Epithelial Regeneration Ability of Crohn's Disease Assessed Using Patient-Derived Intestinal Organoids

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Research

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Abstract

**Background:** Little is known about the ability of epithelial regeneration and wound healing in patients with inflammatory bowel disease, since the ethical issue of clinical trials and the inherent limitations of tumor-derived epithelial cell lines and animal models. We evaluated the epithelial proliferation and wound healing ability of the patients with Crohn's disease (CD) using the patient-derived intestinal organoids.

**Methods:** Human intestinal organoids were constructed in a three-dimensional intestinal crypt culture of enteroscopic biopsy samples from controls and CD patients. The epithelial regeneration ability of intestinal organoids was assessed using organoid reconstitution, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), 5-ethynyl-2'-deoxyuridine (EdU), and wound healing assays. Tumor necrosis factor-alpha (TNFa) is a major pro-inflammatory effector during the CD pathogenesis. Under the TNFa-enriched conditions, the epithelial regeneration ability of CD patient-derived organoids was compared with that of control organoids.

**Results:** The organoid-forming efficiency of ileal crypts derived from CD patients was reduced compared with those from control subjects ($p < .001$). Long-term cultured organoids ($\geq 6$ passages) derived from controls and CD patients showed inseparable morphologic and cultural features. Under the TNFa-enriched conditions, organoid reconstitution assay and MTT assay revealed that the organoid reconstitution and viability of CD patient-derived organoids were significantly lower than those of control organoids ($p < .05$ for each). The number of EdU+ proliferative cells was significantly lower in TNFα-treated organoids derived from CD patients than in TNFα-treated control organoids ($p < .05$). In the wound healing assay, the unhealed area in TNFα-treated CD patient-derived organoids was significantly larger than that of TNFα-treated control organoids ($p < .001$).

**Conclusions:** Under TNFα-enriched conditions, the wound healing ability of CD patient-derived organoids is reduced due to the reduced cell proliferation compared with that of control organoids. The epithelial regeneration ability may be impaired in patients with CD.

**Background**

The intestinal epithelium, which acts as a frontline defense of the human body, is repetitively injured and continuously regenerated [1]. Epithelial regeneration depends on the self-renewal and proliferation of LGR5+ intestinal stem cells (ISCs), which occur in intestinal crypts [2]. Crohn's disease (CD) is a chronic relapsing-remitting inflammatory bowel disease (IBD), characterized by cycles of mucosal inflammation and ulceration, followed by regeneration and restoration [3]. Impaired epithelial regeneration can lead to sustained intestinal inflammation, and could be accompanied by ulcers and complications, such as fibrosis and fistulas.

Nevertheless, little is known about the ability of epithelial regeneration and wound healing in patients with CD. Clinical trials could not be implemented to evaluate this issue due to ethical reasons and clinical heterogeneity. Classical tumor-derived cell lines and animal model systems have inherent limitations [4].
Immortalized cell lines consist of a homogenous cellular component and evade cellular senescence due to the presence of certain mutations [5]. Animal models are difficult to replicate human-specific biological processes [6]. With the advent of intestinal epithelium-derived organoids, it is possible to cultivate all epithelial cellular components and re-create the functional crypt–villus architecture [7–9]; patient-derived intestinal organoids might be appropriate for studying the regenerative ability of epithelial cells, and might prove to be a useful model for studying human intestinal diseases. This study aimed to evaluate the epithelial regenerative ability of CD patient-derived intestinal organoids, compared to that of control subject-derived intestinal organoids.

Inflammation challenges the epithelial integrity and barrier function. The intestinal epithelium needs to adapt to a multitude of signals in order to perform the complex process of maintenance and restitution of its barrier function [10]. Tumor necrosis factor alpha (TNFα) acts as a major proinflammatory and tissue damage-promoting effector during the pathogenesis of CD; this is supported by evidence provided by studies involving experimental mouse models and the therapeutic effects of TNFα-neutralizing reagents in IBD treatment [11–13]. Recently, mucosal healing has been considered as a therapeutic target, as it improves the prognosis of patients with CD [14]. We should attempt to understand the TNFα-induced alteration of epithelial regenerative ability in CD patient-derived organoids, compared to that of control organoids.

