Paradoxical decrease of mitochondrial DNA deletions in epithelial cells of active ulcerative colitis patients

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Fukushima, Kouhei, and Claudio Fiocchi. Paradoxical decrease of mitochondrial DNA deletions in epithelial cells of active ulcerative colitis patients. Am J Physiol Gastrointest Liver Physiol 286: G804–G813, 2004; 10.1152/ajpgi.00398.2003.—Ulcerative colitis (UC) is a condition characterized by chronic inflammation targeted at the epithelial layer. In addition to being involved in immune phenomena, UC epithelial cells exhibit decreased oxidation of butyrate, downregulation of oxidative pathway regulatory genes, and overexpression of mitochondrial (mt) genes. We investigated whether these events, which translate an altered energy metabolism, are associated with an abnormal pattern of mtDNA deletions. Highly purified colonocytes were isolated from surgically resected control, involved and uninvolved inflammatory bowel disease mucosa. The frequency, type, and number of mtDNA deletions were assessed by PCR amplification, Southern blot analysis, and cloning and sequencing of amplified DNA fragments. The 4977 mtDNA deletion was less frequent in UC than control and Crohn’s disease (CD) epithelial, regardless of patient age. Several other deletions were detected, but all were less common in UC than control and CD cells. The frequency, variety, and number of mtDNA deletions were invariably lower in colonocytes isolated from inflamed mucosa than in autologous cells from noninflamed mucosa. In conclusion, in the absence of inflammation, UC colonocytes exhibit an mtDNA deletion pattern similar to that of control cells, indicating a normal response to physiological levels of oxidative stress. In active inflammation, when oxidative stress increases, the frequency, variety, and number of mtDNA deletions decrease. Because comparable abnormalities are absent in active CD, the mtDNA deletion pattern of active UC suggests that colonocytes respond uniquely to inflammation-associated stress in this condition. Energy metabolism

Although the etiology of ulcerative colitis (UC) is still uncertain, colonic epithelial cells (EC) have long been considered central to its pathophysiology. In addition to the restriction of the inflammatory process and associated dysplastic changes to the epithelial lining (43), there is abundant experimental evidence pointing to colonocytes as likely targets and mediators of key pathogenic events. Detection of circulating colon antibodies and cytotoxic mononuclear cells in UC patients initially suggested that colonocytes could be targets of autoimmune reactivity (6, 49). Subsequent studies demonstrated that in UC, EC express high levels of human leukocyte antigen D-related antigens (48), secrete abnormal mucin glycoconjugates (41), produce increased amounts of cytokines and chemokines (4, 42, 57), express costimulatory molecules (39), display autoantigens recognized exclusively by UC tissue-eluted antibodies (53), and fail to mediate induction of suppressor T cells (36). Additional reports demonstrated cyclooxygenase 2 and NF-κB activation (46, 51), as well as an increased rate of EC apoptosis in UC (23, 52). These results, coupled to recent evidence of broad changes in metabolic and transport pathways detected by gene expression profiling (32), suggest not only that UC EC are involved in deranged immune and inflammatory responses but are also in a state of abnormal metabolic activity.

The notion of altered colonocyte metabolism in UC is not entirely new. In humans, the large bowel is a significant source of energy (38) that the epithelium consumes in considerable amounts to sustain high proliferative rates and multiple synthetic and secretory processes (19). The major energy source for colonocytes is the short-chain fatty acid butyrate derived from bacterial fermentation of carbohydrates (19). There is evidence that butyrate metabolism is defective in UC. Roediger (44) reported that oxidation of butyrate to CO₂ and ketones by colonic EC isolated from UC tissue was significantly lower compared with that of EC from normal colon, an impairment also found in EC from quiescent UC (8). This oxidative defect has led to the hypothesis that UC is an “energy-deficiency disease” (44). This intriguing possibility finds support in the decreased ATP levels in the mucosa of UC patients (29), the induction of experimental colitis by inhibition of fatty acid oxidation (45), the clinical and histological features of diversion colitis that mimic UC (21), and the beneficial effects of short-chain fatty acid enemas in diversion colitis and UC (5, 21, 47).

In most organs and tissues, the primary source of cellular energy in the form of ATP is oxidative phosphorylation. This process occurs in mitochondria in which mitochondrial (mt)DNA encodes for essential subunits of the respiratory chain (20, 55). Even under physiological conditions, there are large variations in the number of deletions in mtDNA due to its specific structure, function, and location. mtDNA mutates 10 times as frequently as nuclear DNA, has no protective histones or repair system, and is constantly exposed to free radicals generated during the cell’s normal oxidative phosphorylation process (25, 26). When mtDNA mutations occur in muscle or nerve cells, this results in reduced energy production translated into obvious clinical manifestations (20, 55). However, similar defects may be far more difficult to detect when they occur in the intestinal epithelium, particularly under inflammatory conditions. Thus, except for the few above-mentioned studies (8,
44), human intestinal EC have received little attention as a possible source of defective oxidative metabolism due to mtDNA mutations.

