Determinants of Mild Clinical Symptoms in Cystic Fibrosis Patients

Residual Chloride Secretion Measured in Rectal Biopsies in Relation to the Genotype

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Abstract

Previous Ussing chamber measurements of secretagogue-provoked changes in short circuit current in rectal suction biopsies of cystic fibrosis (CF) patients showed that in a minority of patients chloride secretion in response to cholinergic agonists is reduced but not completely absent. To assess a possible relationship between this phenomenon and both the genotype and the phenotype, we performed Ussing chamber experiments on rectal suction biopsies of 51 CF patients. The CF mutation was identified in 89 out of 102 CF alleles. No apparent chloride secretion was found in 30 CF patients (group I). Low residual chloride secretion was found in 11 CF patients (group II), while a relatively high residual secretion appeared in 10 CF patients (group III). Pancreatic function was preserved more frequently in CF patients displaying residual secretion: 0% in group I, 27% in group II, and 60% in group III ($P < 0.001$). The age at diagnosis (mean±SEM) in group III (18.4 ± 6.6) was significantly different from group I (1.2 ± 0.4, $P < 0.01$) and group II (3.5 ± 1.4, $P = 0.05$). Residual chloride secretion was found in some of the 28 dF508 homozygous patients (three in group II, and one in group III), disclosing that other factors than the CF gene defect itself affect the transepithelial chloride transport. The age at diagnosis correlates significantly with the magnitude of the secretory response, even within the dF508 homozygous patients ($r = 0.4$, $P < 0.05$). We conclude that residual chloride secretion in CF is the pathophysiological basis of preserved pancreatic function and delayed presentation of the disease, which is not exclusively determined by the CF genotype. (*J. Clin. Invest.* 1994. 93:461–466.) Key words: cystic fibrosis • intestinal secretion • genotype • phenotype • mutational analysis

Introduction

In cystic fibrosis (CF)[1] conductive chloride transport is defective in epithelial tissues. This results in viscous secretions asso-ciated with pulmonary infections, malabsorption, and intestinal obstruction (1–3). A major gene defect (dF508) represents $\sim$ 70% of the gene mutations in CF (4, 5). The product of the CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR), functions itself as a chloride channel and may therefore be held responsible for the known defect in transepithelial chloride transport in CF (6, 7). In most CF patients, including dF508, the mutation in the CF gene results in a mislocalization rather than dysfunctioning of the CFTR-chloride channel (8–13). In a smaller group of CF patients, however, the channel protein is properly inserted into the apical membrane of the epithelial cells but is functionally impaired or inactive (8, 14).

The severity of the disease ranges widely. Homozygosity for the dF508 mutation was found to correlate with pancreatic insufficiency (15–18), early diagnosis (16–19), poor lung function (16, 18), chronic pseudomonas colonization (18), and high mortality (18). A relationship between clinical manifestations of the disease and the genotype implies that abnormalities in transepithelial electrolyte transport may vary with specific mutations. More than 230 different CF mutations have now been described (20).

In contrast to other epithelia of CF patients, intestinal tissues not only show defective chloride secretion in response to cAMP-linked secretagogues, but also in response to Ca-linked secretagogues, including cholinergic stimulation (21–23). As shown by electrophysiological studies in isolated colonic crypts (24) and in colonic epithelial cell lines (25, 26), a major stimulating effect of carbachol is on the basolateral potassium conductance, creating an increased driving force for chloride exit through CFTR-chloride channels in the apical membrane (24, 25). Furthermore, the protein kinase C component of the carbachol response may activate the CFTR-chloride channel by direct phosphorylation (27–29). However, in contrast to their prominent role in other epithelial tissues in the intestine, the contribution of calcium-activated chloride channels (different from CFTR) to the apical chloride secretion is negligible (26, 30, 31).

Recently, we demonstrated that in a minority of CF patients chloride secretion provoked by cholinergic agonists was reduced but not completely absent (32). This residual transepithelial chloride transport could help to explain the clinical heterogeneity among CF patients (32, 33). To assess a possible relationship between this phenomenon and both the genotype and the phenotype, we extended our Ussing chamber experiments on rectal suction biopsies to a larger group of CF patients and found a relationship between residual chloride secretion and both a milder phenotype and the CF genotype.

