ARTICLES

Amelioration of Intestinal Disease Severity in Cystic Fibrosis Mice Is Associated with Improved Chloride Secretory Capacity

KATALIN GYÖMÖREY, RICHARD ROZMAHEL, AND CHRISTINE E. BEAR

Programme in Cell Biology [K.G., C.E.B.] and Genetics [R.R.] in the Research Institute, Hospital for Sick Children, and the Physiology [K.G., C.E.B.] and Pharmacology [R.R.] Departments, University of Toronto, Toronto, Ontario M5G 1X8, Canada

ABSTRACT

The variability in intestinal disease severity in patients with cystic fibrosis (CF) has been associated with the expression of secondary modifier genes. The locus containing these modifier genes in CF patients is syntenic with a modifier locus previously associated with survival in CF transmembrane conductance regulator–knockout mice. These previous studies showed that the proportion of CF mice that survive weaning (mildly affected mice) *versus* those that succumb to obstruction of the small intestine (severely affected) is related to their genetic background and the expression of modifier genes. In the present work, we show that the basal transepithelial chloride transport measured across jejunum obtained from mice of mixed genetic backgrounds segregates into two groups, some mice having low and others having high, near normal chloride transport. Further, we report that the segregation of mice with respect to intestinal chloride transport correlates with their predicted segregation on the basis

of genotype at the “modifier locus.” These findings support the hypothesis that intestinal disease modification in CF mice correlates with improved chloride transport through non-CF transmembrane conductance regulator chloride channels. (*Pediatr Res* 48: 731–734, 2000)

Abbreviations:

PD, potential difference
NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid
DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
CF, cystic fibrosis
CFTR, cystic fibrosis transmembrane conductance regulator
MI, meconium ileus
Wt, wild type
DPC, *N*-diphenylanthranilic acid

Disease severity in CF is variable. Although CF is caused by mutations in the CFTR, secondary genes likely influence disease severity in certain organs. MI, a severe form of intestinal disease, occurs only in 10 to 15% of CF patients (1, 2). It is considered likely that secondary genes influence the presentation of MI as this complication recurs in families yet it is not related to any particular class of mutation in the CFTR gene itself (1, 2).

Supporting this hypothesis, a recent study identified the presence of a genetic CF modifier of MI on human chromosome 19 (3). This modifier locus in CF patients is syntenic with

a region on mouse chromosome 7, which was previously linked to intestinal disease amelioration in CFTR knockout (CF) mice (4). In the mouse study, Rozmahel *et al.* showed that CF mice fall into three classes, based on survival: class I (CI) mice die of intestinal obstruction at birth, class II (CII) die at 4 wk during weaning, and class III (CIII) survive for a normal life-span (4). The proportion of CIII *versus* CII mice at weaning varies depending on the murine strain as the genetic modifiers are linked to certain strains. Only about 10% of CF mice in the outbred UNC population (comprising an undetermined mix of four strains) are long living (CIII) (5), and in the inbred BALB/cJ × 129/Sv background, approximately 50% of the mice are CIII (Fig. 1) (4).

The purpose of this work is to determine whether the classification based on survival at weaning correlates with chloride secretion through an alternate path, as a primary function of CFTR in the intestine is to mediate chloride

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Correspondence and reprint requests: Christine E. Bear, Ph.D., Programme in Cell Biology, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto M5G 1X8, Canada.

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secretion (6). Therefore, we tested the hypothesis that the chloride secretory capacity of CIII CF mice exceeds that of CII CF mice.

METHODS

Mice. Wt *Cfr* (+/+) and CF *Cfr* (-/-) mice were compared at 6–8 wk of age in the inbred BALB/cJ $\times$ 129/Sv background and the outbred UNC background of an undetermined mix of BALB/cJ $\times$ 129/SvJ $\times$ DBA $\times$ C57BL/6J strains. A significant number of CIII mice can be generated in the BALB/cJ $\times$ 129/Sv background for Ussing chamber studies. Weaning CF mice onto a liquid diet (5) allowed us to keep severely affected CII in addition to CIII CF mice alive beyond 6–8 wk of age. Approximately 90% of liquid-fed CF mice in the outbred population and 50% in the inbred background are predicted to be severely affected CII (Fig. 1) (4,5). All animal studies were approved through the Animal Care Committee at The Hospital for Sick Children, Toronto.

