0031-3998/07/6205-0564
PEDIATRIC RESEARCH
Vol. 62, No. 5, 2007
Printed in U.S.A.

Recovery of Exfoliated Cells From the Gastrointestinal Tract of Premature Infants: A New Tool to Perform "Noninvasive Biopsies?"

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ABSTRACT: To gain insight into specific gene expression in the gastrointestinal (GI) tract of preterm infants, we adapted a method to isolate exfoliated epithelial cells. Gastric residual fluid aspirates (n = 89) or stool samples (n = 10) were collected from 96 neonates (gestational age, 24–36 wk). Cells were characterized by microscopic observation, cytokeratin-18 immunodetection, and expression of transcripts. The human origin of cellular DNA was confirmed by amplification of specific X and Y chromosome sequences. Isolation yielded 100–500 cells per sample for gastric aspirates (n = 8) and 10–20 cells for fecal samples (n = 5). Epithelial origin was confirmed by immunodetection of cytokeratin 18. Analyses of reverse transcribed products, using two independent methods, from 15 gastric fluid and two stool samples showed that 18S-rRNA and transcripts of beta-actin, glyceraldehyde-3-phosphate dehydrogenase (gapdh), and period1 were in quantities corresponding to at least 10 cells. On 59 aspirates, we found beta-actin transcripts (all but one), cytokeratin 18 (eight positive of eight samples), SLC26-A7-1 (13 positive of 19 samples), period2 (17 positive of 17 samples), and clock (25 positive of 26 samples). Exfoliated cells can be recovered from gastric aspirates and fecal samples and serve as a tool to investigate the impact of therapeutic and nutritional regimens on the maturation of GI functions. (***Pediatr Res* 62: 564–569, 2007**)

Investigation of gene expression in the gastrointestinal (GI) tract commonly relies on the analysis of tissue biopsies. The performance of tissue biopsies, however, is unethical in human neonates. Exfoliated or sloughed cells are naturally lost by mammals in their environment and have been used as surrogates for target cells to predict the response to bioactive food components (1) or to detect biomarkers of cancer in children with inflammatory bowel disease (2) and cancer in adults (3). Yet, the feasibility of this approach has not been assessed for the exploration of neonatal GI tract.

Our aims were to determine the feasibility of this approach in human infants and assess the number, quality, and lineage of cells exfoliated that can be recovered from gastric and intestinal lumen in premature infants and to evaluate whether these cells can be used to measure the expression of specific genes of interest in a "noninvasive" fashion and thus serve as surrogates for true invasive tissue biopsy specimens. The ability to use exfoliated cells instead of biopsy or autopsy material would be highly useful to evaluate disease biomarkers in therapeutics and to explore GI functional maturation (4). We have chosen to assay clock genes as well because (1) these genes are believed to be ubiquitous in somatic cells of mammals and may be modulated in the GI tract by food intake (5) and (2) we detected the expression of these genes in cells from intestinal biopsy specimens obtained in adults in earlier studies (6). To our knowledge, even though the ability of feeding to induce and synchronize the expression of these genes has been extensively studied in peripheral tissues in laboratory rodents (7), this has not been explored in human infants, whether full term or preterm. In newborn rats, a common animal model used in neonatology (8), breast-feeding was shown to alter the circadian rhythm of the clock in the liver, with the beginning of rhythmic oscillations as early as postnatal d 2 for rev-erb-alpha and bmal1 transcripts to reach a complete organization at d 30 (9). As clock genes have been shown to regulate cell proliferation (7), they may play a role in cell homeostasis in an epithelium that undergoes tremendous cell growth in the first few weeks of life.