**Methods**

**Sampling**

To establish intestinal organoids, we used human intestinal tissue from controls and patients with CD using biopsy forceps during single-balloon enteroscopy at the Samsung Medical Center, Seoul Korea, between November 2016 and December 2018. At least four biopsy samples were obtained from mucosal tissues in the jejunum (100–150 cm distal of the ligamentum of Treitz), ileum (50–100 cm proximal to the ileocecal valve), and colon (transverse colon). Patients with CD were diagnosed according to the guidelines [15]. In patients with CD, biopsies were performed at least 5 cm away from the ulcers. All samples were acquired with informed consent. This study was approved by the institutional ethical committee of the Samsung Medical Center (IRB No. 2016-02-022).

**Crypt isolation from the biopsy specimens**

Endoscopic biopsy samples were incubated in PBS with 10 mM ethylenediaminetetraacetic acid (Thermo Fischer Scientific, San Jose, CA, USA) and 1 mM dithiothreitol (Thermo Fischer Scientific) at 4°C for 30 minutes, and then vortexed for 30–120 seconds. The supernatant containing crypts was filtered through 70-µm cell strainers (Corning, Bedford, MA, USA) and suspended in the basal medium advanced Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fischer Scientific) supplemented with antibiotic–antimycotic solution (Thermo Fischer Scientific), HEPES (Thermo Fischer Scientific), GlutaMAX (Thermo Fischer Scientific), N2 (Thermo Fischer Scientific), B27 (Thermo Fischer Scientific), and N-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA)).
**Three-dimensional (3D) intestinal crypt culture** (Fig. 1)

Isolated crypts were resuspended in Matrigel (Corning) and plated in 48-well culture plates (Corning). After incubation at 37°C for 15 min, 250 µl of maintenance medium (50% Wnt3a-conditioned medium (ATCC#CRL-2647, Manassas, VA, USA) and 50% of 2× basal medium supplemented with recombinant human EGF (Sigma-Aldrich), recombinant human noggin (R&D Systems, Minneapolis, MN, USA), recombinant human R-spondin1 (PeproTech, Cranbury, NJ, USA), nicotinamide (Sigma-Aldrich), p160ROCK inhibitor (Selleck Chemicals, Houston, TX, USA), p38 MAP kinase inhibitor (SB202190, Sigma-Aldrich), and Prostaglandin E2 (PGE2, Cayman Chemical, Ann Arbor, MI, USA) was added to the wells. A GSK3 inhibitor (Stemgent, Cambridge, MA, USA) was added in the medium during the first 2 days.

Depending on the shape, organoids can be classified into spheroids and enteroids. Spheroids are defined as round- or oval-shaped organoids with a thin wall composed of a single layer of undifferentiated cells. Enteroids are defined by the presence of visually sharp borders (buddings) along their basolateral (antiluminal) side or irregularly thickened walls, which consist of all components of epithelial cells [16].

**Organoid-forming efficiency**

One hundred crypts obtained from control subjects and CD patients were plated in 25 µL of Matrigel in maintenance medium. The organoid-forming efficiency was calculated as the percentage of viable organoids per 100 intestinal crypts.

**Organoid subculture, maintenance, and differentiation**

After 7 days of the culture process, the organoids were mechanically disrupted and suspended in the cell dissociation buffer (Thermo Fischer Scientific). Single cells and small cell clusters were resuspended in Matrigel and plated in 48-well culture plates (Corning). The medium was changed every 2 days and the organoids were passaged at a ratio of 1:2–1:4 on day 7. After 6 passages, most organoids in the maintenance medium formed uniform spheroids that were able to stable subculture for a long time.