In studies of inflammatory bowel disease (IBD)-associated gene expression by differential screening of colonocyte cDNA libraries, we recently detected a quantitative overexpression of clones encoding for mitochondrial genes in UC (16). In studies of gene expression profiling of UC and Crohn’s disease (CD) tissues using high-density oligonucleotide microarrays, we also detected a marked downregulation of various oxidative pathway regulatory genes in UC, including mitochondrial HMG Co A synthase 2 (X83618), cytochrome oxidase 17 (L77701), and cytochrome oxidase 5B (M19961) (18–9, 53.6–, and 3.1-fold decrease, respectively) (32). These data, combined with earlier observations of decreased oxidative phosphorylation in UC colonocytes (8, 44), support the hypothesis that mtDNA abnormalities might be present in UC colonocytes and explain the previously reported defects in energy production involved in disease pathogenesis. Therefore, we investigated the presence and types of mtDNA mutations in EC isolated from control, UC, and CD colonic specimens and possible differences between normal and inflamed colonic epithelium. We initially investigated the 4977-bp deletion because of its high frequency and known association with defective energy production in typical mitochondrial myopathies. The results showed that control and UC and CD EC contained similar amounts of total mtDNA and that all displayed the 4977-bp deletion, but this was less frequent in UC mucosa than CD mucosa. When autologous EC from inflamed and noninflamed UC mucosa were compared, the 4977-bp deletion was far less common in EC from involved mucosa than in EC from uninvolved mucosa. Several other mtDNA deletions were also detected, and their frequency was also considerably diminished in UC compared with control and CD EC.

MATERIALS AND METHODS

Patient population. Intestinal EC were isolated from freshly resected colonic surgical specimens obtained at University Hospitals of Cleveland and the Cleveland Clinic Foundation. Histologically normal samples were derived from 17 patients (12 men and 5 women; 36–82 yr old; mean age 60.7 yr) undergoing large bowel resection because of adenocarcinoma (n = 13), adenoma (n = 2), squamous cell carcinoma (n = 1), and sigmoid volvulus (n = 1). Fourteen UC patients (8 men and 6 women; 20–66 yr old; mean age 43.3 yr) with active disease who had submitted to colectomy were evaluated. Paired samples from both involved and uninvolved mucosa were obtained from 6 patients. Thirteen CD patients (3 men and 10 women; 17–76 yr old; mean age 42.9 yr) with active disease were also examined. All diagnoses were confirmed by clinical, radiological, endoscopic, and histological findings. Additionally, strate muscle and liver tissues were obtained from surgical specimens and used as positive control for the 4977-bp deletion. The project was approved by the Internal Review Boards of University Hospitals of Cleveland and the Cleveland Clinic Foundation.

Purification of EC and lamina propria mononuclear cells. Intestinal EC were prepared as previously described (56). Brieﬂy, mucosal strips were treated at room temperature for 30 min in 1.5 mM DTT (Sigma, St. Louis, MO) and then gently stirred in a 1-mM EDTA (Worthington Biochemical, Freehold, NJ) solution for three 60-min periods. The supernatants were collected and centrifuged, and the resulting pellets were suspended in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD). Cell suspensions were digested with 3 mg/ml dispase (Boeringer Mannheim, Indianapolis, IN) and 0.5 mg/ml DNase I (Worthington Biochemical) for 30 min at 37°C. ECs were purified over a 50% Percoll (Pharmacia Biotechnology, Piscataway, NJ) gradient, spun at 1,500 rpm for 20 min. ECs equilibrated at the interface were collected and washed. The purity and viability of ECs were consistently >97%, with <3% contaminating mononuclear cells (56). Preparation of lamina propria mononuclear cells (LPMCs) has been previously described in detail (13). After sequential steps with DTT and EDTA to remove mucus and EC, the dissected intestinal mucosa was digested with collagenase (Worthington Biochemical) and DNase I. The resulting crude cell suspension was purified by using Ficoll–Hypaque gradient and resuspended in RPMI 1640.

Extraction of total and mtDNA. To obtain total DNA, cells were lysed with guanidine isothiocyanate buffer (containing 50% guanidine isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, and 0.7% 2-mercaptoethanol) followed by a 5.7-M cesium chloride gradient. This was centrifuged at 30,000 rpm for 18 h (model L7–55 ultracentrifuge; Beckman, Palo Alto, CA). After removal of the protein phase, viscous DNA was collected and dialyzed by using 1× Tris–EDTA buffer with constant stirring at 4°C. Finally, DNA was phenol-extracted, ethanol-precipitated, and quantified by absorbance at 260 nm.