In vitro measurement of transepithelial PD and membrane resistance. Freshly excised intestinal tissue was mounted onto an Ussing chamber with a surface area of 0.28 cm² and bathed in a buffered solution of the following composition: 140 mM NaCl, 10 mM KHCO₃, 1.2 mM K₂HPO₄, 2 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose in the apical bath and 5 mM mannitol in the serosal bath. The low chloride buffer was prepared as above, except that the 140 mM NaCl was replaced with 140 mM sodium gluconate and 5.8 mM CaCl₂ to account for the calcium-chelating effect of gluconate (7). The solutions were adjusted to pH 7.4, heated to 37°C, and gassed with 95% O₂ plus 5% CO₂. PD measurements reached a steady state after 8 min following placement in the Ussing chamber. Membrane resistance was measured by passing a 1- μ A current at 1-min intervals and applying Ohm's law. NPPB, glibenclamide, DIDS, and bumetanide were dissolved in DMSO. The final DMSO concentration did not exceed 0.2%, and we confirmed that this DMSO concentration did not have an effect on the basal PD. DPC was dissolved in equimolar NaOH. The optimal concentration of each drug was predetermined in

jejuna from Wt animals. The potential toxic effect of the final drug concentrations used was assessed by comparison of the PD response evoked by glucose uptake via the electrogenic sodium glucose cotransporter before and after drug treatment. In intestinal tissues from three CIII CF mice, addition of 30 mM glucose to the apical solution produced the same change in PD before and after 0.5 mM NPPB treatment: 0.3 ± 0.09 mV and 0.2 ± 0.09 mV, respectively, suggesting that NPPB is not toxic to the tissue.

Statistics. The significance of differences between paired experimental groups was assessed using the *t* test or one-way ANOVA where appropriate.

RESULTS

Jejuna of CIII CF mice generate a negative transepithelial PD. We measured the basal PD across the jejuna of 6- to 8-week-old CIII CF mice from the inbred background (*i.e.* those that survived weaning on solid food) and their Wt siblings. The boxes shown in Figure 2 delineate the range covered by 50% of the PD measurements for a certain group of animals, with the line inside the box indicating the median value. The single lines extending from the box perimeter indicate the total range covered by these measurements. The

Figure 1. The proportion of CIII or mildly affected CF mice depends on mouse strain. The pies represent the population of CF mice at the time of weaning at 4 wk of age. *Left*, in the outbred background, only 10% of the CF mice belong to the CIII group (black), a group defined on the basis of survival curves (5). The remaining mice belong to CII. *Right*, in the inbred background, 50% of the mice survive when weaned onto a solid diet, and are hence CIII mice [(4), black portion of pie].

Figure 2. CIII mice from inbred background can generate negative transepithelial PD across jejunal epithelium Basal PD across the jejunum of age-matched (6–8 wk old) Wt mice ($n = 20$) and CIII CF mice ($n = 22$) with inbred background. Both groups exhibit a negative basal PD. The basal PD in CIII CF mice is less negative than that of their Wt siblings. Boxes show range of 50% of each population. The median is shown as a line within the box, and the range of the entire data set is indicated by single lines extending from the box perimeter. * indicates significant differences between CF and Wt values. The dotted line indicates the mean -1 SD of CIII CF values.

basal PD across the jejunum of Wt animals is negative at the luminal epithelial surface relative to the basolateral surface (Fig. 2). Jejuna obtained from CIII CF mice also exhibit a negative PD, albeit it is less negative than that observed in jejuna from Wt animals (Fig. 2). The mean $\pm$ SE of the PD values obtained from Wt animals and the CIII CF animals were -3.0 ± 0.9 mV, and -2.4 ± 0.6 mV, respectively ($p < 0.05$). The mean $\pm$ SE of the short circuit current values obtained from wild type animals and CIII CF animals were -48.2 ± 4.5 μ A/cm² and -22.2 ± 2.2 μ A/cm², respectively ($p < 0.05$). The transepithelial resistance in the jejunum of CIII CF mice (115 ± 5.8 Ω /cm²) was higher than that seen in Wt animals (69 ± 3.9 Ω /cm²), indicating that the less negative PD and short circuit current measurements in CF mice are not caused by loss of epithelial integrity.

