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Isolation of human intestinal defensins from ileal neobladder urine

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Abstract We describe the isolation of naturally occurring human intestinal defensins HD-5 and HD-6 from ileal neobladder urine and ileal mucosa. Using an antibody-based detection assay, we found multiple N-terminally processed forms of HD-5. The predominant HD-5 forms in tissue were longer than those in neobladder urine (amino acid (aa) 23–94 and 29–94 versus aa 36–94, 56–94 and 63–94) suggesting that Paneth cells store prodefensin that is processed to mature defensin during or after degranulation. Search for mature HD-6 yielded aa 69–100 as the predominant form in both sources. The ileal neobladder is a promising model to study human Paneth cell secretion.

Key words: Small intestine; Mucosal immunity; Antimicrobial peptide; Cryptdin; Phospholipase A2; Ileal neobladder

1. Introduction

In humans and other mammals, host defense of the small intestine is attained by both innate and adaptive immune responses. Of particular interest are the Paneth cells, specialized epithelial cells implicated in innate host defense. Paneth cells are located in the intestinal crypts, and originate from the intestinal stem cells that also generate enterocytes, goblet cells, and enterochromaffin cells (reviewed in [1]). Paneth cells contain numerous secretory granules that are released into the intestinal lumen upon cholinergic stimulation and bacterial challenge [2,3]. In mice and rats, lysozyme, group II phospholipase A2 (PLA2) and intestinal defensins (cryptdins) have been well studied as antimicrobial components of the Paneth cell granules [2–6]. These antimicrobial polypeptides could reach high concentrations within the tight confines of the crypt and could prevent the colonization and invasion of the stem cells located adjacent to the Paneth cells (reviewed in [7]). Unfortunately, the extrapolation from animal models to the human is complicated by the known variability of Paneth cell distribution when diverse mammalian species are compared, with some mammals lacking Paneth cells altogether [8].

The properties of human intestinal mucosa as an antimicrobial barrier have been relatively inaccessible to experimental investigation. Studies of live human Paneth cells have been limited by the lack of an immortalized Paneth cell line and the restricted availability of fresh tissue. In particular, although two human Paneth cell defensin genes have been cloned [9,10] and their gene structure and developmental expression characterized [11], the corresponding peptides have not been isolated. In search of a feasible human model of Paneth cell function, we studied urine voided through ileal neobladders [12,13], documented that it contains Paneth cell associated polypeptides, and for the first time isolated the naturally occurring human Paneth cell defensins HD-5 and HD-6.

2. Materials and methods

2.1. Ileal neobladder urine

We studied patients (UCLA IRB #97-11-026-01A) who were treated with radical cystoprostatectomy and ileal neobladder reconstruction for transitional cell carcinoma of the bladder. In all patients, the tumor was confined to the bladder. The Studer procedure was used with modifications to fashion a continent ileal reservoir using a 40–60 cm segment of ileum that was at least 10 cm proximal from the ileocecal valve [12,13]. From 3 days to 2 weeks after surgery, urine was collected through abdominal drains first, and later, when possible, as voided through the urethra.

2.2. Peptide extraction from urine – preparative scale

Larger tissue and mucous particles in urine collected from ileal neobladders were removed by sedimentation and filtration through 100 μm polypropylene meshes (SpectraPor, Spectrum, Houston, TX). Cellular particles were removed by centrifugation at $3600\times g$ for 10 min and subsequent vacuum-aided filtration through filter paper (No. 4; Whatman, Clifton, NJ). The cleared urine was adjusted to pH 7.0 with ammonium hydroxide or concentrated glacial acetic acid, and cationic components were adsorbed with a weak cation exchange matrix (Macro-Prep CM (carboxymethyl) support, Bio-Rad Laboratories, Hercules, CA) added at a 1:500 ratio of matrix to urine volume. In preliminary experiments we had found that HD-5 binding to the CM matrix was optimal at pH 6.5–7.5. After overnight incubation under constant gentle stirring at 4°C the CM matrix was sedimented at $1\times g$. The supernatant was reextracted as before and batches of matrix from both extractions were further processed in the same manner but separately. The CM matrix was pelleted at $1000\times g$ for 5 min and washed once with 10–100 volumes of 10 mM sodium phosphate buffer, pH 7.0 for 15 min at room temperature under constant agitation followed by centrifugation as above. Thereafter a 50% slurry of the CM matrix was prepared with 10 mM sodium phosphate buffer, pH 7.0, poured into a column and washed with 10 mM sodium phosphate buffer, pH 7.0 until a stable absorbance at 280 nm was achieved. Absorbed cations were eluted with 5% acetic acid. Fractions containing HD-5 were identified by Western blot with polyclonal antibodies against recombinant HD-5 [14], lyophilized, pooled in 5% acetic acid and subjected to further purification as described below.