To obtain mtDNA, purified intestinal epithelial cells (IECs) were suspended in 0.25 M sucrose, 10 mM Tris–HCl, pH 8.0, 3 mM MgCl2, and homogenized. Homogenates were centrifuged at 800 g for 10 min, and the resulting supernatants were centrifuged at 10,000 g for 10 min. Pellets were resuspended in 0.25 M sucrose, 10 mM Tris–HCl, pH 8.0, 50 mM MgCl2, and digested with DNase I (Worthington Biochemical) for 30 min at 37°C. This solution was mixed with a 2.5 volume of 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 100 mM EDTA solution, and centrifuged at 10,000 g for 10 min. The pellets were stored at −70°C until extraction of mtDNA. mtDNA was extracted by using a Wizard miniprep purification kit (Promega, Madison, WI) according to the manufacturer’s protocol based on a selective alkaline denaturation procedure.

Quantification of mtDNA. Quantification of mtDNA in each EC preparation was determined by dot blot analysis as previously described (2). Five hundred nanograms of total DNA were denatured with 3 M NaOH at 65°C for 45 min and neutralized by adding an equal volume of 2 M ammonium acetate. With the use of a slot blotter, duplicate samples of the DNA solution were dotted onto a prehybridized nylon membrane and hybridized at 42°C for 2 h with the same 32P-labeled probe [13234–13852 nucleotide pairs (np)] used for semi-quantification of the 4977-bp mtDNA deletion (see below). The membrane was washed with 2× standard SSC at room temperature for 15 min twice, 1× SSC containing 0.1% SDS at 55°C for 2 h, and 2× SSC at room temperature for 15 min twice. The membrane was then dried, each dotted area was cut out, and contained radioactivity was measured in a scintillation counter.

Detection of the 4977-bp mtDNA deletion. One microgram total DNA from IECs was digested with PstI (6910 and 9020 np) and HindIII (6203, 11680, and 12567 np), which cut normal mtDNA three times within the 4977-bp deleted site (from 8483 to 13459 np). One-tenth volume of digested mixture was used as a template for PCR amplification across either the deletion breakpoint using primer 1 (5′-CCCCCTCTAGGCCCCACTGTAAAGC-3′, 8282–8305 np) and primer 2 (5′-AGTTGAGTCTAGGCGTCT-3′, 13851–13832 np) (10). When the 4977-bp deletion is present, a 593-bp product is generated by this primer set. In the absence of the 4977-bp deletion, no product is generated except in the case of primer misannealing because normal mtDNA is digested by the restriction enzymes and the distance between the primer binding sites is too long to allow amplification of normal mtDNA. Amplification was performed at a final concentration of 1× PCR buffer (in mM: 10 Tris–HCl, 1.5 MgCl2, 50 KCl, and 0.1 mg/ml gelatin, pH 8.3), 0.15 μM each primer,
0.1 mM NTP, and 0.75 units Taq polymerase (Promega) in a total volume of 25 μl. The amplification profile involved denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min, and extension at 72°C for 1 min for 35 cycles preceded by denaturation at 94°C for 2 min and final extension at 72°C for 5 min. The resulting products were electrophoresed and stained with ethidium bromide.

Quantification of 4977-bp mtDNA deletion. Southern blot analysis was used to detect the 593-bp amplified product resulting from the specific mtDNA deletion. Ten micrograms total DNA from IECs, LPMC, muscle, and liver were digested with the restriction enzymes described above. After phenol-chloroform extraction and ethanol precipitation, digested DNA fragments were quantitated with a spectrophotometer. Serial amounts of digested DNA (50, 5, 0.5, 0.05 ng) were then used as PCR template under the same condition described above except that 32 cycles were employed based on preliminary study showing that amplification remained within log phase. Ten microliters of reaction mixture were electrophoresed and transferred onto nylon membrane (GeneScreen; New Research Products, Boston, MA). For probe preparation, a plasmid containing the insert encoding mtDNA fragment confirmed by sequencing (from 13234 to 13852 bp) was prepared and 32P labeled with a nick translation kit (Boehringer-Mannheim). This probe hybridized to the 593-bp amplified product but not the 720-bp product cogenerated during amplification with primers I and 2 under high-stringency conditions (Fig. 1). Blots were prehybridized for 3 h at 42°C in 50% deionized formamide, 5× SSC solution, 1% SDS, 5× Denhardt’s solution, 25 mM NaPO4·K2PO4, and 50-μg salmon sperm DNA. Hybridization was performed over 16–18 h at 42°C. The blots were then washed twice with 2× SSC at room temperature for 15 min, followed by high-stringency washing with 0.2× SSC and 0.1× SDS at 65°C for two 1-h periods. Finally, membranes were rinsed with 2× SSC, air-dried, and exposed for 2–16 h to Kodak XAR-2 films. For semiquantification, all membranes were hybridized and exposed together at the same time.