Methods

Patients. This study was approved by the Hospital Medical Ethical Committee and by patients or their parents by written informed con-

sent. During the study period of 2 yr, 173 pediatric and adult CF patients (78 male, 95 female) with a mean age of 14.8 yr (range, 0.1–69.9 yr) who regularly visited our University Hospital were asked to have a rectal suction biopsy taken. Supplemented with 2 patients homozygous for the G542X mutation who came from another clinic, a total of 51 CF patients (25 male, 26 female) with a mean age of 16.5 yr (range, 0.1–69.9 yr) were enrolled in the study. Each patient's diagnosis was confirmed by at least two abnormal sweat tests (> 70 mmol sodium/liter or > 60 mmol chloride/liter).

The medical records were evaluated for a history of meconium ileus, pancreatic insufficiency, distal intestinal obstruction syndrome (DIOS), chronic pseudomonas colonization, and nasal polyp extraction. Pancreatic sufficiency was defined as normal fecal chymotrypsin (> 6.3 U/g) and fat content values (< 5 g/d) without pancreatic enzyme supplements. The date of onset of chronic pseudomonas colonization was defined as the date of the first isolation of at least two consecutive sputum samples from which pseudomonas could be cultured. Records of forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) were measured and expressed as a percentage of predicted normal values for age, height, and sex. The results of each patient's most recent pulmonary function tests were used. At the end of the 2-yr study period, each subject was assigned a clinical score with the use of the Shwachman and Kulczycki clinical scoring system as modified by Doershuk et al. (34). This is based on general activity, pulmonary physical findings, growth and nutrition, and x-ray findings, resulting in a score from 20 (worst) to 100 (best). Furthermore, each patient's age at diagnosis and maximal derived (current age at the end of the 2-yr study period or age at death) were recorded. Four patients died during the study period.

Electrophysiological study. The procedure of short circuit current (I_{SC}) measurements on rectal tissue has been described earlier (32). Briefly, fresh rectal suction biopsies were mounted in an Ussing chamber with an exposed area of 1.13 mm^2. After stabilization of the basal I_{SC}, various substances were added to the mucosal (M) and/or serosal (S) baths: (a) glucose (10^{-2} mol/liter) was added to M and S; (b) to reduce the contribution of electrogenic sodium absorption to the I_{SC}, specific sodium channels were blocked by amiloride (10^{-4} mol/liter) added to M; (c) the endogenous prostaglandin synthesis that is possibly linked to cAMP-mediated chloride secretion was inhibited by adding indomethacin (10^{-5} mol/liter) to M and S; (d) by adding carbachol (10^{-4} mol/liter) to S the cholinergic activation of chloride secretion was initiated. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

To investigate the reproducibility of the experiments without increasing discomfort for the participants, we analyzed two biopsy specimens simultaneously in a subgroup of 18 CF patients.

The mean carbachol-provoked change in I_{SC} values (±SEM) was 35.1±2.6 μA/cm^2 in 47 healthy volunteers < 18 yr old, and 22.4±4.3 μA/cm^2 in 8 subjects > 18 yr old. This difference tended to be statistically significant ($P = 0.07$, Mann-Whitney; Pearson's correlation between age and I_{SC}: $r = -0.23$, $P = 0.10$). The inward current provoked by carbachol in controls (I_{SC} positive; Fig. 1) reflects transcellular chloride transport (serosa to mucosa) through the Na-K-Cl cotransporter in the basolateral membrane and the CFTR-chloride channel in the apical membrane (32). The observed magnitude of this response in controls may well be underestimated by underlying potassium secretion (32).

