DNA fragmentation is a feature of cystic fibrosis epithelial cells: a disease with inappropriate apoptosis?

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Abstract Cystic fibrosis (CF) is a single-gene disease caused by mutations in the *CFTR* gene, which result in disrupted chloride secretions with inspissated mucous secretions by exocrine glands. Nick-end labelling was used to assess DNA fragmentation in 14 CF and 24 control duodenal samples, and in two CF and two control lung tissues. In CF small intestine median 46% (range: 30–82) villus enterocytes show DNA fragmentation (vs. 3% (range: 1–7) in controls $P < 0.001$) and median 37.5% (range: 23–79) crypt enterocytes show Ki67 antigen ($P < 0.001$). In CF airways 57% (range: 54–70) of epithelial cells show DNA fragmentation. Inappropriate high DNA fragmentation is a feature of various CF epithelia. This could have great impact in understanding the mechanisms leading to disease.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; DNA fragmentation; Apoptosis; Programmed cell death

1. Introduction

Cystic fibrosis (CF) is a single-gene disease and is the most common, lethal, inherited disease in Caucasians, with an incidence of 1 in 2000–3000 births.

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which result in its altered expression and function [1]. Defective electrolyte transport, particularly defective chloride (Cl^-) transport, is the hallmark abnormality in CF epithelia. This disrupted epithelial Cl^- secretion, which is critical for the production of properly hydrated secretions, has as a consequence that many exocrine organs become clogged with thick, inspissated secretions. In the lung the unusually viscous nature of the secretions is associated with chronic infections, airway obstruction, and eventually respiratory failure. In the gastrointestinal tract, obstruction of pancreatic ducts by hyperconcentrated secretions results in destruction of the pancreas and insufficient delivery of pancreatic enzymes.

Although it has been demonstrated that CFTR is a Cl^- channel [2], it has been postulated that CFTR may influence other cellular functions. Numerous phenotypic abnormalities have been observed in CF epithelia, including increased electrogenic Na^+ absorption [3], increased sulfation of glycoconjugates [4], abnormal regulation of outwardly rectifying Cl^- channels [5], overexpression of annexin V [6], and impaired cell volume regulation in intestinal crypt epithelia [7]. It is not currently understood how these multiple phenotypic abnormalities could be controlled by a defect in a Cl^- channel, and, thus, additional functions of CFTR have been proposed. Several studies have suggested that CFTR may affect the endocytosis and exocytosis of membrane vesicles and that CFTR may play a role in controlling membrane recycling [8]. Recently it has been reported that CFTR may participate in the apoptotic process by influencing the intracellular pH [9].

In this paper we demonstrate that inappropriate DNA fragmentation is a feature of CF epithelia never described before, thus indicating that mechanisms other than those involved in determining inspissated secretions of exocrine glands may be involved in the pathogenesis of CF.

2. Materials and methods

2.1. Patients

Fourteen patients (8 male, 6 female; mean age: 11.3; range: 3–23 years) (Table 1) affected by CF underwent duodenal endoscopy for diagnostic purposes. Two patients (cases #1 and #6) carried the ΔF508/ΔF508 mutation, 10 carried ΔF508/other, and two (cases #12 and #14) carried other/other (Table 1).

All but two patients received pancreatic enzyme supplementation (1000 U lipase/g fat) at the time of biopsy; 11 of them showed mild to moderate pulmonary disease with *Pseudomonas* infection. Nine of 14 patients underwent gastric and/or duodenal biopsies because of recurrent vomiting, and 5 of them showed increased serum levels of antigliadin IgA antibodies. Only one patient (case #14, Table 1) showed severe chronic diarrhoea. In two patients (cases #10 and #11, Table 1) intestinal biopsy was performed before diagnosis of CF because of failure to grow. One patient (case #5, Table 1) received a second biopsy 15 days after withdrawal of pancreatic enzyme, and another (case #14, Table 1) received a second biopsy after 3 months of elemental diet because of severe chronic diarrhoea.