2.3. Peptide extraction from urine – analytical scale

Urine from ileal neobladders was cleared as described in the previous section. Normal urine and urine from urinary tract infection (UTI urine; #1: $>10^5$/ml *Citrobacter freundii*, #2: $>10^5$/ml *Proteus mirabilis*; kindly provided by Dr. David A. Bruckner, Clinical Microbiology, UCLA Medical Center) were first centrifuged at $1500\times g$ for 10 min and subsequently sterile filtered (0.2 μm Nalgene, Nalge Nunc

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International, Rochester, NY). If necessary, the urines were adjusted to pH 7.0 with ammonium hydroxide or concentrated glacial acetic acid. 20 ml of urine was incubated with 100 μl of the CM matrix overnight under constant gentle agitation at 4°C followed by centrifugation at 1500×*g* for 5 min. The matrix was washed three times in 10 mM sodium phosphate buffer, pH 7.0 for 5 min each at room temperature under constant gentle agitation and subsequently centrifuged for 5 min. Absorbed cations were eluted in two steps. First 200 μl of 10% acetic acid was added for 1 h, followed by centrifugation as above. Then 1 ml of 5% acetic acid was added to the matrix overnight at 4°C under constant gentle agitation and the supernatant collected after centrifugation as above was pooled with the 10% acetic acid eluate. Aliquots of the combined eluate were lyophilized and subjected to acid urea-PAGE (AU-PAGE) followed by Western blot analysis.

2.4. Dot blot and Western blot

PVDF membrane (Immobilon P or for sequencing Immobilon PSQ, Millipore, Bedford, MA) was briefly wetted in methanol and rinsed in dH_2O. For dot blot, peptides were lyophilized, resuspended in 2 μl 0.01% acetic acid, deposited onto the membrane, and allowed to adsorb for 30 min at 4°C in a humid chamber. Recombinant proHD-5/HD-5, phospholipase A2 from human tears (kindly provided by Dr. Xiao-Dan Qu, UCLA, Department of Medicine) and human breast milk lysozyme purified in our laboratory were included as standards. For Western blot, peptides were electroblotted from AU-PAGE to the membrane in 0.7% acetic acid/10% methanol at RT with 0.18 A for 10 min omitting prior soaking of the gel in transfer buffer to minimize peptide losses [15]. Longer blotting times caused migration of peptide bands through the membrane. After fixation with 0.05% glutaraldehyde in TBS for 1–2 h the blots were blocked for 30 min with 3% gelatin in TBS and incubated overnight at RT with polyclonal rabbit serum against either HD-5 (1:2000) or PLA2 (1:5000; a generous gift from Dr. Timo J. Nevalainen [16]) or human lysozyme (1:100, DAKO, Carpinteria, CA) diluted in 1% gelatin in TBS containing 0.01% thimerosal as preservative. Thereafter blots were briefly rinsed with water, washed three times with TBS containing 0.05% Tween 20, incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) 1:2000 in 1% gelatin in TBS, washed as before and developed as described previously [14].

2.5. Isolation of human defensin HD-5

Initially, CM-macroprep extracts were directly subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC) with a 7.8 mm×300 mm Waters C18 column (Delta Pak) eluted by a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) from 1% to 21% during the first 10 min, followed by an increment to 31% during the following 40 min and an increment to 61% within 15 min. Fractions eluting between 21% and 41% acetonitrile were analyzed by dot blot assay for HD-5. The presence of HD-5 was confirmed by AU-PAGE Western blot and fractions containing at least 3 μg of HD-5 peptide as estimated by reactivity of recombinant HD-5 and proHD-5 standards were subjected to a second RP-HPLC on a 3.9 mm×150 mm Waters C18 column (Delta Pak) with 0.1% heptafluorobutyric acid (HFBA) as pairing agent and an acetonitrile gradient from 1% to 35% during the first 17 min, followed by an increment to 41% during the following 24 min and an increment to 61%/min during the last 10 min. All peak fractions were again analyzed as above and HD-5 containing fractions of at least 80% homogeneity were subjected to sequencing and mass spectrum analysis. In samples of $<80\%$ homogeneity, peptides were electroblotted to Immobilon PSQ membrane, briefly soaked in amido black stain (0.4% naphthol blue black, Sigma, 25% isopropanol, 10% acetic acid), destained with water and bands corresponding to HD-5 reactive bands in a duplicate Western blot were cut out and subjected to sequencing.