In the majority of CF patients, hereafter classified as group I, carbachol induces only an outward current response (I_{SC} negative; Fig. 1). Most plausibly this reversed current results from apical potassium secretion that is unmasked in the case of absent or largely reduced chloride secretion (32, 35, 36). The carbachol-provoked I_{SC} changes in CF patients, and possibly also in controls, may therefore be the net result of two opposite currents. In a subclass of CF patients, usually after a negative peak response, a separate and small positive peak response (i.e., inward current) was observed (Fig. 1, groups II and III). To facilitate comparison with the data from group I and controls, the net maximal change in I_{SC} was defined as the sum of the negative and positive

peak responses (Fig. 1, *neg + pos*). This calculated response was either negative (low residual secretion, group II) or positive (high residual secretion, group III).

DNA analysis. We analyzed for the R117H, R347P, A455E, 621 + 1G → T, dI507, dF508, 1717 – 1G → A, V520F, G542X, S549N, S549I, G551D, R553X, R1162X, S1251N, W1282X, and N1303K mutations by PCR amplification of the appropriate exon sequences. Specific mutation detection involved the use of allele-specific oligonucleotides or amplification refractory mutation system (ARMS) (5, 37, 38). In addition, PCR-amplified exons 10 and 11, which are known to contain several mutations, were subjected to single strand conformation polymorphism as an aspecific screen for sequence alterations in these exons (39).

Statistics. Differences were considered significant if $P \leq 0.05$. The Pearson's correlation coefficient was calculated between short circuit current and age at diagnosis. Comparing the maximal derived age, a survival analysis was performed using the log-rank test for testing significancy. All statistical tests were two tailed.

Results

The reduction in basal I_{SC} in response to amiloride (mean±SEM) in CF patients ($-9.2±1.6$ μA/cm^2; $n = 51$) was not different from age-matched control values ($-8.7±1.5$ μA/cm^2; $n = 50$). This outcome strengthens our previous conclusion that the strong increase in apical sodium channel activity seen in CF airway does not occur in rectal epithelium (32).

Three groups of CF patients could be distinguished on the basis of the electrophysiological response to carbachol. Group I consisted of 30 patients who failed to demonstrate residual intestinal chloride secretion. In group II, 11 patients showed low residual chloride secretion, and group III consisted of 10 patients displaying high residual chloride secretion. From group I to III, the CF patients had a milder clinical presentation of the disease. This is demonstrated by the age at diagnosis, which is significantly higher in CF patients with high residual secretion compared with groups I and II (Table I). The difference in the age at diagnosis between groups I and II indicates that even CF patients with a low residual secretion are less affected by the
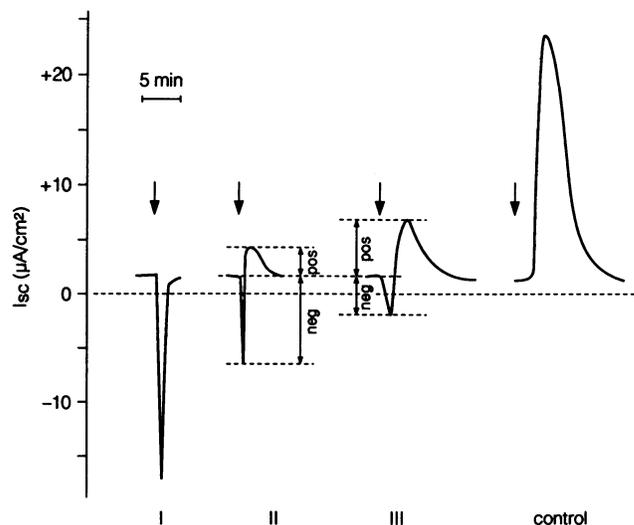

Figure 1. Different patterns of carbachol-induced I_{SC} responses in rectal epithelium in the presence of amiloride and indomethacin. Additions are marked by arrows. For concentrations and site of additions, see Methods.