To recreate the physiological parameters of the intestinal epithelium, the spheroids were cultured in a differentiation medium (maintenance medium without Wnt3A conditional medium, SB202190, nicotinamide, and PGE2). The differentiation medium was changed every 2 days and enteroids were grown for 7–12 days.

**Organoid reconstitution assay**

After more than six passages, the organoids were mechanically disrupted and suspended in the cell dissociation buffer. Single cells were resuspended in Matrigel and plated in 48-well culture plates. The intestinal organoids were cultured in maintenance medium for 2 days to obtain a stable number of organoids before inducing organoid differentiation; then, the maintenance medium was changed to a differentiation medium every 2 days. Different concentrations of human recombinant TNFα (R&D Systems) were added to the culture medium every 24 hours. The organoid reconstitution rate was
calculated as a percentage of the number of viable organoids on day 9–10 proportional to the number of viable cells on day 2.

3-(4,5-Dimethylthiazolyl-2)-2, 5-Diphenyltetrazolium Bromide (MTT) assay

Ten microliters of MTT (Sigma-Aldrich) were added to each well of the culture plates incubated for 3 h until purple precipitate was visible. After 100 µl of detergent reagent was added, the organoids were incubated at room temperature in the dark for 2 h, prior to recording the absorbance at 570 nm. The optical density (OD) of organoids containing well is expressed in ratio based on the values of TNFα-free control organoids containing well.

5-Ethynyl-2′-Deoxyuridine (EdU) assay

Two hundred micrograms of EdU (Abcam, Cambridge, UK) were added to the culture medium 2 h before fixation with cold 4% paraformaldehyde (Biosesang. Seongnam-si, South Korea). Incorporation of EdU into DNA was detected using the Click-iT™ EdU Alexa Fluor® 488 imaging kit (Thermo Fischer Scientific). The number of EdU (+) cells was measured in 10 organoids (size > 100 µm) selected from TNFα-free and -treated enteroids derived from the controls and CD patients.

Wound Healing Assay

3D cultured organoids were digested into single cells using TrypLE Express (Thermo Fischer Scientific) and 5 × 10^4 cells were seeded into 24-well plates containing CytoSelect™ 24-Well Wound Healing Assay inserts (Cell Biolabs, San Diego, CA, USA) [17]. Organoid monolayers were cultured in maintenance medium until confluence was reached. The inserts were then carefully removed to produce 0.9 mm diameter wounds and fresh differentiating medium was added to each well. The area of the un-healed wound was measured in three different areas. The area of the un-healed wound is expressed in %, based on the observed area of TNFα-free control organoids.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

One-step qPCR was performed using One Step PrimeScript™ III RT-qPCR Mix (Takara, Kusatsu, Japan) with primers as follows; LGR5 primer (Forward: 5’-aactttgcattgtggaagg-3’, Reverse: 5’-acacattggggttaggaaca-3’), BMI1 primer (Forward: 5’-cgtgtattgttctctctatggtgcagga-3’, Reverse: 5’-ttcagtagtggctgctgtttg-3’), ATOH1 primer (Forward: 5’-cagctgcatgacttccccc-3’, Reverse: 5’-tttagcagcctggacaagga-3’), and HES1 primer (Forward: 5’-ttcttcctatctcaccggg-3’, Reverse: 5’-ctggaaggtgacactgttc-3’).

RNA sequencing

RNA sequencing was conducted on endoscopic biopsy tissue samples from controls and patients with CD and organoids derived from these samples. Total RNA was extracted from intestinal organoids using
the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was isolated from endoscopic biopsy tissue samples from controls (n = 2) and patients with CD (n = 2, non-inflamed area, during infliximab maintenance treatment). In addition, organoid culture was conducted with intestinal crypts derived from these specimens. Organoids were sub-cultured at least 6 passages. RNA was isolated from the organoids on day 3, day 6, and day 9, and organoids treated with TNF\(^\text{30 ng/ml}\) on day 3, day 6, and day 9. RNA sequencing was performed using total RNA samples with > 10 µg of RNA and an integrity number > 8. The libraries were constructed for whole-transcriptome sequencing using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) and sequenced using the 100-bp paired-end mode of the TruSeq Rapid PE Cluster Kit and the TruSeq Rapid SBS Kit (Illumina).