To obtain highly purified HD-5, CM-macroprep extracts were first desalted on a G10 Sephadex column (Pharmacia Biotech, Piscataway, NJ), then subjected to continuous preparative gel electrophoresis as described before [14] and fractions containing HD-5 as determined by Western blot analysis were subjected to RP-HPLC with TFA and – if necessary – with HFBA according to the procedure above.

2.6. Isolation of human defensin HD-6

Peptides that eluted from C18 HPLC columns at 25–35% acetonitrile in 0.01% TFA (characteristic of most defensins) and had a defensin-like electrophoretic mobility in AU-PAGE but were not lysozyme or the defensins HD-5, HNP1–3, HBD-1 as determined by dot blot analysis, were subjected to N-terminal amino acid sequencing and mass spectrum analysis. For further purification, RP-HPLC fractions eluting between 24 and 29% acetonitrile in 0.1% TFA and containing a peptide comigrating with lysozyme (elution at ∼39% acetonitrile in 0.01% TFA) in AU-PAGE were subjected to a second RP-HPLC using HFBA as pairing agent and an acetonitrile increment as described above. Peak fractions were analyzed by AU-PAGE and fractions containing a peptide with lysozyme-like mobility were subjected SDS-Tricine PAGE. If homogeneity was $>80\%$, N-terminal sequencing and mass spectrum analysis were performed. Fractions of lower purity were subjected to AU-PAGE, electroblotted to Immobilon PSQ membrane, stained with amido black as above and bands comigrating with lysozyme standard were cut out and subjected to N-terminal amino acid sequencing. When continuous gel electrophoresis was employed, fractions comigrating with lysozyme in AU-PAGE were reserved for further purification of HD-6.

2.7. Purification of HD-5 and HD-6 from human intestinal tissue

Ileal mucosal fragments of approximately 0.5–2 g from four different donors (kindly provided by D. Scott Strong, Department of Colorectal Surgery, CCF, IRB # 1155) were ground under liquid nitrogen using a mortar and pestle, 5–10 ml of 5% acetic acid was added, the suspension was sonicated on ice and extracted at 4°C for 24 h under constant agitation. Insoluble particles were removed by centrifugation at 1500×*g* for 10 min. The supernatant was diluted 1:10 with water, supplemented with 5 mM EDTA and 2 mM PMSF and the pH was increased to 7.0 with ammonium hydroxide followed by a second centrifugation to remove newly formed precipitates. Then CM matrix was added at a ratio of matrix to tissue extract of 1:50 and cationic peptides were allowed to bind to the matrix overnight at 4°C under constant gentle agitation. After centrifugation at 1500×*g* for 5 min the matrix was washed three times with 10 mM sodium phosphate buffer, pH 7.0, and peptides were eluted with acetic acid as described for the analytical urine extractions, first with twice the CM matrix volume of 10% acetic acid and then five times the CM matrix volume of 5% acetic acid. Pooled eluates were analyzed by AU-PAGE and

Table 1
Biochemical characterization of purified human intestinal defensin forms

Defensin (encoded aa)	Peptide form	Source	% ACN elution (C18 RP-HPLC)		N-terminal sequence	Mass	
			TFA	HFBA		Expected	MALDI-TOF
HD-5 (1–94)	20–94	r	29–31	37–39	ESLQER	8103	8106
	23–94	IL	31.5	37.25	QERADEA	7774	7780
	29–94	IL	31.5	37.25	ATTQKQS	7045	7052
	36–94	NB	30	38	GEDNQDL	6296	6293
	56–94	NB	26	35	TSGSQA	4271	4270
	63–94	NB	26.5	35	ATCYCR	3582	3586
	64–94	r	24–25	33–35	TCYCR	3511	3510
HD-6 (1–100)	69–100	IL, NB	25	ND	AFTCHCRR	3708	3711

Shown are the predominant naturally occurring forms in ileal neobladder urine (NB) and ileum tissue extract (IL) and, for comparison, the recombinant forms (r). Amino acid sequences are in standard single letter code, and the expected masses are based on all cysteines forming disulfide bonds. The accuracy of MALDI-TOF is approximately 0.1% of the measured mass. ND = not determined.

subsequent Western blot and selected samples further purified as described above by RP-HPLC.