Screening for additional mtDNA deletions. To detect additional mtDNA deletions (40), 50 ng of mtDNA or 250 ng of total DNA were amplified by using another mtDNA-specific primer set (primer 3: 5’-TTCTAGTATTTGAGAAGCCTT-3’, 7311–7330 nt; primer 4: 5’-CTAGGGTAGAATCCGATGATGTG-3’, 13928–13905 nt). Primer 4, which anneals just laterally to the primer 2 binding site, was used because of primer 2 misannealing. To distinguish true amplified products due to deletion from those generated by primer misannealing, amplification by PCR was carried out by using the same conditions mentioned with or without 2 μCi [32P]dCTP (‘hot’ and ‘cold’ experiments) in a total volume of 12.5 μl. The amplification profile involved initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, primer annealing at 58°C for 2 min, and extension at 72°C for 3 min for 35 cycles, followed by final prolonged extension at 72°C for 5 min.

In “cold” experiments, resulting products were utilized for Southern blotting and hybridized with the same mitochondrial probe described above, which is predicted to bind to the downstream end of amplified products (Fig. 1). In “hot” experiments, resulting products were electrophoresed on 6 or 8% nondenaturing polyacrylamide gel, which was exposed for 4 h to Kodak XAR-2 films. The size of the bands resulting from “hot” and “cold” experiments were compared and classified as deletions or misannealing products based on the finding of same-size bands in both experiments or neither, respectively. Other combinations of primers (primer 1 and primer 5: 5’-GTACTACAGGTGGTCAGAG-3’, 16159–16142 nt; primer 3 and primer 5) (40) were also used in hot experiments. In preliminary experiments, IECs were isolated from two separate areas in two control specimens. Total DNA was extracted from two areas of the first specimen and mtDNA from two areas of the second specimen, and mtDNA deletion patterns were compared by PCR. No differences were observed in the patterns displayed by the paired autologous IEC samples in each of the two specimens.

Cloning and sequencing of amplified DNA fragments. In preliminary studies investigating the 4977-bp deletion, each amplification yielded one or two bands of 720- and 600-bp size which were cloned into the pBluescript KS vector. Briefly, the bands were cut out and purified with Wizard PCR Preps DNA purification system (Promega) according to the manufacturer’s protocol. After phosphorylation with T4 polynucleotide kinase (GIBCO-BRL, Gaithersburg, MD), these products were ligated to the vector by using DNA Blunting Kit (Takara Biochemical, Berkeley, CA). Each clone was sequenced with mtDNA Sequencing System (Promega).

In studies of additional deletions, amplified DNA fragments were selected by comparison of Southern blotting and electroeluted from polyacrylamide gel. Briefly, pieces of dried gel containing bands thought to represent deletion or misannealing products were apposed to Whatman DE 81 paper on 0.6% agarose gel and eluted at 120 V for 45 min. The paper was washed in low-salt buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl) three times, eluted in high-salt buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM NaCl), phenol-chloroform extracted, and ethanol precipitated. After a final wash with 70% ethanol, the pellet was resuspended with 20 μl diethyl pyrocarbonate water. Two microliters of solution were used as PCR template. The amplified product was electrophoresed on 1% agarose gel, cut out, and purified with Wizard PCR Preps DNA purification system. Purified DNA was directly sequenced with a mtDNA Sequencing System.

RESULTS

Levels of mtDNA in total EC DNA. When radioactivity from the probe hybridized to mtDNA was measured and compared in samples of total DNA from control and UC and CD EC, no statistically significant differences were observed (Fig. 2). Although comparison between EC from inflamed and uninflamed UC mucosa failed to detect significant differences, even when carried out with autologous cells, the amount of mtDNA varied more in UC than control and CD isolates, suggesting that distinct portions of total mtDNA contain deletions in any given sample as described below.