Reads from files in the FASTQ format were mapped to the hg19 human reference genome using TopHat version 2.0.6, with default parameters (http://tophat.cbcb.umd.edu/). Raw read counts mapped to genes were measured using the BAM format file in HTSeq version 0.6.0 (https://htseq.readthedocs.io/), to quantify transcript abundance. The coding genes were selected, and raw read counts were normalized to trimmed mean of M-values. Differential expression analysis of RNA-seq experiments was conducted with edgeR (version 3.28.1). Unsupervised hierarchical clustering analysis with the Euclidean distance and complete linkage algorithm was used to create a heatmap with the associated dendrogram.

**Availability of Data and Materials**

The datasets presented in this study can be found in GEO (accession number = GSE173294, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173294)

**Results**

Intestinal crypts isolated from duodenal (CD, n = 5; controls, n = 5), jejunal (CD, n = 9; controls, n = 13), ileal (CD, n = 43; controls, n = 15), and colonic biopsy samples (CD, n = 12; controls, n = 8) from controls (n = 34) and patients with CD (n = 51) were cultured in Matrigel with maintenance medium and then organoid-forming efficiency was evaluated. Patient characteristics for enrolled patients are listed in Table 1. In both groups, the organoid-forming efficiency of duodenal crypts was the highest, followed by jejunal, ileal, and colonic crypts. The organoid-forming efficiency of the ileal crypts obtained from patients with CD was significantly lower than that of ileal crypts obtained from controls on day 3 (53.4% ± 4.0% vs. 39.6% ± 2.4%, \(p = 0.005\)), day 5 (38.9% ± 3.0% vs. 21.1% ± 1.7%, \(p < 0.001\)), and day 7 (29.2% ± 2.8% vs. 15.7% ± 1.5%, \(p < 0.001\)). The organoid-forming efficiency on day 7 of jejunal crypts obtained from CD patients was numerically lower than that of those obtained from controls (46.8 ± 2.6% vs. 38.1% ± 3.8%, Fig. 2).
Table 1
Characteristics of enrolled patients and number of attempts to construct organoids.

| Total number of enrolled patients | 85 | Number of attempts to construct organoids | 110* |
|----------------------------------|----|------------------------------------------|------|
| Patients with CD (n)             | 51 | Number of sub-culture (≥ passage 3) enabled organoids | 61   |
| Age, years (mean ± S.D)          | 36.2 ± 13.4 | CD-derived organoids (n) | 69   |
| Male (n)                         | 43 | Duodenal organoids (n of attempts / n of subculture) | 5 / 4|
| Montreal classification (n)      |     | Mucosal healing / no duodenal involvement | 5 / 4|
| Age at diagnosis (A1/A2/A3)      | 0 / 42 / 9 | Active ulcer | 0 / 0|
| Location (L1/L2/L3)              | 1 / 34 / 16 | Jejunal organoids (n of attempts / n of subculture) | 9 / 8|
| Behavior (B1/B2/B3)              | 20 / 27 / 4 | Mucosal healing / no jejunal involvement | 4 / 4|
| Medication at sampling           |     | Active ulcer | 5 / 4|
| None (sampling before treatment), (n) | 6 | Ileal organoids (n of attempts / n of subculture) | 43 / 23|
| Immunomodulator user, (n)        | 25 | Mucosal healing / no ileal involvement | 8 / 5|
| Anti-TNFα user, (n)              | 25 | Active ulcer | 35 / 18|
| Sampling modality                |     | Colonic organoids (n of attempts / n of subculture) | 12 / 2|
| Colonoscopy                      | 11 | Mucosal healing / no colonic involvement | 4 / 1|
| Single-balloon enteroscopy (n)   | 40 | Active ulcer | 8 / 1|
| Controls (n)                     | 34 | Control-derived organoids† | 41 / 24|
| Age, years (mean ± S.D)          | 50.6 ± 17.9 | Duodenal organoids (n of attempts / n of subculture) | 5 / 4|
| Male sex (n)                     | 24 | Jejunal organoids (n of attempts / n of subculture) | 13 / 11|
|                                  |     | Ileal organoids (n of attempts / n of subculture) | 15 / 8|