Table I. Characteristics of CF Patients with a Different Pattern of Carbachol-induced Changes in I_{sc} in Rectal Suction Biopsies in the Presence of Amiloride and Indomethacin

	Intestinal chloride secretion		
	I: No apparent residual secretion	II: Low residual secretion	III: High residual secretion
CF patients (n)	30	11	10
Carbachol induced secretion:			
Net change in I_{sc} (μA/cm^2)	-8.8 ± 2.1	-5.8 ± 1.5	5.2 ± 1.4*
Genotype:			
1. dF508/N1303K	2	0	0
2. dF508/dF508	24	3	1
3. dF508/G542X	1	1	0
4. G542X/G542X	0	2	0
5. dF508/A455E	0	1	3
6. A455E/other	0	0	1
7. R553X/other	0	1	0
8. dF508/other	3	3	5
Age at diagnosis (yr)	1.2±0.4	3.5±1.4	18.4±6.6[‡]
Died in study period (n)	2	2	0
Maximal derived age (yr)	14.3±2.0	13.0±2.4	26.9±6.4[§]
Shwachman score	69.0±4.2	74.1±7.6	74.0±7.0
Pulmonary function:			
CF patients (n)	19	8	7
Age (yr)	19.1±1.7	15.4±1.4	33.9±6.8
FVC (% predicted)	68.1±4.2	75.4±10.3	81.8±7.3
FEV1 (% predicted)	51.9±5.4	63.9±12.4	58.5±10.7
Pancreatic insufficiency (n [%])	30 [100]	8 [73]	4 [40][‖]
Meconium ileus (n [%])	10 [33]	2 [18]	1 [10]
DIOS (2n [%])	4 [14]	0 [0]	0 [0]
Pseudomonas colonization (n [%])	21 [72]	7 [64]	4 [40]
Nasal polyp extraction (n [%])	9 [31]	1 [9]	3 [30]

Values are expressed as mean±SEM unless otherwise indicated. Differences of group III from groups I or II: *I, II: $P < 0.001$; [‡] I: $P < 0.01$, II: $P = 0.05$, Mann-Whitney; [§] I: $P < 0.05$, II: $P = 0.06$, log-rank test; [‖] $P < 0.001$, Fisher's exact test.

disease ($P = 0.07$, Mann-Whitney) than those without. The proportion of pancreatic insufficient CF patients decreases significantly from groups I to III (Table I). Additionally, the mean carbachol-provoked I_{SC} response in the 9 pancreatic sufficient patients (3 of group II and 6 of group III) was significantly higher (2.6±2.5 μA/cm^2) than in the other 42 pancreatic insufficient patients (-7.1 ± 1.6 μA/cm^2; $P < 0.01$, Mann-Whitney). The proportion of CF patients with other intestinal symptoms (meconium ileus and DIOS) was also lower in group III, but the differences were not significant (Table I). The prevalences of pseudomonas colonization, and nasal polyp extraction are summarized in Table I. Comparing the lung functions and Shwachman scores (Table I), as well as the previously mentioned prevalence of pancreatic status, DIOS, pseudomonas colonization, and nasal polyp extraction, we have to take into account that the time for CF patients of group III to develop more advanced disease was longer. This is demonstrated by their average maximal derived age, which was significantly higher in group III than in the other groups (Table I).

The distribution of genotypes among the three groups defined on the basis of electrophysiological response is indicated in Table I. Of all genotypes the dF508 homozygous patients are most frequent (55%). Most, but not all, of them lack a positive I_{SC} component in their response (Table I). CF patients carrying

one A455E mutation are predominantly found in group III. Considering the variability in I_{SC} responses, even within the dF508 homozygous patient group, we also asked ourselves whether or not a relationship existed between the magnitude of the secretory response and the phenotypic expression of the disease. A positive correlation ($r = 0.37$, $P < 0.01$) was found between age at diagnosis and the net I_{SC} response (Fig. 2). This correlation was also significant ($r = 0.40$, $P < 0.05$) for the dF508 homozygous patients.

Two CF patients combined dF508 with G542X; one of these showed low residual secretory activity. The two CF patients homozygous for the G542X mutation both displayed low residual secretion (Table I). All CF patients with one A455E allele showed residual activity (Table I). Compared with all other genotypes in this study, these CF patients are diagnosed at a significantly older age, have a higher I_{SC} response, have a higher maximal derived age, and are less frequently pancreatic insufficient (Table II).