MATERIALS AND METHODS

Patients and Samples. Gastric residual fluid aspirates (0.5–1.5 mL; 89 samples) and fecal samples (0.5–3 g; 10 samples) were anonymously collected at random (and only once per infant) from a total of 96 premature infants. Gestational ages were between 24 and 36 wk and postnatal ages ranged from 1 to 90 d. Samples were immediately processed to isolate exfoliated cells by immunocapture using a procedure adapted from Bandaletova *et al.* (10). Briefly, gastric residual fluid aspirates or fecal samples were resuspended by vigorous hand shaking in 20 mL phosphate-buffered saline solution without Ca^{2+} and Mg^{2+} (PBS0) containing 1 g/L D-glucose and antibiotics (penicillin and streptomycin) with 0.05 mM dithiotreitol (DTT) at room temperature. Cell suspensions were gently sieved on a cell dissociation sieve (CD1-1KT, Sigma Chemical Co., St.-Quentin-Fallavier, France) through two grids of 40 and 60 mesh, respectively. Sieved suspensions were centrifuged for 10 min at 200× g at 4°C, and cellular pellets were resuspended in 4 mL PBS0 containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) and 0.05 mM DTT with 80 μL of CD326 (EpCAM) Microbeads

Received March 15, 2007; accepted June 12, 2007.

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This work was supported in part by an ANS (Action Nouvelle Soutenue 2006) grant from the AlimH Department, INRA, France.

Abbreviations: GI, gastrointestinal; **PBS0,** phosphate-buffered saline solution without Ca^{2+} and Mg^{2+}

(Miltenyi Biotec, no. 130-061-101, Bergisch Gladbach, Germany). Cell suspensions were incubated for 40 min at 4°C. Immunolabeled cells for human epithelial antigen (or CD326 (EpCAM); (11) were loaded and separated on MACS column through a high-gradient magnetic field (Miltenyi Biotec, MiniMACS Starting kit, no. 130-90-312). Total cells were eluted in 1 mL final volume of PBS0. Thereafter, cells were separated by centrifugation (190× *g*, 5 min, 4°C) and the pellet resuspended in buffers suitable for immunocytochemistry, Western blotting, and nucleic acid extraction for sex determination or reverse transcriptase-polymerase chain reaction (RT-PCR). The specific number of analyses and number of patients per analysis performed are summarized in Table 1. All samples were collected from the Hôpital de la Mère et de l'Enfant in Nantes, France, EU. The protocol was approved by the Nantes Hospital Ethics Committee.

Cell line and adult cells used as controls. Colon cancer cells (Caco-2 cell line purchased from European Collection of Animal Cell Culture, UK; ECACC#860120) with an enterocytic differentiation (12) were derived from a human colon cancer and were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin (Invitrogen, Cergy Pontoise, France). These cells were used as control in parallel experiments to check for cellular capture and detection of specific glycoprotein (11) by the kit, Western blot analysis, and RT-PCR. Total RNA samples were extracted from Caco-2 cell monolayers in 1 mL of TRIzol (Invitrogen) on ice followed by storage at −80°C. Two micrograms of each sample was denatured for 5 min at 70°C with 1 μg of random primers (Promega, Charbonnières, France). Reverse transcription was then performed for 60 min at 37°C and 5 min at 95°C with 10 mM deoxynucleotides and 2.5 μL AMV (Promega). cDNA were also obtained after Rneasy preparations of total RNA extracted with Qiagen MicroRneasy kit (Courtaboeuf, France) from clones of Caco-2 cells containing 50–500 cells harvested by cloning cylinder (Sigma Chemical Co.) to allow recovery by trypsinization of colonies grown from single-cell and from oral cells of oral mucosa swabs obtained in human adult volunteers.

Microscopic observations of exfoliated cells. Cell phenotypes were analyzed by microscopic examination with Hoechst 33258 staining for nuclear DNA.

Isolation of total DNA and sex determination. Genomic DNA was extracted from exfoliated cells of two independent samples with Nucleospin tissue kit (Macherey-Nagel, Germany) and compared with Caco-2 cell DNA extracts. Sex determination was performed on aliquots of Rneasy Lysis buffer with β-mercaptoethanol containing epithelial cells isolated from gastric fluids or from oral mucosa of a male and a female adult human volunteer.