*patients having samples from two (n = 21) and three (n = 2) different sites.
| Total number of enrolled patients | 85 | Number of attempts to construct organoids | 110* |
|----------------------------------|----|------------------------------------------|------|
|                                  |    | Colonic organoids (n of attempts / n of subculture) | 8 / 1 |

*patients having samples from two (n = 21) and three (n = 2) different sites.

Organoids grown from intestinal crypts were sub-cultured in maintenance medium and the control organoids became uniformly spheroid (>90% of total organoids) after 2–4 passages. However, organoids derived from patients with CD had both enteroid and spheroid forms in the early passages and became uniformly spheroids after 4–6 passages. After six passages, the organoids derived from the controls and patients with CD exhibited consistent spheroid features and culturing behaviors (Fig. 3). The shapes of organoids cultured in the maintenance medium were similar regardless of their location; however, those cultured in differentiation medium tended to have different shapes, depending on their origin. The murine enteroids tended to have more budding structure compared to human enteroids. Ileal organoids typically exhibited budding, whereas jejunal organoids formed thick-walled structures (Fig. 4).

Previous studies have identified the cytotoxicity of intestinal organoids occurred in a concentration-dependent manner in response to TNFα [18–20]. To address the appropriate concentration of TNFα and the interval of administration to assess the epithelial regenerative ability, control organoids were tested for the changes in expression of the ISC and progenitor marker after TNFα treatment and organoid survival at the various concentrations of TNFα. The organoid viability—measured using MTT and the enteroid/spheroid ratio—decreased significantly with a gradual increase in the TNFα concentration. At TNFα concentrations of ≤ 10 ng/ml in the differentiation medium, changes in cell viability and morphology were negligible; however, the changes observed at TNFα concentrations of ≥ 30 ng/ml were notable (Supplementary Figures S1). The expression of LGR5 (active ISC), BMI1 (reserve ISC), HES1 (absorptive progenitor), and ATOH1 (secretory progenitor) increased within 24 h after TNFα treatment and then decreased (Fig. 5).

Based on these results, organoids derived from controls and CD patients—cultured over the long-term (≥ 6 passages)—were treated with 30 ng/ml TNFα every 24 hours for 10 days (Fig. 6A). The epithelial regenerative ability of intestinal organoids was evaluated using organoid reconstitution, MTT, EdU, and wound healing assays.

The organoid reconstitution rate of TNFα-treated organoids was significantly lower than that of TNFα-free organoids (jejunal organoids: 68.9% ± 12.4% vs. 47.4% ± 13.6%, p < 0.001; ileal organoids: 55.2% ± 12.3% vs. 32.8% ± 10.1%, p < 0.001). There was no significant difference in the organoid reconstitution rate between TNFα-free controls and CD patient-derived organoids; however, the organoid reconstitution rate of TNFα-treated CD patient-derived organoids was significantly lower than that of TNFα-treated control organoids (jejunal organoids: 55.5% ± 11.5% vs. 39.3% ± 10.6%, p = 0.011; ileal organoids: 40.2% ± 6.9% vs. 25.3% ± 6.6 %, p = 0.027; Figs. 6B and 6C).
The organoid viability was assessed using MTT; results showed that the formazan absorbance values of viable TNFα-treated organoids were significantly lower than those of TNFα-free organoids (OD of jejunal organoids: 0.96 ± 0.16 vs. 0.71 ± 0.12, p < 0.001; OD of ileal organoids: 0.83 ± 0.16 vs. 0.56 ± 0.16, p < 0.001). In the TNFα-enriched condition, the viable cells of jejunal and ileal CD patient-derived organoids was significantly decreased compared with those of the control organoids (OD of jejunal organoids: 0.79 ± 0.10 vs. 0.62 ± 0.07, p = 0.135; OD of ileal organoids: 0.66 ± 0.13 vs. 0.45 ± 0.10, p = 0.019; Fig. 7).