Basal transepithelial PD in the jejunum of CIII CF mice in the inbred background reflects chloride secretion. To assess the contribution of chloride secretion to jejunal basal PD, we assessed the effect of chloride ion substitution on these measurements. We found that reduction of chloride ion concentration from 146 mM to 12 mM reduced the basal PD across the intestine of Wt mice from 4.7 ± 0.8 mV ($n = 6$) to 2.3 ± 0.2 mV ($n = 5$; $p < 0.03$). These findings support previous findings by Grubb and colleagues (8) and suggest that basal PD measurements primarily reflect chloride secretion. Furthermore, the basal PD was inhibited by blockers of chloride secretion. We applied bumetanide to the basolateral epithelial membrane, so as to inhibit chloride uptake via the Na⁺/K⁺/Cl⁻ cotransporter. The chloride channels inhibitors NPPB, DPC, glibenclamide, and DIDS were applied to the apical bath to block apical channels. All of these inhibitors, with the exception of DIDS, diminished the basal PD in Wt mice. Importantly, these same blockers also inhibited the basal PD across jejuna obtained from CIII CF mice (Fig. 3). These findings suggest the jejunal epithelium of CIII CF mice can produce chloride secretion.

CII and CIII CF mice can be segregated on the basis of jejunal chloride secretion. In the previous studies, we found that inbred CIII CF mice, identified by survival on a solid diet, exhibit a mean basal PD of -2.4 ± 0.6 (SD) mV (Fig. 2) and that this PD reflects mostly chloride secretion (Fig. 3). If the expression of modifier genes correlates with basal chloride secretion, we would expect CII CF mice to exhibit decreased chloride secretion when compared with their CIII siblings. To investigate this hypothesis, we weaned CF mice in the inbred and outbred backgrounds onto a liquid diet. This experimental maneuver will maintain CII mice alive by alleviating obstruction (5) and is unlikely to cause direct changes in the secretory capacity of the jejunal epithelium. For example, in preliminary studies, we determined that the chloride channel blocker NPPB inhibited the jejunal PD measured in solid diet- and liquid diet-fed Wt animals to a similar extent, by 1.8 ± 1.2 (SD) and 1.8 ± 0.4 (SD) mV, respectively.

We classified CF mice fed liquid diet into those that are similar to CIII animals and those which are not similar to CIII mice on the basis of the jejunal PD measurements. The CIII bioelectric phenotype was defined as any PD value more negative than -1.8 mV as this is the mean PD measured for

this group $+1$ SD (Fig. 2). Figure 4 shows that the proportion of mice which exhibit negative PD values in the same range as the CIII-CF mice varies depending on the genetic background as expected (4, 5). These proportions mirror those predicted on

Figure 3. The basal PD in the jejunum of CIII CF mice and Wt siblings is sensitive to inhibitors of chloride secretion. *A*, sample tracings show that the basal PD in Wt and CIII CF mice is diminished by apical application of NPPB. *B*, bar graphs show mean PD ($\pm$ SE) before (–) and after (+) application of 200 μ M bumetanide (B), 500 μ M NPPB (N), 1 mM DPC (P), 500 μ M glibenclamide (G), and 500 μ M DIDS (D) in 6 CIII CF mice and 8 Wt mice. * indicates the inhibition was statistically significant ($p < 0.05$, paired t test); NS indicates that the inhibition was not significant ($p > 0.05$, paired t test).