Table 1. *Analyses and number of patients per analysis performed on gastric residual fluid aspirates and fecal samples collected from neonates (24–36 wk)*

Samples	Type of analysis	No.
Gastric residual fluid aspirates (total no. = 89)	Microscopy (four in double staining for immunofluorescent detection of cytokeratin 18)	8
	Immunodetection by Western blot (immunocaptured cells of samples of seven infants were pooled to allow detection)	7
	DNA extraction	2
	RNA extraction and double analysis of reverse transcribed products	13
	RNA extraction and single analysis of reverse transcribed products	59
Stool samples (total no. = 10)	Microscopy (two in double staining for immunofluorescent detection of cytokeratin 18)	5
	Concurrent extraction of RNA and proteins	3
	RNA extraction and double analysis of reverse transcribed products	2

Exfoliated cells were immediately isolated by immunocapture, and analyses were performed within 3 wk after the date of collection. All samples were independent and anonymously collected. Gastric residual fluid aspirates were collected from four to 13 infants per month for 13 months. The low numbers of cells obtained per sample preclude their use in two different assays except for sex determination realized on aliquots of four samples. Attempting concurrent extraction of RNA and proteins from the same column was unsuccessful due to the low number of cells recovered in stool samples.

Primers used in polymerase chain amplification for sex determination were adapted from Pascal *et al.* (13) and specific for repetitive sequences found in the X chromosome: X forward CGAGAATCTGCAAGTGGACG and X reverse GTTCAGCTCTGTGAGTGAAA. These primers amplified a 305-base pair fragment.

A second set of primers was designed for Y chromosome: Y forward ATTCGATTCCGTTTCATTGC and Y reverse CGAAGAGAATGGAAACAAATGG. These primers amplified a 260-base pair fragment.

Our PCR conditions were in a final volume of 20 μL, with 2 μL taq DNA polymerase buffer 10× containing 15 mM $MgCl_2$ (Promega), 2 μL dNTPs 2 mM (0.2 M final), 1 μL of primer F 10 μM, 1 μL of primer R 10 μM, 0.1 μL of *Taq* polymerase Promega (0.5 unit), genomic DNA 10 to 20 ng in H_2O.

Products were amplified according to the following PCR cycle: 7 min at 95°C, 30 cycles [30 s at 95°C, 30 s at 55°C, 30 s at 72°C] and 7 min at 72°C. Revelation was performed by agarose gel electrophoresis containing SYBRGreen, and bands were visualized under ultraviolet light.

Immunocytochemistry. Exfoliated and Caco-2 cells were fixed in PBS0 with 4% formaldehyde overnight at 4°C to preserve the cell morphology. The next day, fixative was replaced by 70% ethanol for cell permeation. Samples were then rehydrated by replacing ethanol with PBS0 with 0.2% bovine serum albumin (Fraction V, Sigma Chemical Co.). Anti–cytokeratin 18 mouse MAb (DC-10, sc-6259, Santa Cruz Biotechnology Inc., Heidelberg, Germany) or anti–cytokeratin 18 rabbit polyclonal antibody (H-80, sc-28264, Santa Cruz Biotechnology Inc.) was used in 1/10 to 1/50 dilution range on fixed-cell monolayers for immunofluorescence (revelation by goat anti-mouse fluorescein isothiocyanate conjugate (F 4018) or goat anti–rabbit FITC conjugate from Sigma Chemical Co., used in 1/50 dilution, respectively). Samples were visualized under a Nikon Eclipse 400 microscope equipped for epifluorescence (double-labeled by Hoechst 33258).

Western blot analysis. For Western blot analyses, cell monolayers containing 10^7 Caco-2 cells or exfoliated cell preparations pooled from seven infants were washed twice and collected in ice-cold PBS0. After incubation in cell lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 150 mM NaCl, 0.2% Nonidet P-40, and anti-proteases cocktail) on ice for 5 min, the cells were centrifuged for 5 min at 3000× *g* to obtain cytoplasmic fraction. Protein extracts (loaded at 20 μg total proteins per sample) were electrophoresed through sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using standard procedures. Cytokeratin 18 was detected by primary antibodies [mouse MAb (DC-10), sc-6259, Santa Cruz Biotechnology, Inc., Tebu, France] and secondary antibody conjugated with horseradish peroxidase. Revelation was performed with an ECL chemiluminescence kit (Amersham Biosciences, Freiburg, Germany). Cytokeratin 18 molecular weights were evaluated with a molecular weight calibration kit. Relative quantification was carried out with ImageJ software (http://rsb.info.nih.gov/ij/; accessed March 8, 2007) to obtain a rough estimate of cell numbers.