2.8. Peptide identification

For defensins, N-terminal amino acid sequencing was performed by the UCLA Protein Microsequencing Facility, mass spectrum analysis was performed in the UCLA Center for Molecular and Medical Mass Spectrometry by laser desorption Voyager RP Instrument (PerSeptive Biosystems, Framingham, MA) and electrospray measurements were recorded on Sciex API III (Perkin-Elmer, Foster City, CA). Lysozyme and PLA2 were identified by Western blot.

2.9. Western blot analysis of human plasma

Blood anticoagulated with 5 mM EDTA was centrifuged at $2000\times g$ for 10 min at RT and plasma was acidified to final 5% acetic acid to ensure dissociation of any smaller protein bound to a macromolecular carrier proteins [17,18], transferred to a 30 kDa molecular weight cut off centrifugal concentrator (Omega Macrosep, Pall-Filtron, Northborough, MA), and centrifuged at $5000\times g$ at 4°C to separate macromolecules from the peptides of interest. Filtrate was collected, the retentate diluted five-fold in 5% acetic acid followed by repeat centrifugation as above to ensure complete pH-dependent dissociation of small peptides. Resulting filtrates were pooled, transferred to a 3 kDa molecular weight cut off centrifugal concentrator (Omega Macrosep, Pall-Filtron, Northborough, MA) and centrifuged at $5000\times g$. Dialysis and concentration of the sample was achieved by repeated addition of 5% acetic acid followed by centrifugation as above with a final 300-fold reduction of eluates smaller than 3 kDa. The sample was lyophilized and subjected to AU-PAGE followed by Western blot analysis as described above using polyclonal antibodies against HD-5.

3. Results and discussion

3.1. HD-5 and HD-6 appear as multiple N-terminally processed forms

Using a polyclonal antibody we detected multiple HD-5 forms in the ileal neobladder urine of each patient. The most abundant forms were identical in multiple donors (Fig. 1A and Table 1). The shortest form identified differed from the engineered version (rHD-5) by only one additional N-terminal amino acid. We next compared the secreted forms found in ileal neobladder urine with the forms present in ileal tissue. Numerous tissue forms were detected, overall longer than those found in urine, indicating that human intestinal defensin 5 is stored as a precursor in the Paneth cell granules and then further processed during or after secretion. This is in contrast to the other granule-associated defensins in humans, human neutrophil defensins 1–4, that are stored as terminally processed mature forms [19].

In general, the activity of antimicrobial peptides correlates with their net cationic charge [20–22] and defensin proforms consisting of a negatively charged propiece attached to the cationic mature peptide are much less active than the mature peptides. Thus, the inactive recombinant prodefensins proHNP-1 and proHD-5 both exhibit a net charge of +1. When these propeptides are chemically cleaved, the resulting mature forms HNP-1 (net charge +3) and rHD-5 (net charge of +4) exhibit broad spectrum antimicrobial activity [23,24]. The major HD5 forms isolated from the ileal neobladder urine have net charges between +3 and +5 and are all likely to be antimicrobial. Detailed antimicrobial testing of each form will be the focus of future studies.

For other antimicrobial peptides that are stored as inactive precursors, the inactive propeptide and activating enzymes are produced by the same cell [25,26]. Proteases found in Paneth cells include trypsin [27] that cleaves after basic amino acid residues and matrilysin which has isoleucine and leucine cleavage sites [28–30]. Two of the major HD-5 forms found in neobladder urine start C-terminal to an arginine consistent with a trypsin cleavage site. The possibility that trypsin is a key enzyme involved in defensin processing is indirectly supported by presence of trypsin inhibitors in the Paneth cell granules [31,32] that could prevent the premature activation of Paneth cell trypsin. Aminopeptidases from urine [33] or the intestinal brush border [34,35] could further process HD-5 and generate some of the forms found in the ileal neobladder. Conversely, the appearance of uncleaved human intestinal defensin aa 36–94 in the ileal neobladder urine could indicate incomplete processing due to rapid dilution and washout or unfavorable composition of the neobladder fluid.