Detection of the 4977-bp mtDNA deletion in EC. Epithelial cell-derived total DNA was amplified by using the above-described primers (Detection of the 4977-bp mtDNA deletion) to detect the 593-bp product indicative of the 4977-bp mtDNA common deletion. Two amplification products were observed by ethidium bromide staining of Southern blots (Fig. 3A): a larger (720 bp) and a smaller molecular weight (593 bp) product, the latter being of the expected size resulting from the digestion of mtDNA containing the 4977-bp deletion (2). Initial screening showed that the frequency of detection of the two products varied depending on the source of EC. The 720-bp product was detected in all control and IBD samples. In contrast, the 593-bp product was present in all isolates from control mucosa (7 isolates), half (4 of 8) of the UC isolates, and in two of five CD isolates.

The larger molecular weight band could represent an additional deletion product distinct from the expected 593-bp product or could simply result from misannealing of the primers. To exclude the latter possibility, cloning and sequencing was carried out in five 720-bp products (1 control, 3 UC, and 1 CD) and three 593-bp products (1 control, 1 UC, and 1 CD). All 720-bp products represented misannealing of downstream primer to an mtDNA segment positioned at 8985 through 9002 nt without deletion (not shown). All smaller bands represented actual deletion products, displaying the same known deletion breakpoint and a molecular weight of 593. The deletion was 4977 bp long, with the breakpoint on the left side at nucleotide

G806 MITOCHONDRIAL DNA DELETION IN IBD EPITHELIUM
position 8483 within the ATPase 8 gene and on the right side at nucleotide position 13460 within the NDS gene (Fig. 3B).

Because large numbers of fresh human EC cannot be recovered as absolutely pure populations, we investigated the possibility that detection of the 4977-bp deletion was due to contamination with other cell types. Because the only numerically relevant contaminating cells are mononuclear cells, the presence of the 593-bp deletion product was sought in LPMC. Utilizing the same amplification conditions followed by Southern blotting, 593-bp deletion products from one control and one UC isolate strongly hybridized the probe derived from an mtDNA fragment. In contrast, no hybridization was detected when autologous LPMC were used in the same experiments (Fig. 4). Skeletal muscle and liver tissues were used as positive controls, and all displayed the 593-bp product (not shown).

Finally, to investigate whether site-dependent differences existed in the detection of EC mtDNA deletion along the gastrointestinal tract, hybridization was carried out seeking the 593-bp amplification product in normal duodenal, ileal, cecal, and rectal EC. Evidence for the 4977-bp deletion was detected

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**Fig. 1.** Top: relative position of the primers and the probe used for screening of mitochondrial (mt)DNA deletion in epithelial cells (EC). Nucleotide position (np) was confirmed by sequencing. The probe encodes the nested sequence just close to the downstream primer in experiments with primers 1 and 2 and with primers 3 and 4. Bottom: results of amplification. The 593-bp deletion product, detected with primers 1 and 2, was present in all EC from control mucosa (seven isolates), 4 of 8 of the ulcerative colitis (UC) isolates, and 2 of 5 Crohn’s disease (CD) isolates. The 720-bp products represented misannealing of the downstream primer to an mtDNA segment positioned at 8985–9002 np. Deletions detected with primers 3 and 4 and with 3 and 5 are individually listed. No deletions were detected in control, UC, or CD isolates using primers 1 and 5.
in all samples, although quantitative differences of 593-bp product were present relative to each other (Fig. 4).

Quantification of the 4977 mtDNA deletion. The differential detection of the 593-bp product between control and IBD IECs suggested that the presence of inflammation could have an effect on the frequency of the 4977-bp deletion in the various cell isolates. To investigate this, a semiquantitative analysis of the detectability of the 593-bp product was developed on the basis of amplification of four serial 10-fold dilutions of EC DNA followed by Southern blot hybridization. With the use of this protocol, detection of each amplified product correlated with the original amount of EC DNA regardless of the source of the isolates (Fig. 5A).

On the basis of this semiquantitative assay, we investigated whether any differences existed in the level of the 593-bp product among control, CD, and UC IECs. In undiluted samples, the deletion product was detected in all 16 control and all 10 CD isolates, whereas the same product was undetected in 5

Fig. 3. A: products of amplified EC total DNA after 35 cycles. Lane 1: 100-bp DNA molecular weight (MW) ladder; lanes 2 and 3: UC; lanes 4 and 5: CD; lanes 6 and 7: control (C). The upper band shows a 720-bp product common to all samples that represents downstream primer misannealing. The lower band shows the expected 4977-bp deletion product of 593-bp size. This product is absent in both UC samples but present in CD and control samples. Figure is representative of 7 control, 8 UC, and 5 CD isolates. B: autoradiogram of a DNA sequencing gel in the region of the 4977-bp deletion breakpoint. The sequence (dideoxy reactions A, G, T, C) represents the L-strand of mtDNA, reading 5’ to 3’ being performed from bottom to top. The deletion is 4977 bp long, with the breakpoint on the left side at nucleotide position 8483 within the ATPase8 gene and on the right side at nucleotide position 13460 within the ND5 gene.