Two patients (1 male, 24 years old; 1 female, 17 years old) underwent pulmonary lobectomy because of lobar atelectasia. Both carried ΔF508/other mutation and had severe respiratory disease with recurrent pulmonary infections.

Twenty subjects (8 male, 12 female; mean age: 16.4 years; range: 3–42 years) suffering from oesophagitis ($n = 9$), gastritis ($n = 3$) and chronic non-specific diarrhoea ($n = 8$), were used as non-CF controls.

Four non-CF patients with chronic pancreatitis and exocrine pancreatic insufficiency (2 male, 2 female; mean age: 27 years; range: 16–35 years) were also investigated.

Two subjects (2 male; aged 63 and 57 years) underwent pulmonary lobectomy because of bronchial cancer.

Informed consent was obtained from all patients.

2.2. Intestinal tissues

Intestinal specimens were obtained at the duodenal–jejunal flexure by peroral biopsy from all patients. Immediately after explants, biopsy

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PCD, programmed cell death; TUNEL, terminal deoxynucleotidyl transferase-mediated D-UTP-digoxygenin nick-end labelling

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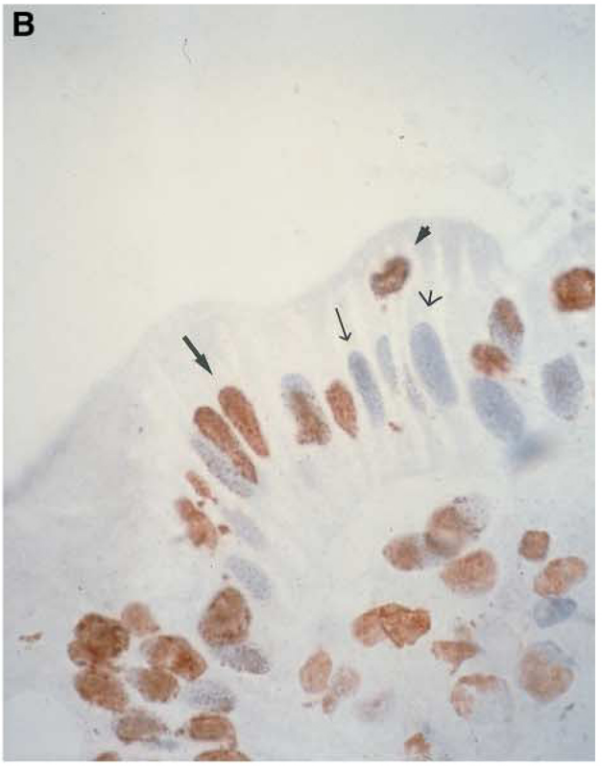