Two hours after EdU administration, EdU+ cells were confirmed in the buds, which represented the intestinal crypt (Supplementary Figure S2). The number of EdU+ cells was higher in TNFα-free organoids than in TNFα-treated organoids (91.9 ± 32.6 vs. 32.3 ± 22.2, p < 0.001). Although there was no significant difference in the number of EdU+ cells between the control and CD patient-derived organoids in the steady state (82.7 ± 33.1 vs. 102.4 ± 30.5, p = 0.653), the number of EdU+ cells were significantly lower in TNFα-treated CD patient-derived organoids than in TNFα-treated control organoids (49.0 ± 19.1 vs. 15.6 ± 7.7, p = 0.001, Fig. 8).

The wound healing assay showed that the unhealed wound area in TNFα-treated CD patient-derived organoids was significantly larger than that in control organoids at 8 h (50.5% ± 10.5% vs. 84.7% ± 12.3%, p < 0.001), 16 h (11.0% ± 4.8% vs. 64.3% ± 14.0%, p < 0.001), and 24 h after insert removal (1.7% ± 1.5% vs. 37.0% ± 8.5%, p < 0.001). There was no significant difference in the wound healing between the organoid derived from controls and CD patients. The unhealed wound area at 24 h was not significantly different between the TNFα-free and TNFα-treated control organoids (1.7% ± 1.5% vs. 16.0% ± 4.6%, p = 0.257), however showed significant difference between the TNFα-free and TNFα-treated CD patient-derived organoids (2.3% ± 2.5% vs. 37.0% ± 8.5%, p < 0.001). In addition, the unhealed wound area at 24 h of TNFα-treated CD patient-derived organoids was significantly larger than that in TNFα-treated control organoids at 24 h after insert removal (16.0% ± 4.6% vs. 37.0% ± 8.5%, p = 0.044). The wound-healing ability of TNFα-treated CD patient-derived organoids was significantly lower than that of TNFα-free control and CD patient-derived organoids, and TNFα-treated control organoids (Fig. 9).

RNA-seq was performed on endoscopic biopsy tissue samples from controls and patients with CD and organoids derived from these samples. The clustering heatmap and principal component analysis identified that the gene expression profile was clearly separated between the endoscopic biopsy tissue samples and the organoids derived from these samples. Furthermore, among the gene expression profile of organoids, there was a clear distinction between TNFα-free and -treated organoids (Figs. 10A and 10B). The epithelial lineage-specific gene expression was evaluated in the tissue samples and organoids derived from these samples (Fig. 10C). In endoscopic biopsy tissue samples, the expression of genes associated with the intestinal microbiota (NOD2, DEFA5, DEFA6, PLA2G2A, MUC2, and NARP6) and differentiated cells [enterocytes (ECs): SI, APOC3, ALPI, and APOA1; goblet cells (GCs): MUC2; Paneth cells (PCs): WNT3, ARG2, DLL1, and DLL4; and enteroendocrine cells (EECs): CCK, CHGA, CHGB, and NEUROG3)] was increased in comparison to organoids. The expression of EC markers such as VIL1, KLF5, and KRT5 and GC markers such as TFF3 and MUC13 were up-regulated in biopsy tissue samples and TNFα-free control organoids (day 6 and 9 organoids). In the TNFα-treated control organoids, the
expression of ISC markers, such as LGR5, OLFM4, and TNFRSF19, was increased compared with those of TNFα-treated CD patient-derived organoids. The alterations of ISC properties can be affect cell proliferation and wound healing ability

**Discussion**

Rapid restoration of epithelial defects following injuries or physiologic damage is indispensable for maintaining gut homeostasis [10]. CD is characterized by chronic and transmural inflammation that can occur along the entire GI tract, but primarily occurs in the small intestine. Nevertheless, the ability of epithelial regeneration and wound healing in patients with CD have not been evaluated, especially by the location of the gastrointestinal tract. To our knowledge, this study is the first to evaluate organoid-forming efficiency using patient-derived organoids in patients with CD according to the location of GI tract.