In 18 CF patients duplicate rectal suction biopsies were studied. When every first result (Fig. 2, x-axis) equals the results of the duplicate registration (y-axis), then a line through zero (β = intercept = 0) with a tangent = 1 can be drawn. In the equation: Result1 = α Result2 + β, the β was -1.5 (not different from 0) and the α was 0.7 (not different from 1).
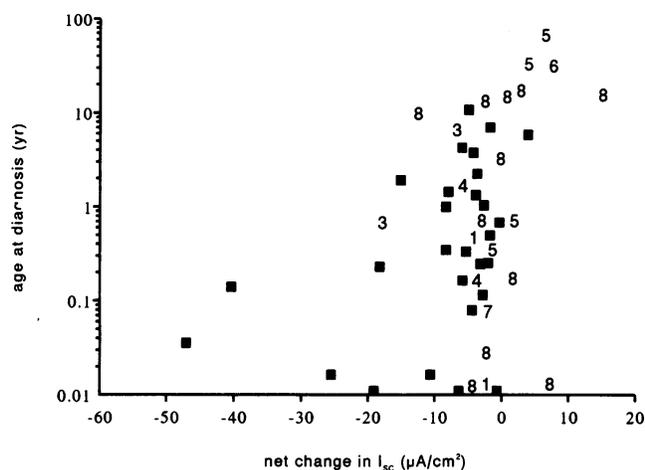
Figure 2. Correlation between age at diagnosis and I_{SC} measurements on rectal suction biopsies of CF patients ($r = 0.37$, $P < 0.01$). The correlation was also found in the dF508 homozygous patients (■) ($r = 0.40$, $P < 0.05$). The other genotypes, indicated by numbers, are explained in Table I. Positive net I_{SC} changes implicate net residual chloride secretion while a negative response indicates predominant K^+ secretion perhaps blunted by underlying residual Cl^- secretion.

Discussion

The changes in I_{SC} provoked by carbachol represent the net result of all electrical charge fluxes through both apical and basolateral membranes. A positive change in I_{SC} induced by carbachol in a control biopsy specimen is an indicator for net electrogenic chloride secretion. However, in most CF patients, responses to carbachol are characterized by a change in I_{SC} in

Table II. Characteristics of CF Patients with One A455E Mutation vs. All Other Tested Mutants

	Genotype	
	1, 2, 3, 4, 7, 8 (see Table I)	5 (dF508/A455E) 5 (A455E/other)
Patients (n)	46	5
Carbachol induced secretion:		
Net change in I_{sc} ($\mu A/cm^2$)	−6.4±1.6	3.8±1.7*
Age at diagnosis (yr)	2.7±0.7	26.3±12.4‡
Died in study period	4	0
Maximal derived age (yr)	14.5±1.5	35.0±11.4§
Shwachman score	67.0±12.5	71.5±3.4
Pulmonary function:		
CF patients (n)	30	4
Age (yr)	18.4±1.2	42.9±9.8
FVC (% predicted)	72.7±4.1	72.5±8.0
FEV1 (% predicted)	57.9±5.1	42.1±7.4
Pancreatic insufficiency (n [%])	41 [89]	1 [20]‖
Meconium ileus (n [%])	13 [28]	0 [0]
DIOS (n [%])	4 [9]	0 [0]
Pseudomonas colonization (n [%])	29 [64]	3 [60]
Nasal polyp extraction (n [%])	11 [24]	2 [40]

Values are expressed as mean±SEM unless otherwise indicated.
* $P < 0.01$, ‡ $P < 0.05$, Mann-Whitney; § $P < 0.001$, log-rank test; ‖ $P < 0.01$, Fisher's exact test.

the opposite direction (32). Apparently, this inversed response reflects potassium secretion through apical potassium channels, most likely unmasked by the chloride channel defect associated with the CF condition (32). In both normal and CF tissue the magnitude of the chloride and potassium current, respectively, may well be underestimated when apical potassium channels are also functioning in normal tissue (36) and if apical chloride channels retain a residual activity in CF cells. However, the relative magnitude of chloride and potassium currents for each biopsy is difficult to estimate. First, the small size of the biopsy as well as the transient character of the changes in I_{SC} provoked by carbachol prevent accurate assessment of unidirectional ^{36}Cl or ^{86}Rb flux rates. Also, specific inhibitors of apical chloride channels (i.e., CFTR) or apical potassium channels are not yet available (31). The positive I_{SC} responses remain representative for residual chloride secretion while negative I_{SC} responses indicate predominant potassium secretion, perhaps blunted by underlying residual chloride secretion.