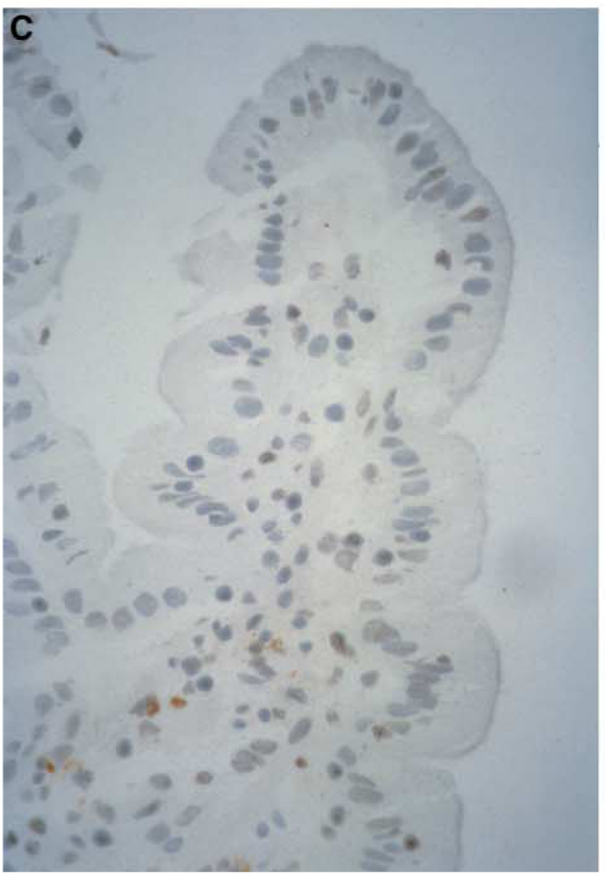

Fig. 1. DNA fragmentation in CF and control small intestine. DNA fragmentation (arrows) is detected in most of villous enterocytes of CF tissues (A): the staining is evident in the nucleus of morphologically normal cells: some of them show a thin granular pattern involving only a part of the nucleus (thin arrow), others show uniform and tense nuclear staining (arrow); some cells show features of shedding (arrowhead); stained bodies are present within the cytoplasm of some lamina propria mononuclear cells. Some villous enterocytes are devoid of any staining at the nuclear level (thin arrowhead) (B). No staining was observed in non-CF control tissues (C). 3 μ cryostat tissue sections: peroxidase staining technique (A–C). Original magnification: ×110 (A,C); ×240 (B).
←

specimens were embedded in OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C until cryosectioning. In two cases intestinal specimens were also fixed in formalin and embedded in paraffin according to routine histological procedures. Duodenal biopsies showed normal morphology in all cases at routine histological examination. Gastric biopsies were obtained from three CF patients and three controls.

2.3. *Bronchial tissues*

Tissues from CF patients were formalin-fixed and paraffin-embedded according to routine histological procedure. Immunohistochemical analysis was performed in the peripheral part of the tissue, far from the atelectasic area; inflammatory findings were evident in peribronchial areas.

Tissues from non-CF patients were collected far from the bronchial lesion; inflammatory findings were scattered in peribronchiolar areas, as in CF tissues. These specimens were embedded in paraffin according to routine diagnostic procedure.

2.4. *In vitro tissue culture*

Duodenal biopsies from three CF patients (cases #6, #8 and #11, Table 1) and 10 controls were also cultured in vitro for 24 h. Mucosal samples from each patient were placed on a stainless steel mesh positioned over the central well of an organ culture dish, with the villus surface of the biopsy specimens uppermost. The cultures were made as

previously reported in the presence of the culture medium [10]. After incubation the specimens were harvested, snap-frozen in liquid nitrogen, and prepared for cryosectioning as described earlier.

2.5. In situ detection of DNA fragmentation in epithelial cells

Fresh intestinal specimens from all CF patients and controls were immersed in OCT compound, rapidly frozen in liquid nitrogen, and 5 μm sections were cut in a cryostat. Sections were air-dried and fixed in acetone. To detect cells with DNA fragmentation, we used terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling (TUNEL) method as modified by Surh and Sprent [11]. DNA fragmentation was visualised by peroxidase or alkaline/phosphatase staining technique; both procedures gave the same results. In two intestinal samples cryostat and paraffin-embedded tissues, according to Moss et al. [12], were simultaneously tested and the results compared afterwards. After validation of the TUNEL method on paraffin-embedded intestinal samples, paraffin-embedded lung sections were similarly analysed.

2.5.1. Control experiments. For control of specificity we have used: (i) unfixed tissues compared with acetone-fixed tissues, to exclude that DNA fragmentation observed in our study was due to manipulation after explants; (ii) incubation with TdT enzyme alone or with labelled nucleotides alone under the same experimental conditions; (iii) incubation with TdT enzyme added with unlabelled nucleotides; (iv) incubation of normal tissues with DNAse I (Sigma Chemical Co., 1 mg/ml for 15 min) under the same experimental conditions; (v) detection of the reaction product by two different technical approaches (peroxidase staining or alkaline phosphatase staining technique); (vi) processing of the biopsy samples in parallel with negative (control patients) and positive (atrophic coeliac biopsies, as well as DNAse treatment of normal biopsies) controls. All the experiments were performed at least 3 times each with the same results.