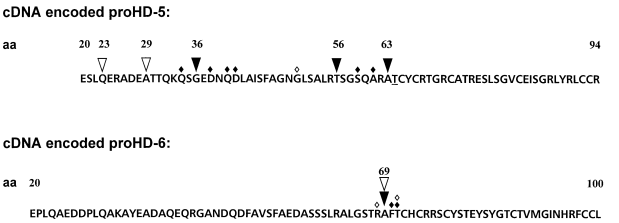

Fig. 1. Amino acid (aa) sequences (single letter code) of cDNA-encoded human intestinal prodefensins HD-5 and HD-6 and the N-termini of purified peptides. Symbols: predominant natural forms ▼, ▽; less abundant natural forms ◆, ◇; filled: from ileal neobladder urine; open: from ileal mucosa extract. The N-terminus of mature recombinant HD-5 is underlined (residue 64).

We also isolated one major form of the other known intestinal defensin, HD-6, in various ileal neobladder urines and in both intestinal tissue samples that were fractionated (Fig. 1 and Table 1). Like two of the major forms of HD-5, the major form of HD-6 is generated by cleavage C-terminal to an arginine residue. Multiple, less abundant, N-terminally processed forms of HD-6 were also identified, suggesting that HD-5 and HD-6 may be processed by the same enzymes. For HD-6, the strategy for isolation was based on chromatographic properties, and the spectrum of HD-6 forms isolated cannot be compared to HD-5 forms, since the latter was detected using a polyclonal antibody that recognizes both pro- and mature defensin forms.

We did not find any defensin peptides that deviated from the sequences of HD-5 and HD-6 as predicted from their cDNAs and genes, consistent with our previous study that confirmed the absence of additional defensin genes in human small intestine [11]. The limited diversity of human intestinal defensin genes contrasts with the seventeen known murine intestinal defensin (cryptdin) sequences encoded by a correspondingly large number of genes [36,37]. In humans, diversity of intestinal defensin forms might be generated by differential posttranslational processing, a situation also seen with the processing of the human urinary β-defensin HBD-1 [18].

3.2. Human intestinal defensin HD-5 and phospholipase A2 in ileal neobladder urine are Paneth cell associated

In the mucosa of the small intestine, HD-5 is detectable only in Paneth cells [9,14]. Because of recent findings that HD-5 is also expressed in other epithelial cell types in different tissues [38,39] we explored the possibility that this defensin could be produced in the normal or infected urinary tract. We performed Western blots for HD-5 and two other fre-