Isolation of total RNA. Exfoliated cells were resuspended in Rneasy lysis buffer containing β-mercaptoethanol and stored at −80°C until further purification (MicroRneasy, Qiagen). As <5000 cells were expected, we added 20 ng poly-A RNA per sample as carrier RNA.

The final sample volume was of 75 μL. We proceeded according to kit requirements by ethanol precipitation of nucleic acid onto silica gel membrane of MinElute spin column with a step of digestion by Dnase-I in Rnase-free Dnase digestion buffer. Elution volume was 12 μL in Rnase-free water. The samples were stored at −80°C less than 3 wk before reverse transcription and RT-PCR analyses.

Reverse transcription of total RNA. The efficiency of total RNA amplification and cDNA production was compared using Sensiscript (Qiagen) and MessageBooster (Epicentre Biotechnologies, Madison, WI) kits. Both kits use a modified procedure from Phillips and Eberwine (14) to produce an amplified RNA and a final cDNA product. Sensiscript reactions were performed on 6 μL total RNA and MessageBooster reactions on 2 μL total RNA as required by suggested standard procedures. Exfoliated cells isolated from gastric fluids ($n = 15$) and stool samples ($n = 2$) were analyzed by both assays to detect 18 S ribosomal RNA, beta-actin, and gapdh and period1. Thereafter, 59 reverse transcribed samples from gastric residual fluid aspirates were obtained by Sensiscript reactions and analyzed.

RT-PCR. We designed forward and reverse primers with Beacon Designer or PerlPrimer (15) software; the specificities were assigned independently online with the Blast software (Table 2). Primers were also tested on total RNA extracts of Caco-2 cell monolayers, of oral mucosal at cells of an adult volunteer, and single intact colonic crypts isolated from healthy human adult subjects [the latter as we described (6)].

For RT-PCR, an aliquot of cDNA was used in the presence of 250 nmole/L specific forward and reverse primers designed for the target genes and 7.5 μL

Table 2. *Human primer sequences used in real-time quantitative RT-PCR and determined using GenBank database and Beacon Designer or Pearlprimer software*

Gene	Accession no.	Nucleotides	Sequence
ribosome 18-S	*X03205*	R 1224–1206	5′-CTCCTGGTGGTGCCCTTCC-3′
		F 1099–1117	5′-GATGCGGCGGCGTTATTCC-3′
beta-actin	*NM_001101*	R 37–58	5′-TGCTATCCAGGCTGTGCTATCC-3′
		F 177–194	5′-GCCAGGTCCAGACGCAGG-3′
gapdh	*XR_0237891*	F 883–903	5′-CACCCACTCCTCCACCTTTGAC-3′
		R 994–973	5′-GTCCACCACCCTGTTGCTGTAG-3′
slc26-A7-v1	*NM_052832*	R 4336–4358	5′-AAT CCA GCA TCA AGA CCA CTA CAC-3′
NM		F 4486–4509	5′-TTC CAC ACA CCA AGG CTA CAA TG-3′
cytokeratin 18	*NM_000224*	R 1403–1380	5′-GCC TCA GAA CTT TGG TGT CAT TGG-3′
		F 1257–1275	5′-GCC GCC TGC TGG AAG ATG G-3′
egf-r	*NM_005228*	R 2013–2031	5′-TGTGCCACCTGTGCCATCC-3′
		F 2145–2164	5′-ACCACCAGCAGCAAGAGGAG-3′
oct-4-pg-5-v1	*NM_002701*	R 1056–1076	5′-TCA GGG AAA GGG ACC GAG GAG-3′
		F 909–931	5′-CAA GCG ATC AAG CAG CGA CTA TG-3′
period-1	*AF022991*	R 412–431	5′-AGGCAACGGCAAGGACTCAG-3′
		F 516–537	5′-AGGAGGCTGTAGGCAATGGAAC-3′
period-2	*NM_003894*	R 646–667	5′-TGGAGGAGATGGAGAGCGTTAC-3′
		F 761–782	5’-GGATGCAACCTGGTCAGAGATG-3′
clock	*AF011568*	R 2823–2844	5′-GTCTCAGCAACAGCAGCAACTC-3′
		F 2942–2961	5′-GCCATCCCCTTCCTCCCTTG-3′
rev-erb-alpha-1	*NM_021724*	R 996–975	5′-GGT GAT GTT GCT GGT GCT CTT G-3′
		F 881–902	5′-CTT CCT CGT CGT CAT CCT CCT C-3′