2.5.2. Immunohistochemistry. Cryostat sections (3 μm) of each sample were incubated with the monoclonal antibody (mAb) anti-Ki67 (Dako M722, working dilution, 1:25, Copenhagen, Denmark) as previously reported [10].

2.5.3. Morphometry and statistical analysis. At least five slides for each sample were evaluated. The number of dividing (Ki67+) cells in crypts was calculated for 100 crypt enterocytes. The number of cells with DNA fragmentation was calculated within the epithelial compartment on the villus as percentage of 100 enterocytes by counting at least 500 enterocytes in each sample. In lung tissues the number of epithelial cells with DNA fragmentation was expressed as percentage of total epithelial cells. The counts were performed at the microscope with a calibrated ocular graticule aligned parallel to the muscolaris mucosae and independently analysed in a blind manner by two observers; the results were compared afterwards [10].

Student's two-tailed t test for independent samples was used to compare CF with control samples. Non-parametric tests (Wilcoxon two-tailed) also were applied and the results are concordant with those obtained using parametric tests.

Table 1
Epithelial features of CF small intestine

Patients	Age	Sex	Number of positive cells (per 100 enterocytes)	
			Villous (DNA fragmentation)	Crypts (Ki67)
1	23	M	48	40
2	8	F	30	42
3	7	M	41	45
4	15	M	51	37
5	19	M	57	79
5[a]			54	71
6	11	F	44	46
7	18	M	71	24
8	14	F	41	38
9	7	F	35	51
10[b]	4	M	82	23
11[b]	3	F	52	34
12	15	M	4	4
13	11	M	23	16
14	4	F	49	31
14[c]			51	35

[a]After 2 weeks of pancreatic enzyme withdrawal.
[b]Without pancreatic enzyme supplementation at the time of biopsy.
[c]After 3 months of elemental diet.

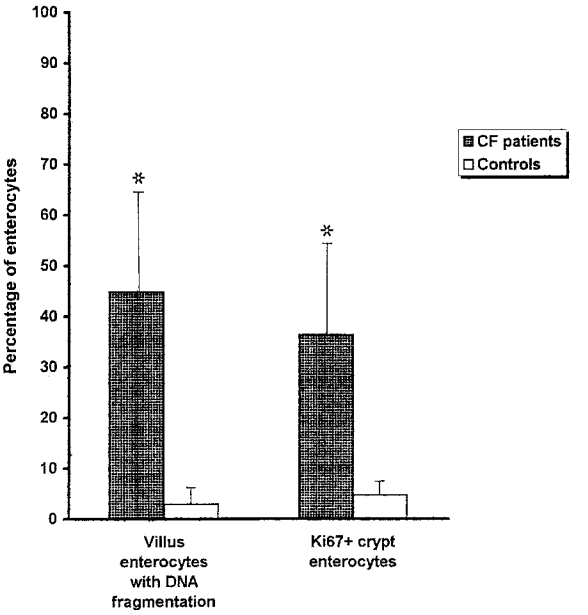

Fig. 2. Expression of DNA fragmentation in villus enterocytes and of Ki67 antigen in crypt enterocytes in CF and control subjects. Black bars, CF tissues ($n=14$); white bars, control tissues ($n=24$). *$P<0.001$ vs. controls.

3. Results

3.1. DNA fragmentation in villus enterocytes

All the mucosal samples showed normal morphology; in all cases the villus height as well as the crypt depth were normal [13] and the villus height/crypt depth ratio was higher than 3.

In mucosal samples from CF patients, the number of villus enterocytes showing DNA fragmentation was higher than in controls in all but one case (case #12, Table 1) (median: 46/100 villus enterocytes; range: 4–82; mean: 44.8, SD 19.2, vs. median: 3; range: 1–7; median: 2.95, SD 1.5 in controls; $P<0.0001$) (Table 1 and Fig. 1A,B,C, Fig. 2). The cells that underwent DNA fragmentation were distributed along the villus at the top as well as in the medial region. Most of them appeared morphologically normal, with normal expression of brush border enzymes such as alkaline phosphatase and sucrase–isomaltase (not shown). Distinct patterns of staining, from a thin granular distribution involving only a part of the nucleus to a uniform and intense nuclear staining were observed in different cells (Fig. 1B); other enterocytes showed, on the contrary, features of cellular shedding. Stained bodies were detected in the cytoplasm of lamina propria macrophages located near to the surface epithelium (Fig. 1B).