Considering the recent finding that most mutant CFTR is functionally active but is inappropriately targeted to the plasma membrane (8–13), one possible reason for a variable level of residual chloride secretion, in particular among patients of identical genotype, may be a variable degree of mislocation of the CFTR protein in the enterocytes. Alternatively, the variability may arise from a variable expression level of other chloride channel species in the apical membrane, e.g., calcium-activated chloride channels, which normally do not contribute much to the carbachol-provoked changes in I_{SC} in intestine (26, 30, 31). Furthermore, several missense mutations located in the transmembrane domain of CFTR are characterized by an incomplete loss of chloride channel function and may also lead to residual chloride secretion (14).

In Table I, we demonstrate a relationship between residual chloride secretion and clinical parameters. The positive I_{SC} responses observed in group III CF patients are not the result of the more advanced mean age in this group, since control values normally decline with increasing age (see Methods). The high residual secretion found in these CF patients might very well be the pathophysiological basis of their milder clinical presentation, as indicated by the age at diagnosis and the preserved pancreatic function. The data from from Table I additionally indicate that, in comparison with groups I and II, and in contrast of what could be expected from their higher current age, CF patients of group III also reach a higher Shwachman score and have a more preserved lung function. The preserved pulmonary function in group III suggests that residual chloride secretion may also occur in airway epithelium. Some of the CF patients (Fig. 2) have a calculated response approaching age-matched control values. However, the appearance of their responses was abnormal, due to the (preceding) transient deflection of the I_{SC} that was never observed in controls.

A more continuous relationship between the I_{SC} and the age at diagnosis is illustrated in Fig. 2. Even within the group of dF508 homozygous patients, a positive correlation between the net I_{SC} response and the age at diagnosis was found. As discussed above, the variability in phenotypic expression of the disease within patients of identical CF genotype can be explained by interindividual differences in the extent of missorting of mutant CFTR or in the expression of other (compensatory) chloride channels. It has been suggested before that genetic factors other than the CF gene may modify the expression

of the disease (17). Prediction of the clinical prognosis in individual CF patients may thus not rely on the CF genotype alone.

The previously described genotype–phenotype relationship (16–19) implies that the (residual) function of CFTR may vary with different mutations. In *Xenopus oocytes* it was demonstrated that the chloride conductance depends on the CF genotype (9). In this study we demonstrate that most, but not all, dF508 homozygous patients are found in group I and that CF patients with one A455E mutation can be found predominantly in group III. The latter mutation presumably affects the sorting and/or activity of CFTR to a lesser extent, thus allowing for residual secretion and milder clinical symptoms (Table II). The A455E mutation was also characterized by others as a mild mutation associated with preserved pancreatic function (40). The two dF508/N1303K patients both had pancreatic insufficiency and failed to show apparent residual chloride secretion. This is in agreement with others who characterized the N1303K mutation as a severe one (41).

Interestingly, two patients homozygous for the nonsense mutation G542X had a low residual secretion (in duplicate). Since in other stop codon mutants no CFTR mRNA was detectable (42), the residual chloride secretion in the two homozygous G542X patients most likely results from compensatory non-CF-affected chloride channel species (26) in the apical membrane, rather than from the presence of some functional CFTR.

We conclude that the residual chloride secretion in CF is the pathophysiological basis of preserved pancreatic function and delayed presentation of the disease. Compared with dF508, the A455E mutation can be characterized as a mild mutation allowing for residual chloride secretion. The occurrence of residual chloride secretion in the intestine is not exclusively determined by the CF genotype.

Acknowledgments

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