F, forward; R, reverse.

SybrGreen buffer (Qiagen). Conditions of amplification on our iCycler were a first step at 95.0°C (14 min) followed by 45 cycles at 95.0°C for 30 s and then a step at 60.0°C for 30 s followed by 80 cycles at 95.0°C for 10 s and a final cycle of return to 15°C (all steps supposed a decrease set point temperature after cycle 2 by 0.5°C). At the end of each run, a melting curve analysis was performed to ascertain the presence of a single amplicon, but products of amplification were not sequenced. As recommended by suppliers, we used some PCR primers that prime within 500 bases of the 3′-end of the mRNA (Table 2). RT-PCR analyses on reverse-transcribed products from oral mucosa cells of an adult volunteer gave by two independent RT methods that 18S ribosomal RNA [count (ct) of 23.7 with Sensiscript and of 23.6 with Messagebooster and transcripts of beta-actin (ct of 27.8 with Sensiscript and of 24.7 with Messagebooster), gapdh (ct of 32.3 with Sensiscript and of 21.8 with Messagebooster), period1 (ct of 31.5 with Sensiscript and of 43.3 with Messagebooster).

RESULTS

Under a microscope, we enumerated approximately 100–500 cells per gastric residual fluid aspirate (on eight samples) and 10–20 cells per fecal sample (on five samples). As shown in Figure 1 on cells from gastric residual fluid aspirates, only two phenotypes were observed: epithelium-like or occasional nonepithelial cells. Most cell nuclei were nonapoptotic with no difference by sex and age at sampling (approximately 1% were apoptotic). In stool samples, cells displayed similar phenotypes but the proportion of apoptotic nuclei was of 20%. Exfoliated cells from the gastric residual fluids of premature infants fed with probiotics were recovered with adherent bacteria, whereas cells from infants fed a regular formula were bacteria free. We did not observe any effect of breast-feeding on the appearance of bacteria.

To confirm the epithelial origin of recovered cells, we obtained consistent immunodetection of cytokeratin 18 by Western blot on an extract of cell preparations pooled from gastric fluid aspirates of seven infants (Fig. 2) and on immunolabeled cell preparations observed by epifluorescence.

Both cellular genomic DNA and mRNA can be recovered in fresh stools from adult patients (16). If this is the case in infants as well, there is every reason to believe exfoliated cells might potentially be used to monitor the expression of genes reflecting the maturation of the GI tract of premature infants. There is, however, a risk of contamination of gastric residual fluid aspirates with exogenous cellular material. To confirm the human origin of exfoliated cells from gastric residual fluid aspirates, we verified the presence of Y and X sequences in these cells. DNA extraction of two infants was compared with Caco-2 cells (Fig. 3) and Y sequences were identified in samples from male infants, indicating that the cells isolated indeed originated from the patient.