These epithelial changes were also observed in those two patients (cases #10 and #11, Table 1) who underwent intestinal biopsy in the absence of pancreatic enzyme supplementation, in patient #5 (Table 1) after 2 weeks of pancreatic enzyme withdrawal, and in patient #14 (Table 1) after 3 months of elemental diet.

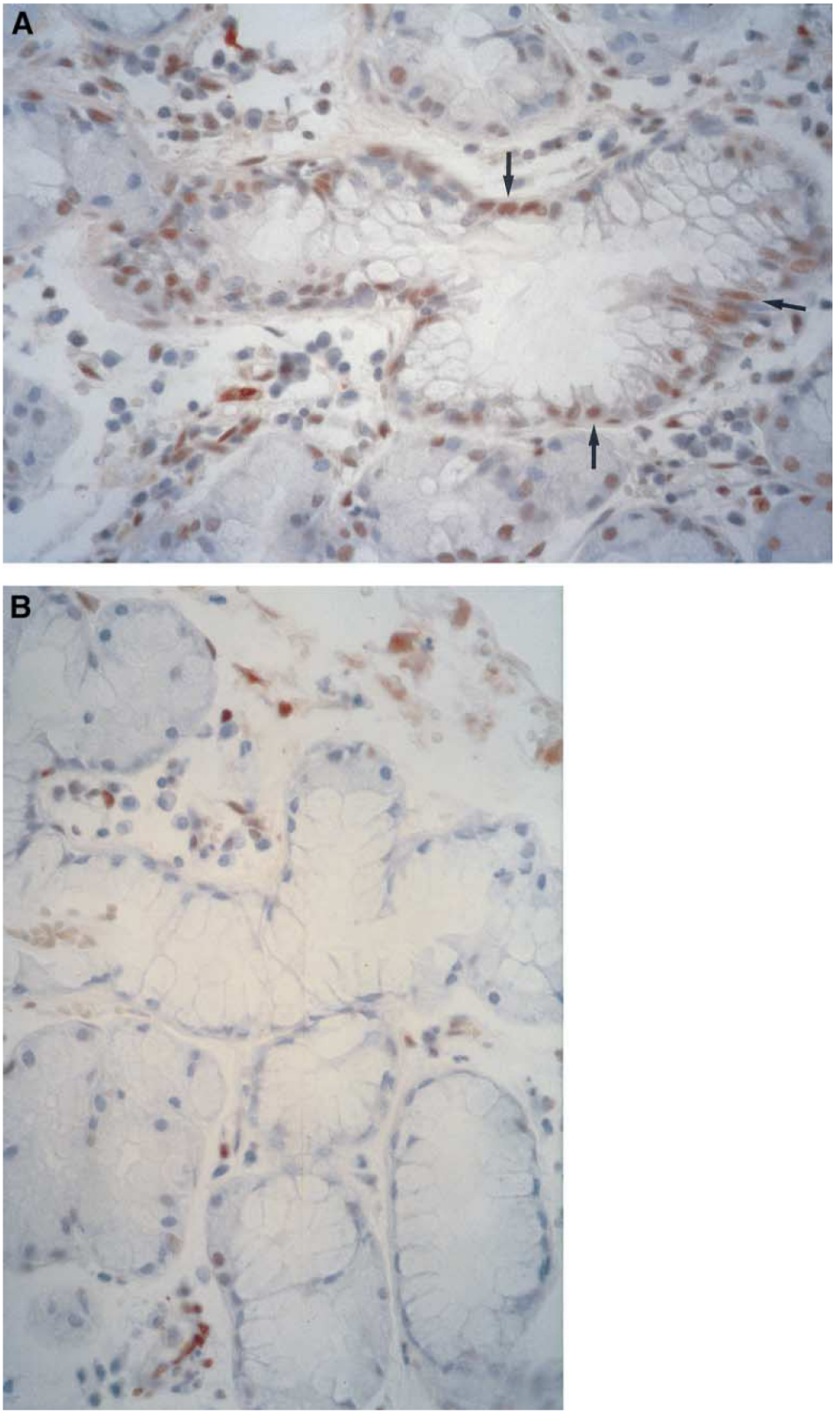

Fig. 3. DNA fragmentation in bronchial tissues of CF patients and non-CF controls. Most of epithelial cells in submucosal glands show DNA fragmentation in CF tissues (arrows) (A), whereas only scattered stained cells are found in non-CF controls (B). 3 μm paraffin embedded tissue sections: peroxidase staining technique (A–B) (original magnification: ×150 (A,B)).

No differences in the pattern of DNA fragmentation were found between subjects with non-CF chronic pancreatitis and the other controls.

3.1.1. Control experiments. (i) No differences were observed between acetone-fixed and unfixed sections. (ii) No staining was detected after incubation of all CF tissues in the presence of TdT enzyme alone or with labelled nucleotide alone. (iii) No staining was detected after incubation with TdT enzyme added with unlabelled nucleotides. (iv) Strong staining of all the mucosal cells (both in lamina propria and epithelial compartment) was detected after incubation of control biopsies with DNAse. (v) No differences of staining were observed after detection of the reaction product with peroxidase or alkaline phosphatase staining technique. (vi) Differences between healthy controls and patient's tissues were always observed even when the biopsies were processed at the same time and samples were studied simultaneously in the same experiment. Moreover, concordant results were always obtained when tissue samples were repetitively studied. Finally identical results were obtained when we compared cryostat and paraffin-embedded tissues from the same individual.

