetal nephropathic cystinosis cell strain was mutated with ethyl methanesulfonate and then, a 6-thioguanine-resistant hypoxanthine/aminopterin/thymidine (HAT)-sensitive cell was cloned. This cell strain was then transfected with pSV3-neo plasmid DNA and selected in the aminoglycoside G418. We refer to the resulting cell strain (TG1-neo) as a "universal parent," since it can be fused with any cell that is not "neomycin" (G418) resistant or HAT sensitive (presumably hypoxanthine phosphoribosyltransferase negative). Only the hybrid can exist in G418 and HAT medium.

TG-1 neo was fused with five other cell types: a normal, an intermediate cystinotic, two benign cystinotics, and another infantile nephropathic cystinotic. In addition to cystine measurement, additional characterization included chromosome studies, HLA marker analysis, and, in one set of hybrids, glucose-6-phosphate dehydrogenase isoenzyme analysis.

MATERIALS AND METHODS

Fibroblast Cell Strains. Human diploid fibroblasts were grown on culture dishes (Lux) in Coon's modified Ham's F-12 medium (Irvine Scientific) with 10% fetal bovine serum (Irvine Scientific) (15). Hybrids were cultured in the same media with HAT (16) and G418 (0.1 mg/ml) added (17). The fibroblast cultures used were from a fetus with infantile nephropathic cystinosis (18), from a Black patient with the same type of cystinosis (19), from a patient with intermediate cystinosis, and from two unrelated patients with benign cystinosis. The normal control fibroblast culture was started from the foreskin of a normal male infant.

Mutation and Transfection. Fetal skin fibroblasts from a patient with nephropathic cystinosis (F.S.J.) (18) were treated with 2.15 mM ethyl methanesulfonate for 24 hr and selected in 30 μM 6-thioguanine. One clone from this selection was then used for the transfection. This clone was resistant to 6-thioguanine and sensitive to HAT. For transfection, 10^6 cells per 100-mm plate were treated with 20 μg of pSV3-neo plasmid DNA (without carrier DNA) in the presence of calcium phosphate (17) but without glycerol shock. Plasmid pSV3-neo was provided by Peter Southern (Scripps Clinic and Research Foundation, La Jolla, CA). To select for G418-resistant clones, cells were plated at 10^5 cells per 100-mm dish in G418 (0.1 mg/ml). G418 was obtained from GIBCO. The transfection frequency was 1.6 clones per 10^5 treated cells. The transfected cells had increased, but not

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Abbreviation: HAT, hypoxanthine/aminopterin/thymidine.

infinite, life spans. For this reason, cells from several G418-resistant clones were combined for fusion experiments and are referred to as TG1-neo.

Hybrid Formation. Hybrid cells were formed with TG1-neo and all other cell lines using 50% polyethylene glycol (M_r, 6000) (20). All fusions were done 24 hr after plating 10^6 cells in 60-mm dishes in a 1:1 mixture of TG1-neo and the other cell type. Twenty-four hours after polyethylene glycol treatment cells were replated at 10^5 cells per 100-mm plate in HAT and G418 (0.1 mg/ml). Growth medium was changed every 3–5 days and hybrid clones were pulled from the dish after 2 weeks with cloning rings. Each clone was then expanded in the HAT and G418 medium and analyzed as cell number permitted.

Analysis of Hybrid Clones. Cystine measurements were done on $\approx 10^5$ cells (35-mm plates) by the method of Oshima *et al.* (21). Chromosome analysis was done, at the same passage, in cells cultured either on chamber slides at 5×10^4 cells per slide or on coverslips placed in 35-mm tissue culture dishes. *In situ* chromosome harvests were done using colchicine as mitotic inhibitor and 0.8% sodium citrate for 30 min at room temperature to swell the cells (Hsiao-chen Chang, personal communication). Glucose-6-phosphate dehydrogenase analysis was done on at least 2×10^5 cells using agarose gel electrophoresis in a Tris/EDTA/borate buffer, pH 6.8 (22). HLA typing was performed at the Histocompatibility Lab of the University of California, San Diego, Medical Center on at least 10^6 cells by lymphocyte cytotoxicity.

RESULTS

Characterization of Hybrid Clones. In all fusions, unmixed parental cells were treated with polyethylene glycol and placed in selection medium as controls for the actual hybrid crosses. No clones were seen after 2 weeks in these controls, whereas clones were evident in all hybrid crosses. The earlier the passage number of the cell crossed with TG1-neo, the more vigorous were the clones that grew out.

Although the selection procedure itself ensured that only hybrid cells would survive, additional proof of hybridization was obtained. HLA markers were identified from both the parental and hybrid cells (Table 1). In four of the five hybrids constructed, at least one HLA marker from each parent was identified.