Fig. 4. Right: detection by Southern blot analysis of the 593-bp deletion product PCR-amplified from total DNA extracted from EC of various portions of the intestinal tract. From left to right, the product is detected in normal duodenal (D), ileal (I), cecal (C), and rectal (R) EC from control mucosa. Left: detection by Southern blot analysis of the 593-bp deletion product PCR-amplified from total DNA extracted from EC and lamina propria mononuclear cells (LPMC). From left to right, the product is detected in control and UC EC but not in the autologous LPMC.
of 11 (45%) UC isolates (Fig. 5B). When the same samples were serially diluted, clear differences in deletion detectability were noticed among the three groups: deletions were frequent in diluted and highly diluted (up to 1:100) samples of control and CD samples but less in diluted (up to 1:10) and none in highly diluted UC samples (Fig. 5C).

Because the frequency of the 4977-bp deletion increases with age (11, 27), we investigated whether the lower detection in UC IECs was related to this variable. This was accomplished by analyzing the data in Fig. 5, B and C according to the patient’s age (decades of life, 20–70 yr old). No correlation with age was observed in any of the control, CD, or UC patient groups. Similarly, the number of mtDNA deletions was not influenced by the use of anti-inflammatory drugs, including 5-aminosalicyclic acid, corticosteroids, or immunosuppressive agents.

Screening for additional mtDNA deletions. The different detection of the 4977-bp deletion in EC from normal and IBD mucosa suggested that other deletions might exist depending on the presence or type of inflammation. This possibility was explored by using PCR and Southern blot analysis. Because the probe used for 4977-bp quantification recognizes a nested sequence close to the downstream primer site, detection of smaller-than-predicted size bands (6.6 kb with primers 3 and 4) hinted at the presence of additional mtDNA deletions. Indeed, other deletion products were found, ranging in size from the predicted 6.6 to 0.3 kb. Bands of 6.6 and 0.7 kb were found in almost all samples (Fig. 6). Sequencing showed that 0.7-kb product was generated by the misannealing of upstream primer (primer 3) to the 0.7-kb upward site of downstream primer binding site (not shown). A 1.7-kb band was commonly detected, but less frequently in UC EC, and corresponded to the 4977-bp deletion as confirmed by sequencing (not shown). The number of types of deletion products varied substantially among samples, but the number was considerably smaller in UC compared with control and CD EC (Fig. 5C). To exclude that these products derived from cells contaminating EC isolates, autologous LPMC were also tested. As was the case for the 4977-bp deletion (Fig. 4), all additional bands were detected only in EC (not shown).

In “hot” experiments, the number of detected bands was greater than that seen with Southern hybridization, probably because products caused by misannealing of the primers were also displayed (not shown). The number of bands was strictly
dependent on the primer set used (Fig. 1). For instance, several bands were generated by the combination of primers 3 and 4, but a few by combining primers 3 and 5, and none by primers 1 and 5. The patterns displayed in autoradiographs of total DNA and mtDNA from the same EC were essentially identical, indicating that the primers annealed to mtDNA regardless of the sample preparation.

Sequencing of DNA deletion products. Having detected several additional products representing potential deletions, it was critical to ensure that they represented actual deletions and not misannealing as well as to investigate potential differences among control, CD, and UC EC. By comparison of patterns displayed with Southern blots and “hot” experiments, 26 bands were tentatively classified as true deletion and 17 as misannealing products, and all were eluted and sequenced. Twenty-three (88%) of the suspected 26 bands were confirmed as true deletions (Fig. 1), and only three, including the previously observed 0.7-kb band, were the result of primer misannealing. The length of the deletions varied from 4977 to 8441 bp, spanning more than the half of the human mitochondrial genome. An example derived from an UC EC isolate is shown in Fig. 7. The distribution of the breakpoints occurred randomly along the 10-kb fragment between light- and heavy-strand origins of replication. No unique pattern of deletions was associated with control or IBD EC. All of the 17 bands tentatively classified as resulting from misannealing were confirmed to be misannealing products by sequencing.