Figure 4. Liquid diet-fed CF mice in the outbred and inbred background can be segregated into two groups based on the basal PD. *Left*, the PD measurements of 19 CF mice in the outbred background and maintained on a liquid diet are displayed (gray bar). Of these 19 mice, only two exhibit a basal PD more negative than -1.8 mV, typical of the CIII group (black bar). The basal PD of most mice (17 of 19) in this background is less negative than -1.8 mV (like CII). *Right*, the PD measurements of 15 CF mice in the inbred background and maintained on a liquid diet are displayed (gray bar; open circle represents an outlier). Of these 15 mice, seven exhibited a basal PD that is more negative than -1.8 mV (like CIII, black bar), and eight exhibited a PD less negative than -1.8 mV (like CII, white bar). The cut-off for the CIII phenotype was determined as the mean of the CIII group in Figure 2 $+1$ SD. These proportions of CIII vs CII mirror those predicted on the basis of survival curves (Fig. 1).

the basis of published survival curves for CF mice on these backgrounds (Fig. 2) and support the notion that CII and CIII mice can be segregated on the basis of basal transepithelial PD values. Figure 5 reports the mean transepithelial resistance across jejunal segments obtained from CF mice, and it is clear that differences in basal PD between groups do not reflect differences in tissue integrity. Further, the chloride channel blocker NPPB effectively inhibits transepithelial PD in both groups (Fig. 5). Thus, lumenally directed electrogenic chloride transport can be mediated by channels other than CFTR in the small intestine, and there is a correlation between the extent of this transport and predicted survival of CF mice.

DISCUSSION

In summary, these studies show that there is a correlation between the basal PD across the jejunum and the predicted long-term survival in CFTR-knockout mice. Further, our studies show that this basal PD primarily reflects chloride secretion. As survival is conferred by the expression of a modifier gene(s) (4), the identity of which remains unknown, we suggest that this modifier gene(s) may mediate or regulate chloride secretion by this tissue. Although we lack direct proof at this juncture, it is conceivable that enhanced basal chloride secretion in the small intestines of these mice may alleviate the accumulation of obstructive mucus and fecal material that leads to mortality in the severely affected animals.

The molecular basis for the apical, constitutively active chloride conductance path characterized in these studies remains unclear. In contrast to our previous studies in rectal epithelium (9), we could not stimulate further chloride secretion in jejuna obtained from CIII CF mice by treatment with agonists of cellular calcium accumulation; *i.e.* histamine, carbachol, or bradykinin (data not shown). Further as the constitutively active chloride conductance path in the intestines of CF animals is not inhibited by DIDS, we suggest that this pathway in the small intestine is not mediated by the recently cloned members of the family of calcium-activated chloride channels (10, 11). Our future studies will focus on understanding the mechanisms that underlie enhanced chloride secretion in the small intestine of CFTR-knockout mice, as this is the site which can become obstructed in these animals (4, 6).

Finally, we speculate that amelioration of disease severity in humans may also correlate with the activity of an alternative, apical chloride conductance path. It is well known that the severity of intestinal disease in CF patients is highly variable and tends to be mirrored by variation in the measurements of intestinal ion transport capacity (12). We suggest that identification of the molecular basis for enhanced apical chloride conductance in intestinal epithelia from mice with mild disease may guide our efforts to develop therapies for intestinal disease in CF patients.

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Figure 5. Inbred CF mice with a high basal PD exhibit increased chloride secretion when compared with the CF mice in this group exhibiting a low basal PD. *A*, sample tracings show the basal PD and its sensitivity to inhibition with NPPB (500 μM) in the jejunum of a liquid diet-fed Wt mouse and a CF mouse from each of the two groups (CIII and CII) identified in the inbred strain. *B*, bar graphs show the mean basal PD before (–) and after (+) application of NPPB ($\pm$ SE) in Wt ($n = 5$) mice and CIII ($n = 7$) and CII ($n = 8$) CF mice. *C*, bar graphs show the mean resistance ($\pm$ SE) in these animals; they are not significantly different. * indicates significant differences between the basal PD values of CII vs Wt and CII vs CIII; [dagger] indicates significant differences between the basal PD before and after application of NPPB ($p < 0.05$, one-way ANOVA).