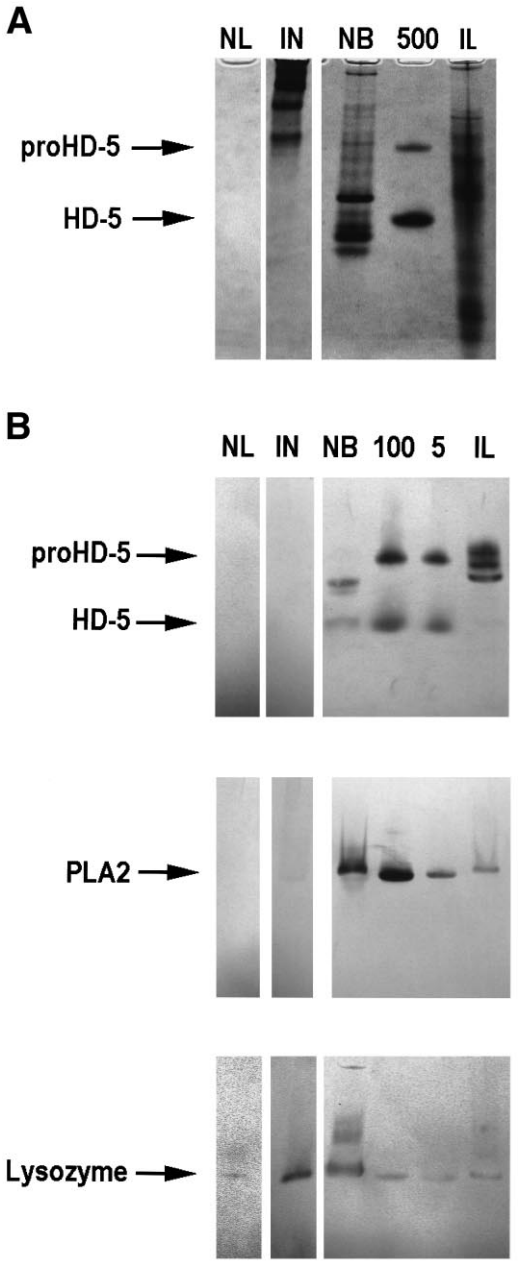

Fig. 2. Detection of human intestinal defensin HD-5, phospholipase A2 and lysozyme in ileal neobladder urine. Cationic peptides from normal urine (NL), infected urine from urinary tract infection (IN), ileal neobladder urine (NB) and ileal mucosa extract (IL) were subjected to AU-PAGE, transferred to PVDF membrane, probed with anti-HD-5, anti-PLA2 and anti-lysozyme antibodies and developed with alkaline phosphatase conjugate. Each lane contained the equivalent of 3 ml of urine or 7.5 mg of mucosa, except for the anti-PLA2 blot where 6 ml urine equivalent and 15 mg mucosa equivalent were loaded. A: Coomassie-stained AU-PAGE with 500 ng recombinant proHD-5/HD-5 as standard. B: Western blot analysis with anti-HD-5 (top), anti-PLA2 (middle) and anti-lysozyme (bottom) and with 100 and 5 ng standard of the respective antigens as indicated by arrows.

quently used Paneth cell markers, PLA2 [16] and lysozyme, on normal urine and urine from patients with urinary tract infection (Fig. 2A), and also compared ileal neobladder urine to urine obtained from same patients before they underwent surgery (data not shown). We detected HD-5 and PLA2 consistently at >10–100 ng/ml in urine obtained from ileal neobladders. In all other urines HD-5 and PLA2 were absent or inconsistently present at the lowest detection limit (<2.5 ng/ml, in agreement with PLA2 concentrations described previously [40] for normal urine). Because we studied most patients in the immediate postoperative period where the newly constructed neobladder is subject to bleeding, admixture of HD5 and PLA2 from blood was possible. However, reported PLA2 serum levels range from 3.7 to 10.8 ng/ml [41,42] which is approximately 10-fold lower than the levels we observed in the ileal neobladder urine and we were not able to detect HD-5 in plasma. Hence, HD-5 and PLA2 in the ileal neobladder urine originate principally from the ileal mucosa of the neobladder and reflect Paneth cell secretion, as these cells are the only source of both HD-5 and PLA2 in the ileum [9,14,16]. In contrast, lysozyme was not restricted to urine from ileal neobladders but was also readily found in normal urine (~ 10 ng/ml) and at increased levels in urine from urinary tract infection (>50 ng/ml) and cannot be used as Paneth cell marker in the ileal neobladder.

3.3. The ileal neobladder is a promising model to study Paneth cell secretion

The ileal neobladder offers many advantages over human tissue extraction as a source of small intestinal secretions, including repeated non-invasive access to the same source and diminished contamination with non-secreted cellular material. As seen on the Coomassie-stained AU-PAGE (Fig. 2B), ileal neobladder urine contains much more protein than normal and UTI urine including possibly hitherto unknown Paneth cell products, but when compared to extracts of ileal mucosa, the neobladder urine is much less complex. Previously, ileal conduit urine was successfully used to purify intestinal mucus glycoprotein [43]. In the ileal neobladder the original blood and nerve supply is maintained [12,13] and measurements of HD-5 or PLA2 concentrations in neobladder urine could also be used to study the physiology of Paneth cell secretion.

The ileal neobladder has been reported to undergo major epithelial remodeling during the first year after surgery, progressing from early changes such as villous atrophy and increase in crypt length to eventual loss of enterocyte differentiation and function, and the development of a flat mucosa with increased goblet cell and mucus production [44–48]. However, since islands of intestinal mucosa are maintained for many years [48,49] the neobladder could remain a useful source of Paneth cell secretions for a long period after surgery.

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