RT-PCR is considered a robust method to screen for RNA contents recovered from fixed material (17) or to develop methods based on determining expression of individual genes from Rnase-rich environment like GI fluids (18). We used 18S rRNA or housekeeping genes, such as *beta-actin* and *gapdh*, which are optimal for normalization in real-time quantitative RT-PCR (19). RT-PCR analyses on reverse-transcribed products from 15 gastric fluid samples and two stool samples demonstrated by two independent methods, the expression of 18S ribosomal RNA (ct range between 23.8 and 30.6 with Sensiscript and between 26.9 and 37.9 with Messagebooster on 15 samples) and the presence of transcripts of beta-actin (ct range between 22.7 and 27.8 with Sensiscript and between 28 and 41 with Messagebooster), gapdh (ct range between 27.6 and 33.7 with Sensiscript and between 26.8 and 38.2 with Messagebooster), period1 (ct range between 24.7 and 31.6 with Sensiscript and between 38.8 and 42 with Messagebooster). The two stool samples analyzed in duplicate gave similar results. Taken together, these data indicate that we

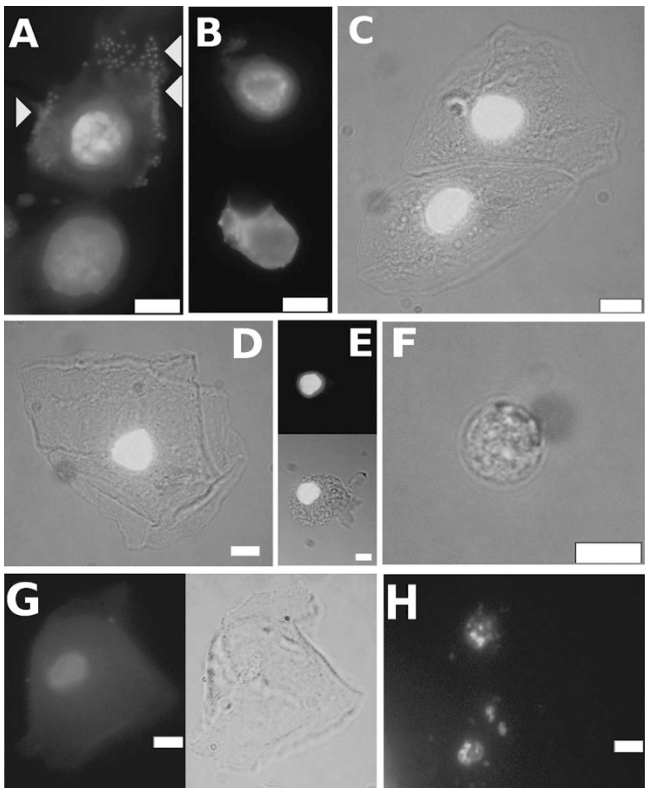

Figure 1. Microscopic analyses of exfoliated cells from gastric residual fluid aspirates (*A–F*) or fecal samples (*G*, *H*) of preterm infants. Cells were labeled by Hoechst 33258 and observed by epifluorescence (*A*, top of *B* or *E*, *G*, *H*) or conventional light with ultraviolet excitation to visualize DNA and cytoplasm (*C–E* bottom, *F*). Note that for infants fed with probiotics, we had adherent bacteria on cells (*A*, *arrowheads*) and quiescent nuclei with similar views for infants fed with regular formula (*B–D*) according to sex and date of sampling. We found typical epithelial morphology (*A*, *C*, *D*) with expression of cytokeratin 18 (*B*, bottom) along with occasional nonepithelial phenotype (*F*) and approximately 1% of apoptotic nuclei. In stool samples, we had typical epithelial morphology (*G*) along with 20% apoptotic nuclei (*H*). Observations were performed at immersion (×1300). White bars = 10 μm.