TG1-neo was fused with fibroblasts from patient N.W., a Black child with infantile nephropathic cystinosis. Patient N.W. has the electrophoretically fast-migrating form (variant A) of the enzyme glucose-6-phosphate dehydrogenase, whereas TG1-neo expresses variant B. Electrophoresis of mixed extracts from both parental cells showed both the A and B isoenzymes, whereas hybrid clones (TG1-neo × N.W.) showed a third band of glucose-6-phosphate dehydrogenase activity between the A and B isoenzymes (data not shown). This provides independent evidence that the cells studied were hybrid cells (16).

Chromosome analysis of TG1-neo showed it to be aneuploid with a median chromosome number of 60. All cells crossed to TG1-neo were diploid (or near diploid). Analysis of chromosome spreads from the hybrid clones showed median chromosome counts ranging from 64 to 91 (mean ± SD; 73 ± 8) in TG1-neo × Bas (the normal control cell) and from 56 to 96 (72 ± 10) in TG1-neo × the various cystinotic cell types used. In the hybrids, many spreads could not be counted accurately because of chromosome "overlap." Since these represented primarily those with the greatest numbers of chromosomes, the reported values are underestimates.

The cystine content of all the cystinosis × cystinosis clones analyzed was in the typical cystinotic range no matter which type of cystinosis was fused with TG1-neo (Table 2). All clones of normal (Bas) × TG1-neo had a cystine content within the range of normal fibroblasts. There was no correlation between cystine content and median chromosome number in any of the hybrid clones.

Table 1. HLA types expressed by parental and hybrid cells

Cell line	Diagnosis	Parental cells A	Parental cells B	Hybrid clones (with TG1-neo) A	Hybrid clones (with TG1-neo) B
TG1-neo (universal parent)		**32,29**	**15,40**	—	—
N.W.	Nephropathic	2,3	35,39	2,3	39,**40**
D.S.	Intermediate	2,3	15	2	**15,40***
J.B.	Benign	2,3	62	2,3	62†
J.O.	Benign	1,24	7,62	1,24	7,**40**,62*
Bas	Normal	3,24	55,62	3	**15,40**‡

*Two clones studied expressed the same HLA type.
†Results from pooled clones.
‡Three clones studied expressed the same HLA type.

DISCUSSION

Since Cogan *et al.* described the first case of benign cystinosis in 1957, many other cases have been reported (23–30). Investigators have attempted to explain why these patients, who have accumulations of cystine crystals in many of the same tissues as patients with nephropathic cystinosis, do not have renal damage. The first biochemical difference noted in patients with benign cystinosis was that the cystine content of their leukocytes, although elevated, was not elevated to the same range as in patients with infantile nephropathic cystinosis (8, 26). Kidney biopsy samples from two patients with benign cystinosis demonstrated no cystine crystals (24, 29), and the cystine content of the one biopsy sample measured was not elevated (29). Thus, it seems that renal function is normal in patients with the benign form of cystinosis because their kidneys do not accumulate cystine.

Patients have also been reported with an intermediate form of nephropathic cystinosis (8–13, 31–33). Unlike patients with benign cystinosis, these patients do have renal dysfunction. They differ from patients with infantile cystinosis because their disease is not apparent until an older age, they frequently do not have the complete Fanconi syndrome, and their glomerular insufficiency progresses more slowly. The age at which their first symptoms are noted has ranged from 4 to 25 years (1). When more than one individual in a sibship has this condition, the age of onset of symptoms is similar. These facts suggest that the intermediate form of nephro-

Table 2. Cystine content of parental and hybrid cells

Cell line	Diagnosis	Cystine content, nmol of ½ cystine per mg of protein: Parental cells	Hybrid clones (with TG1-neo)
TG1-neo (universal parent)		6.9 ± 1.7 (6)	—
N.W.	Nephropathic	10.6 ± 4.2 (8)	8.8 ± 4.6 [5]
D.S.	Intermediate	7.0 (1)	5.0 ± 1.0 [6]
J.B.	Benign	15.8 (1)	6.9 ± 2.6 [10]
J.O.	Benign	4.2 (1)	6.5 ± 1.0 [6]
Bas	Normal	0.2 ± 0.1 (5)	0.2 ± 0.2 [15]

Values are shown as mean ± SD. (*n*) represents the number of parental cultures measured. [*n*] represents the number of clones measured.

pathic cystinosis may represent a range in the spectrum of different types of cystinosis rather than a separate disease. It is possible that these patients are "compound heterozygotes" for the infantile nephropathic and benign forms of cystinosis. Goldman *et al.* (8) pointed out that the range of renal damage in patients with the three forms of cystinosis is proportional to the cystine content of their leukocytes. The leukocyte free cystine is highest in infantile and lowest in benign cystinosis patients (8). Gahl and Tietze (30) recently reported that lysosomal cystine egress is similar in fibroblasts from a patient with intermediate cystinosis and patients with infantile cystinosis. On the other hand, leukocytes from a patient with benign cystinosis displayed a considerable degree of cystine transport ability as measured by transstimulated cystine entry into lysosomes (9% and 29% of mean control values in two experiments) (30).