3.1.2. Ki67 expression in crypt enterocytes. In all but one case (#12, Table 1) a very high number of crypt enterocytes showed Ki67 nuclear expression (median: 37.5/100 crypt enterocytes; range: 4–79; mean: 36.4, SD 17.7, vs. median: 5; range: 1–8; mean: 4.7, SD 1.9 in controls; $P < 0.0001$) (Table 1 and Fig. 2). Ki67+ enterocytes were restricted to the crypt region.

3.1.3. In vitro tissue culture of intestinal explants. In the three patients in whom tissue culture was performed for 24 h in the presence of the medium alone, the number of cells with DNA fragmentation was unchanged with respect to that found before culture (case #6, before culture 44/100 villus enterocytes and after culture 42/100; case #8, 41/100 and 44/100, respectively; case #11, 52/100 and 58/100, respectively); similarly a high number of dividing cells was observed in crypts (case #6, before culture 46/100 crypt enterocytes and after culture 49/100; case #8, 38/100 and 45/100, respectively; case #11, 34/100 and 37/100, respectively). Similarly, in 10 control samples, a period of 24 h of in vitro culture did not alter the pattern of epithelial DNA fragmentation or Ki67 expression observed prior in vitro manipulation (DNA fragmentation in villus enterocytes; median: 2.6; range: 1–6; mean: 2.4, SD 1.1; $P = \text{NS}$ vs. samples before culture; Ki67 expression in crypts; median: 5.1; range: 2–7; mean: 5.6, SD 1.8; $P = \text{NS}$ vs. samples before culture).

3.1.4. DNA fragmentation in gastric epithelium. In three CF patients (cases #4, #6 and #9, Table 1) in whom gastric biopsies were also performed, high epithelial DNA fragmentation was also observed (case #4, 21/100 epithelial cells; case #6, 18/100 epithelial cells; case #9, 22/100 epithelial cells, vs. controls 2/100, 4/100 and 7/100, respectively).