Several previous observations suggested that epithelial regeneration and wound healing capacity was impaired in patients with CD. Patients with CD have a higher rate of postoperative complication, in particular anastomotic complications leading to intra-abdominal sepsis, than patients without inflammatory status [23]. Exposure to ionizing radiation causes cell death of rapidly proliferating cells, leading to acute or chronic gastrointestinal (GI) toxicity in a dose- and time-dependent manner [24]. Patients with CD presented an increased risk of GI toxicity following exposure to therapeutic doses of ionizing radiation [25]. The mutation of CD susceptibility genes, such as NOD2 and ATG16L1, has been associated with ISC dysfunction, leading to impaired epithelial regeneration and wound healing [26, 27]. IBD patient-derived colonoids showed decreased organoid size and number of budding, increased cell death and luminal debris, and inverted polarization [21]. However, the epithelial regeneration ability of CD patients was not directly evaluated in these previous studies, whereas our study evaluated epithelial regeneration capability in a detailed and direct manner.

This study identified that the organoid-formation ability of CD patient-derived ileal crypts was significantly impaired; this was correlated with the fact that the ileum was the most frequently affected area in CD [22]. These ileal crypts were endoscopically obtained from the uninflamed intestine; however, the effect of pre-existing microscopic inflammation on organoid formation cannot be completely excluded. Long-term cultured CD patient-derived organoids were passaged under non-inflammatory culture conditions and showed no significant differences in the morphology and culturing behaviors compared to control organoids, which can mimic the mucosal healing in patients with IBD. Mucosal healing is considered to be an ideal therapeutic target for the long-term remission of IBD [3]. However, while patients achieve mucosal healing, several triggers such as inflammatory cytokines and nonsteroidal anti-inflammatory drugs can cause mucosal damage and inflammation. In this study, TNFα was used as a trigger to mimic the inflammatory milieu. TNFα reduced the cell viability and organoid reconstitution ability of intestinal organoids in a dose-dependent manner. We identified TNF 30ng/ml as an appropriate concentration for the experiment because the cells were not dying too much and presented morphological changes. Previous studies have also suggested that 30 ng/ml of TNFα may
induce cytotoxicity and distinctive cell response in intestinal organoids [18, 20]. In a culture condition with 30 ng/ml of TNFα, both jejunal and ileal organoids derived from CD patients showed the reduced organoid reconstitution ability, cell viability, cell proliferation, and wound healing capability compared with control organoids.

Previous studies have shown that wound healing is accomplished through epithelial restitution, ISC proliferation, and differentiation, although these wound healing processes overlap [23–25]. This study evaluated the epithelial restitution using wound healing assays and achieving organoid cell proliferation using organoid reconstitution, MTT, and the EdU assay. Post-injury wound healing is regulated by a wide range of regulatory factors, including cytokines, growth factors, adhesion molecules, and phospholipids [10, 26]. Inflammatory processes thought to especially interfere with epithelial cell migration and proliferation, and thus modulate intestinal epithelial healing [27]. However, in this study, RNA-seq identified that the expression of active ISC markers such as LGR5, OLFM4, and TNFRSF19 was decreased in CD patient-derived organoids compared with the control organoids under TNFα-enriched condition. Under TNFα-enriched culture conditions, the ISC population may be expanded for reparative proliferation to replace lost cells with TNFα-induced cytotoxicity. However, alterations of ISC properties in organoids derived from CD patient in response to TNFα may affect ISC proliferation and subsequently wound healing ability can be impaired. Previous single cell RNA-seq also identified the alterations of ISC properties in CD patient-derived small intestinal organoids [28], which can support our RNA-seq results. Based on our results, inflammatory processes can particularly interfere with ISC proliferation, and therefore modulate intestinal epithelial wound healing.