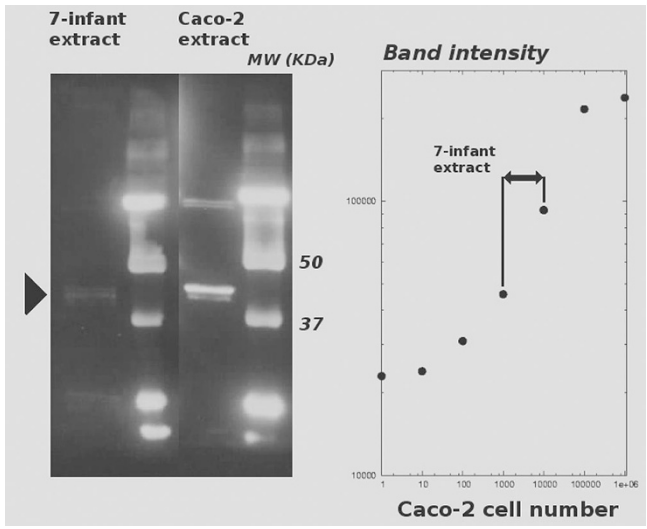

Figure 2. Immunodetection of cytokeratin 18 by Western blot on an extract of cell preparations pooled from seven infants. Exfoliated cells of gastric residual fluid aspirates were immunocaptured and pooled by centrifugation from seven infants collected on the same day. Note that mRNA expression of cytokeratin 18 was confirmed by RT-PCR and our crude estimation of 100–1000 cells per infant close to the range of cell enumeration performed by microscopic observations.

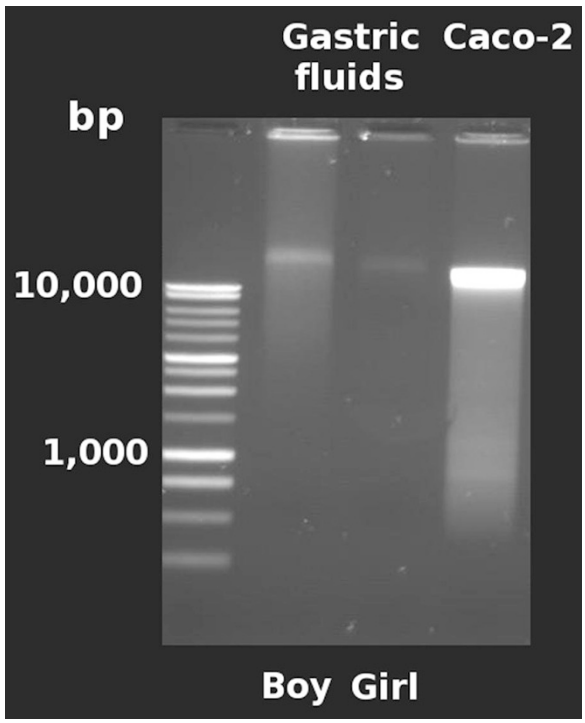

Figure 3. Extraction of total DNA from exfoliated cells of two infants. Exfoliated cells isolated from gastric residual fluid aspirates (one boy and one girl) by immunocapture were extracted for genomic DNA and compared with cultured colon cancer (Caco-2) cell DNA extracts. Note that the difference in band intensities between these two infants was not related to sex or the infant's age at sampling.

recovered amounts of RNA corresponding to 10–50 cells. Yet this estimate should ideally be confirmed by comparing exfoliated cells with biopsy samples because (1) this analysis was carried out on two fecal samples only and (2) Bas *et al.* (19) reported that human intestinal epithelial cells contain 10 times more 18S RNA per cell compared with lymphocytes.

Preliminary data on cell lineage and peripheral clock gene components were obtained by RT-PCR using only the Sensiscript method (Table 3) and examined to explore the influence of the age of infants on cellular loss (the gestational age of infants ranged from 24 to 36 wk). We found the presence of transcripts of beta-actin (ct range between 20.6 and 26.4) in all samples analyzed but one, suggesting that all infants but one were losing some cells. The 58 samples positive for beta-actin were used in independent experiments to search for transcripts related to cell lineage or circadian clock genes. We found transcripts of cytokeratin 18 (ct range between 30.8 and 32.8), egf-r (ct range between 25.6 and 27.1), slc26-A7-v1 (ct range between 33.3 and 35.6), and oct-4-pg-6-v1 (ct range between 27.3 and 32). Detection of cytokeratin 18 and SLC26a 7 variant 1 [associated with anion transporters of gastric parietal cells (20)] transcripts favors an epithelial origin of the exfoliated cells recovered from gastric residual fluid aspirates. Finally, we found transcripts of clock (ct range between 25.3 and 35.4), period2 (ct range between 28.8 and 33) and rev-erb-alpha (ct range between 26.9 and 32.2). These