3.1.5. DNA fragmentation in bronchial tissue. In the lower respiratory tract, 57% (range: 54–70) of epithelial cells showed DNA fragmentation; a high number of such cells was found in submucosal glands, both in collecting ducts where the staining was prominent in the basal epithelium (Fig. 3A) and in serous and mucous tubuli. Conversely, in non-CF bronchial tissue we observed only a few cells with DNA fragmentation (5%; range: 3–7) (Fig. 3B). A similar phenomenon was observed in airway surface epithelium with more than 35% (range: 30–40) of epithelial cells showing DNA fragmentation in CF patients (data not shown) whereas in controls less than 5% (range: 2–8) of epithelial cells were stained.

4. Discussion

We have shown that high DNA fragmentation, and likely apoptosis, is a feature of various epithelia in CF.

CF is a disease caused by the altered function of *CFTR* mutated gene. The CFTR protein has a central role in controlling the electrolyte transport across the epithelia. However, it has been demonstrated that CFTR may also control apoptosis of epithelial cells [9] and it has been hypothesised that DNA fragmentation and therefore apoptosis may be altered in CF [9]. Apoptosis, or programmed cell death, is a mechanism regulating cell function and its pivotal role in the development and maintenance of higher organisms, including humans, is commonly accepted [14,15]. It occurs as a result of the activation of a highly defined and well-controlled pathway [16], and is characterised by nuclear changes such as chromatin condensation, fragmentation and margination, as well as internucleosomal DNA cleavage. DNA fragmentation is commonly considered as the key feature of apoptosis in many cell types and different techniques, including TUNEL [17], have been used to detect apoptosis. TUNEL has been widely accepted as a technique able to detect apoptosis [11] although it only detects DNA fragmentation, a key event of the apoptosis/programmed cell death process. Several reports indicate the fundamental importance of apoptosis in the maintenance of normal function in the gut, and that abnormalities of apoptosis potentially contribute to gastrointestinal diseases [14,18–20]. By using the TUNEL technique, it has been reported that in the small intestine of human and rodents apoptosis at the luminal surface controls the fate of most enterocytes. However, apoptosis is a very rapid process and in normal steady-state conditions it is likely that the majority of enterocytes that underwent cellular death would be rapidly cleared and no longer recognisable. Recently, the TUNEL technique has been used to detect apoptosis in intestinal epithelial cells of coeliac disease (CD) patients [12]. By this approach Moss et al. [12] demonstrated that in the atrophic CD small intestine higher apoptosis occurs in surface epithelium as well as in crypt epithelial cells, compared to that observed in morphologically normal treated CD intestine. In this paper we have demonstrated, by using the same technique (TUNEL), that inappropriately high DNA fragmentation occurs in morphologically normal CF epithelia. This pattern is observed in all but one CF case, irrespective of the nutritional status and the genotype of the patients. In the CF small intestine a high number of villus enterocytes show DNA fragmentation; some of them present normal morphology with normal expression of brush border enzymes, some others show features of cellular shedding. Moreover, stained bodies are also present in lamina propria macrophages. These stained bodies are absent in control tissues, and they likely represent a late event of the cell death pathway.

DNA fragmentation observed in our studies was not due to the in vitro manipulation, as the samples were processed immediately after explants. Increased DNA fragmentation does not seem to be caused by an ongoing inflammatory process in duodenal mucosa, such as we have observed in many CF individuals (unpublished data). Indeed we have found increased DNA fragmentation in villus enterocytes even in those

cases in which no signs of inflammation in the small intestine were observed. Finally the phenomena detected in CF epithelial cells are not related to endoluminal factors since (i) they are still present in a patient after 3 months of elemental diet and (ii) they are observed even in patients who were not treated with pancreatic enzyme supplementation. Moreover, high DNA fragmentation was also observed in villus enterocytes after in vitro organ culture of duodenal explants, indicating that these features are likely not related to environmental factors, but intrinsic to the intestinal mucosa itself. DNA fragmentation is confined to the villus area, since no staining was observed in crypt epithelial cells. Most of the latter cells show, on the contrary, high expression of the Ki67 antigen, thus suggesting that many crypt enterocytes are dividing.