Effect of inflammation on mtDNA deletion. Results demonstrating that mtDNA deletions occurred less frequently in UC than control and CD EC could reflect a primary EC abnormality in UC or, more likely, be the consequence of mucosal inflammation. To distinguish between the two possibilities, the frequency of the 4977 bp and other deletions was determined in autologous EC from involved as well as uninvolved mucosa. In four paired sets of undiluted samples, the expression level of the amplification product in inflamed mucosa-derived EC was consistently lower than that of autologous cells from noninflamed mucosa (Fig. 8A). When a semiquantitative analysis was carried out by progressive dilution of the samples as previously described, the 593-bp band varied from undetectable or detectable at a 1:1 dilution in the involved mucosa to detectable up to 1:1,000 dilution in the uninvolved mucosa (Fig. 8B). Similarly, the number of types of deletions in EC

Fig. 7. Autoradiogram of a DNA sequencing gel in the region of a deletion detected in UC EC. The sequence (dideoxy reactions A, G, T, C) represents the L-strand of mtDNA, reading 5’ to 3’ being performed from bottom to top. The deletion is 6002 bp long, with the breakpoint on the left side at nucleotide position 7449, and on the right side at position 13450.

Fig. 8. A: differential detectability by Southern blot analysis of the 593-bp deletion product amplified from total DNA extracted from EC of involved and noninvolved mucosa in 4 paired UC samples. In each paired set, EC from inflamed mucosa displayed a markedly reduced hybridization signal relative to the signal from the noninflamed mucosa. I, inflamed; NI, noninflamed. B: effect of inflammation on detectability of 593-bp deletion product in total DNA extracted from autologous UC EC from involved and uninvolved mucosa. Each value represents the maximal dilution of individual sample of DNA still detectable by Southern blotting. C: effect of inflammation on the number of types of deletion products in DNA extracted from autologous UC EC from involved and uninvolved mucosa. Each value represents the number of bands detected by Southern blotting in individual sample of DNA.
from involved mucosa was markedly decreased compared with that found in uninvolved mucosa (Fig. 8C).

DISCUSSION

The occurrence of mtDNA deletions is a common phenomenon, and normal tissue homogenates contain a mean concentration of 1% of any particular deletion mutation (40). Nonetheless, the number and type of mtDNA deletions vary greatly in different tissues and organs depending on age and the presence of distinct hereditary or acquired pathological conditions (20, 25, 55). This study is the first to demonstrate that mtDNA deletions are common in EC of the human gastrointestinal tract. Specifically, the results show that multiple types of mtDNA deletions occur in EC from normal and chronically inflamed colon and that both the type and abundance of mtDNA deletions selectively decrease in actively inflamed UC epithelium.

Pathogenic deletions of mtDNA were first described in several uncommon diseases characterized by neuromuscular manifestations, such as the Kearns-Sayer syndrome, chronic progressive external ophthalmoplegia, and the Pearson syndrome (25). A defective energy production due to deletion- and mutation-dependent mitochondrial dysfunction presumably underlies these disorders. Subsequently, mtDNA deletions were also found to be associated with a number of common diseases, including diabetes mellitus (28), hypertrophic cardiomyopathy (35), alcoholism (15), osteoporosis (54), Alzheimer’s and Parkinson’s diseases (26), and exercise intolerance (1). Gastrointestinal manifestations are uncommon and usually secondary to systemic mitochondrial defects, as in mitochondrial neurogastrointestinal encephalomyopathy (22) and sporadic chronic diarrhea with villous atrophy (9). No study has ever been performed seeking direct evidence of mitochondrial abnormalities in primary gastrointestinal diseases. In particular, no search has been conducted for possible mtDNA deletions in gut EC, an extremely metabolically active cell population constantly subjected to oxidative and nonoxidative stimuli. Because of the reported energy defect in UC (29, 44), this condition seemed ideal to investigate the presence of mtDNA defects by using highly purified human EC.

Our preliminary experiments revealed that the 4977-bp common deletion was detectable in normal EC from the small and large bowel as easily as in control skeletal muscle and liver tissue. The initial comparison between EC from control and inflamed colons appeared to indicate that the common deletion was less frequent in EC from IBD than cells from histologically normal control tissue. In contrast, as evidenced by the complete lack of the 593-bp product, the 4977-bp deletion was absent in autologous mucosal lymphoid cells. This is of interest, because it indicates that the factors controlling the expression of mtDNA deletions in normal and inflamed colon selectively affect EC but not all mucosal cells indiscriminately. Comparable findings have been reported in heart failure patients whose cardiac muscle cells but not lymphocytes contain an increased frequency of mtDNA deletions (31).

Quantification by Southern blot hybridization confirmed the differential expression of the 4977-bp deletion in UC compared with normal and CD colonicocytes by showing a markedly decreased frequency of this common deletion in both undiluted and serially diluted samples of EC DNA in UC. The same was true for the other numerous deletions we detected whose frequency was also considerably lower in UC than control and CD EC. A difference in mtDNA deletion frequency between UC and control colonocytes may not be entirely unexpected considering the involvement of UC mucosa by an active inflammatory process. Surprising, however, was that EC from active CD, a condition also characterized by chronic inflammation (43), displayed a large number and diversity of deletions similar to those of control EC. This indicates that the simple presence of inflammation in the mucosa is not solely responsible for the differential expression of mtDNA deletions in UC vs. CD EC and suggests that UC colonocytes respond differently to inflammation-associated stress.