Table 3. *Detection of mRNA by RT-PCR in gastric residual fluid aspirates*

Role	Gene	Accession no.	No. of positive samples/total analyzed
Cytoskeleton	*beta-actin*	NM_001101	58(+)/59
	C ytokeratin 18	NM_000224	08(+)/08
Membrane integrity	*egf-r*	NM_005228	5(+)/5
Anion transporter of parietal cells	*slc26-A7-v1*	NM_052832	13(+)/19
Stem cells	*oct-4-pg-5-v1*	NM_002701	16(+)/19
Circadian clock	*clock*	AF011568	25(+)/26
	period-2	NM_003894	17(+)/17
	rev-erb-alpha-1	NM_021724	4(+)/4

Note that beta-actin transcripts were detected in all (59 samples) but one. Detection of cytokeratin 18 and SLC26a 7 variant 1 [associated with anion transporters of gastric parietal cells (20)] transcripts favors of an epithelial origin.

data suggest that clock genes are expressed in gastric cells of infants.

DISCUSSION

To the best of our knowledge, the current study is first to demonstrate that a significant number of exfoliated epithelial cells can be recovered using a relatively simple procedure from gastric fluid aspirates and fecal samples in preterm infants. This simple method may prove a useful, relatively noninvasive tool to assess the expression of specific genes of interest to monitor the maturation of GI function as a surrogate for true biopsies in preterm infants.

Exfoliated cells have long been found to be shed in abundance in the stools of adult patients with colorectal cancer. These cells are nonapoptotic, and markers in exfoliated cells originating from the tumor are actively studied as a potential tool for cancer screening and prevention (3,21). Although the recovery of exfoliated colonocytes from the stools of normal adults has been described (10), and exfoliated cells from the urinary tract of infants have been studied in the past (22), we are not aware of previous studies in preterm infants with this approach.

As only a few typical apoptotic nuclei were observed in gastric fluid aspirates, we speculate that the recovered cells had been exfoliated shortly before sampling and that the isolation technique performed within <2 h minimizes the risk of RNA degradation during isolation. As collection of gastric residual fluid aspirates are performed as part of the nursing care routine every 3 h in many neonatal intensive care units, the technique may be highly relevant for time series experiments in chronobiology to reassess recent demonstration of the influence of lighting in neonatal intensive care units (23,24) on neonates GI maturation (25). With stools, there is little way to improve cell sampling because colonic transit time differs from one child to the next, and proceeding during routine colonoscopy, as can be done in adults (26), would clearly be unethical. Nevertheless, it can be very useful for other explorations on GI tract maturation, when there is no gastric tubing for less premature infants. However, our microscopic observations are based on staining of nuclear DNA by Hoechst, and further work is needed to evaluate whether immunocaptured cells from gastric aspirates or fecal samples have cytogenetic damage (which may be assessed by micronucleus assay (2) or other cytological analyses) or are normal cells shedding from the delicate balance between proliferation/differentiation and apoptosis of newly formed epithelium.

In contrast to reports on urocytograms (22), neither gestational age nor gender affected the amount of exfoliated cells lost in gastric residual fluid aspirates by premature infants. However, work evaluating the RNA status of postmortem human brain tissue suggests that ribosomal RNA and mRNA have different degrees of degradation depending on postmortem conditions and extraction procedure (27). As exfoliation may happen at random at 3- to 4-h intervals, more work is needed to evaluate integrity of total RNA extracted from gastric aspirates or stools, and its suitability for RT-PCR or microarray analysis. On RNA extracted from fecal samples in adult patients, new markers were screened with the serial analysis of gene expression (SAGE), a method originally used to compare the transcriptomes of normal and colon carcinoma cells in human (28). The collection of exfoliated cells would potentially make a similar approach usable to delineate the time course of gene expression in infants by SAGE.

The current study demonstrates that a sufficient number of exfoliated cells can be recovered from gastric aspirates in preterm infants whatever the term or the postnatal age to measure the expression of specific genes of interest. This innovative approach may prove a useful, noninvasive tool to assess the expression of genes of interest in the GI tract of preterm infants as a surrogate for true biopsies.

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