Increased DNA fragmentation is not exclusive to small intestinal epithelial cells, but also occurs in other gastrointestinal epithelia, as in the stomach. Moreover, it is also evident in the submucosal glands of the respiratory tract. Inappropriate DNA fragmentation is therefore a key feature of CF since it is observed in several epithelia in vivo.

Excessive DNA fragmentation may be related, in an unknown fashion, to the lack of wild-type CFTR protein in epithelial cells. Two considerations point in this direction. First, in airways DNA fragmentation is chiefly observed at the sites of maximal expression of CFTR (submucosal glands) [21]. Second, this phenomenon is less evident in the stomach, where the expression of CFTR is less marked [22]. That the CFTR may play a role in the process of apoptosis has been recently described [9]. This report demonstrated that the CFTR may be involved in the initiation of apoptosis by enhancing the acidification in the epithelial cell milieu [9]. Acidification has been proved to be involved in apoptosis mediated by Fas ligation, cycloheximide, or ultraviolet radiation [23]. A resistance to the induction of apoptosis mediated by these factors was in fact observed in CFTR-mutated transfected cells. Our results in vivo indicate that DNA fragmentation is increased in CF epithelia. One possible interpretation of this apparent discrepancy is that in CF patients DNA is inappropriately nicked in fragments of variable size, but final degradation by endonucleases does not occur, or other apoptosis related processes fail to occur. This would indicate a situation in which the apoptotic process is initiated, but then aborted. That the apoptotic process is interrupted is indicated by the normal morphology of villus enterocytes, despite DNA fragmentation. Moreover, a smaller number of cells with features of shedding was seen than would have been expected on the basis of the high number of cells with DNA fragmentation. The role of CFTR in this process may therefore consist of an inhibitory effect by determining an inability to reach the end-point, cellular death, or by causing delay in the apoptotic pathways. It is also unknown what is the primary event responsible for the high DNA fragmentation. The involvement of a yet unknown other gene responsible for this phenomenon might be a suggestive hypothesis, and it might also explain the lack of correlation genotype/phenotype observed in CF patients (with the exception of pancreatic insufficiency) and in transgenic animals [24]. Experimental on transgenic mice may contribute to clarify these phenomena.

On the other hand it could be supposed that in CF the villus cell death is rapidly compensated by the increased proliferation in crypts. The normal mucosal architecture results from the balance of proliferation, migration, differentiation and death along the crypt-villus axis. If high cell death on the villus is compensated by the replication and migration of crypt enterocytes, a balance is achieved without villus damage and with normal mucosal architecture. In normal conditions there is a 'low-level' balance; in CF patients it may be possible that this balance is achieved at 'higher level', with high cell death in the villus enterocytes and high proliferation in crypts leading to normal villus morphology.

These two different interpretations, excessive DNA fragmentation with a defect (or delay) in cell death, or increased cell death with high cellular turnover, might also be related to each other. In fact the same keys of the signalling pathways of the apoptotic machinery may operate in a different way in different cells (villus or crypt enterocytes) in relation to different phases of the cell cycle [16].

We indeed do not know whether in CF we are dealing with a condition of excessive apoptosis or, on the contrary, we are observing a condition of aborted apoptosis.

Whatever the mechanism, these results demonstrate that a perturbation in the cell death pathway is a feature of CF epithelial cells. Inappropriate apoptosis contributes to the pathology of several human diseases [25–29]. Insufficient apoptosis is associated with many cancers that are now believed to be a consequence of failed apoptotic cell death [26]; in addition flawed apoptosis is a feature of autoimmune disorders [27]. On the contrary disorders of excessive apoptosis have been described, as in Alzheimer's disease, in which hippocampal neurons appear to prematurely commit suicide with consequent neurological damage [28], or in spinal muscolar atrophy, in which the loss of motor neurons in the spinal cord has been linked to a defect in a gene that encodes neuronal apoptosis inhibitory protein [29]. If also CF epithelial cells are characterised by inappropriate regulation of the apoptotic machinery it is likely that the many pathological features of CF might be related to the impaired regulation of the cell death pathways.

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