The decreased frequency of the 4977-bp common deletion in UC EC was independent of the patient’s age, a peculiar observation in view of the well-established notion that the frequency of mtDNA increases progressively with time (27, 33). This lack of correlation between age and deletion frequency, combined with the difference in frequency deletions between UC and CD EC, suggests that induction or regulation of mtDNA deletions is unique in UC, depending not only on the presence but the type and degree of mucosal inflammation. To explore the latter possibility, we investigated the presence of the 4977-bp deletion by directly comparing ECs isolated from paired segments of inflamed and noninflamed mucosa from the same resected specimen. This showed a striking decrease and even absence of deletions in ECs from actively involved but not uninvolved UC mucosa by both visual comparison of the Southern blots as well as by the dilution assay. In addition, as previously noted in random UC EC samples, the number of other mtDNA deletions was also remarkably lower in ECs from involved than from uninvolved mucosa.

In interpreting the above findings, it should be remembered that compared with other organs, the intestine is unique because of the physiological presence of inflammation in the mucosa (12). Consequently, ECs are always exposed to some degree of oxidative stress, a degree that obviously increases in the condition of UC (30, 50). There is evidence for direct oxidant injury to ECs in IBD which is exacerbated by depletion of mucosal antioxidant defenses (7, 37). The deleterious effects of oxygen free radicals to various cellular components are well recognized (24), more so in mitochondria, which are especially susceptible because of their proximity to the respiratory chain, less-efficient DNA repair system, lack of protective histones, and high amounts of direct repeats, all of which may explain why mtDNA has a 10- to 20-times higher somatic mutational rate compared with nuclear DNA (25). In light of these considerations, chronic exposure to reactive oxygen species should lead to a greater number of mtDNA mutation deletions in inflamed epithelium. Therefore, it was surprising to find that the 4977-bp deletion was actually less common in ECs from actively inflamed UC mucosa, whereas the frequency and variety of deletions in ECs from noninflamed UC mucosa was similar to that of control colon. Because of the normally high rate of mutations in mtDNA, postmitotic tissues or those with a slow turnover of DNA accumulate the largest number of tissue-specific somatic mtDNA mutation deletions (22). On the other hand, the degree of accumulation also depends on the intensity and frequency of exposure to reactive oxygen metabolites. Both factors certainly interact in the colon in which EC DNA replication is high as is
the baseline level of oxidative stress under physiological conditions.

The equal frequency and variety of mtDNA deletions in EC from control and noninflamed UC mucosa suggest that, in the absence of active inflammation or during remission, ECs in UC respond normally to local oxidative stress and display a baseline amount of mtDNA deletions. During active inflammation, there is an accelerated migration and enhanced loss of ECs at the luminal surface, resulting in a greatly increased cell turnover (3, 14). Thus there may be not enough time to induce the changes caused by physiological cell aging and oxidative damage, including the normal amount of mtDNA deletions. In addition, the rapid cell turnover alters colonocyte differentiation in active UC (18). In HT29 colon cancer cells, glucose induces a less differentiated phenotype associated with diminished oxygen consumption and mitochondrial oxidative phosphorylation (17), whereas trehalose induces a differentiated, goblet cell-like phenotype with enhanced expression of mitochondrial 16S ribosomal RNA and NADH dehydrogenase subunit 4 (34). The loss of goblet cell characteristic of active UC is compatible with a less differentiated EC phenotype, implying a lower degree of oxidative phosphorylation and impaired free radical generation resulting in less mtDNA damage and fewer deletions. The combination of an accelerated cell turnover and an immature differentiation state may result in the epithelium’s failure to “age properly” and generate effective oxidative phosphorylation, perhaps explaining the inefficient butyrate oxidation and energy production described in UC (29, 44). An entirely different scenario might exist in CD, because in this condition ECs display the same frequency of deletion as normal ECs. This may be explained by lack of exposure of most ECs to increased levels of oxidative stress due to the patchiness of inflammation and the deep transmural rather than epithelial location of the inflammatory infiltrates in CD.

Because mitochondrial function and chronic inflammation are both highly complex events, it is difficult to speculate on the consequences that the decrease of mtDNA deletions in ECs might have in UC. Nevertheless, the detection of mtDNA abnormalities in UC is compatible with and provides a potential mechanism for the reported defects in energy production associated with the pathogenesis of this condition. Further studies are obviously warranted to fully understand the implications of EC mtDNA deletion in the natural history of UC.

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