Studying the development of renal pathology in nephropathic cystinosis during fetal development and infancy might help us understand the differences between the infantile and intermediate forms of cystinosis. The kidney from an 18-week-old fetus with the infantile form of cystinosis contained 100 times the normal cystine content, yet the histology of this fetal kidney was normal (18). When followed very carefully from birth, the earliest age at which any renal abnormalities can be detected in infantile cystinosis is 4–6 months (34, 35). These patients do not usually present for medical attention secondary to renal symptoms until after 8 months of age. It appears that the time at which renal damage occurs in infantile cystinosis is a function of the time the high concentration of cystine exists. The actual cystine level is also likely to be important and the later development of renal pathology in intermediate cystinosis may be due to a lower cystine content in these patients' kidneys.

Genetic heterogeneity is very common in lysosomal storage disorders (36–42). In other such conditions in which genetic complementation was studied by means of somatic cell hybridization, the exact enzyme defect was usually known. Therefore, it was possible to do complementation studies with heterokaryons. If both of the parental cell types have near zero enzyme activity, any increased enzyme activity in the heterokaryon is indicative of genetic complementation. In contrast, fibroblasts can be identified as cystinotic only by demonstrating a 50- to 100-fold increase in their intracellular content of free cystine, or by studying their lysosomal cystine transport compared to normal "cystine-loaded" lysosomes. Both of these methods require large numbers of cells and cannot be interpreted with confidence in heterokaryons.

It was possible to show genetic complementation in heterokaryons between fibroblasts from patients with Tay–Sachs and Sandhoff disease (37, 38). Both of these studies showed apparent return of hexosaminidase activity after such fusion. A study of heterokaryons that were formed between fibroblasts from patients with Hurler, Scheie, and Hurler–Scheie compound syndrome showed no return of α-L-iduronidase activity and thus was taken as evidence that these conditions were allelic mutants (39). Heterokaryon formation between fibroblasts from patients with the infantile, juvenile, and adult onset forms of Gaucher disease yielded similar results. The heterokaryons formed remained deficient in acid β-glucosidase activity, and this was taken as evidence that these mutations occur in a single gene (40, 41). Honey *et al.* (42) described three distinct complementation groups for mucolipidosis III after complementation analysis of 12 fibroblast lines from 10 sibships.

Workers in our laboratory have attempted to study the various forms of cystinosis by complementation analysis for many years. Hybridizing HeLa cells with cystinotic fibroblasts was done as a preliminary step for such studies (16). This study was possible because we used a variant of HeLa (D-98) that is hypoxanthine phosphoribosyltransferase negative and can be selected against in HAT medium. In this experiment, all hybrid clones showed correction of the cystinotic defect. However, it was not possible to do a complementation study between cells from patients with different types of cystinosis in a convincing manner until the availability of a method for inserting dominant selectable markers into mammalian cells made this present study possible (17).

The chromosome loss in hybrid clones was greater in this study, in which cystinotic fibroblasts were fused with TG1-neo, than in our previous study, in which cystinotic fibroblasts were fused with HeLa cells. In the latter study, the hybrids lost $\approx$10% of their parental chromosomes, whereas in the present study the loss averaged $\approx$30%. The lack of complementation in the cystinotic $\times$ cystinotic crosses could only be explained by this chromosome loss if one set of homologous chromosomes (the chromosome that contains a defective gene for cystinosis) from either TG1-neo or the other cystinotic parent was lost in each of the 27 cystinosis $\times$ cystinosis clones. Although this possibility cannot be ruled out, it seems unlikely. Such a pair of homologues were clearly not lost from the normal parent in the TG1-neo $\times$ Bas cross, since this would have prevented the observed correction of cystine content.

Since we constructed only one "universal parent," from a fibroblast from a patient with infantile cystinosis, we were not able to construct every possible hybrid cross. However, we were able to show that no complementation occurred between cells from a patient with typical infantile nephropathic cystinosis, a Black patient with the same defect, a patient with intermediate cystinosis, or two unrelated patients with benign cystinosis. The lack of complementation in any of these crosses strongly suggests that these defects are allelic.

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