The limitation of our study is that the organoid culture system does not reflect the effects of intestinal microbiota, dietary components, and the mucosal immune system. The intestinal microbiota and dietary components contribute to the fine-tuning of ISC survival and differentiation [29]. In addition, only ileal crypts of CD showed a significant reduction in the organoid-formation efficiency compared with those of controls. Because enteroscopy was usually performed for the diagnosis and treatment of CD, ileal sampling was relatively convenient and small sample size of duodenal and jejunal was small. The lack of significance of duodenal, jejunal, and colonic organoid reconstitution rate might be attributed to small sample size.

In conclusion, organoid-forming efficiency of ileal crypt in patients with CD was reduced compared with those of control subjects. When TNFα was used as a trigger to mimic the inflammatory milieu, the epithelial regeneration ability of intestinal organoids was more remarkable in CD patient-derived organoids, compared to that in control organoids. The clinical trials are disabled to settle this issue due to ethical reasons and clinical heterogeneity, our results indicated that the epithelial regenerative ability is impaired in patients with CD, especially in TNFα-enriched conditions. Our findings suggest the underlying unexplained mechanism for the CD that impaired epithelial regeneration results in the defective mucosal integrity and sustained intestinal inflammation, leading to ulcers, fibrosis, and fistulas, which are the main indications for surgery in patients with CD. To improve mucosal healing in patients with CD, additional
treatment option to promote the epithelial regeneration and wound healing ability should be explored and
developed.

**Abbreviations**

ISC, intestinal stem cell

CD, Crohn's disease

IBD, inflammatory bowel disease

TNF α, Tumor necrosis factor alpha

3D, Three-dimensional

MTT, 3-(4,5-Dimethylthiazolyl-2)-2, 5-Diphenyltetrazolium Bromide

OD, optical density

EdU, 5-Ethynyl-2'-Deoxyuridine ()

qPCR, Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

EC, enterocyte

GC, goblet cell

PC, Paneth cell

EEC, enteroendocrine cell

GI, Gastrointestinal

**Declarations**

- **Ethics approval and consent to participate**: This study was approved by the institutional ethical committee of the Samsung Medical Center (IRB No. 2016-02-022).

- **Consent for publication**: Not applicable

- **Availability of data and material**: The datasets presented in this study can be found in GEO (accession number = GSE173294, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173294)

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**Authors' contributions** Conceptualization, C.L. and S.N.H.; Methodology, C.L. and S.N.H.; Validation, C.L. and S.N.H.; Investigation, C.L., E.R.K., D.K.C., Y.H.K., and S.N.H.; Resources, E.R.K., D.K.C., Y.H.K., and S.N.H.; Data Curation: C.L. and S.N.H.; Writing - Original Draft, C.L. and S.N.H.; Writing - Review & Editing, E.R.K., D.K.C., Y.H.K., and S.N.H.; Supervision, Y.H.K.; Project Administration, S.N.H; Funding Acquisition, C.L. and S.N.H.

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Figures

Figure 1
Flow diagram of the patient-derived intestinal organoid model. Scale bar = 100 μm.
Figure 2

Organoid-forming efficiency. Intestinal crypts were isolated from the duodenum (n=5), jejunum (n=9), ileum (n=43), and colon (n=12) of patients with CD and the duodenum (n=5), jejunum (n=13), ileum (n=15), and colon (n=8) of controls. Differences in organoid-forming efficiency between controls and CD patients based on the location of GI tract were assessed by t-test; Scale bar = 200 μm.
Figure 3

Long-term culture of control and CD patient-derived organoids. Bright field and H&E staining images of control and CD patient-derived organoids according to the passages. After six passages, the morphology of control and CD patient-derived organoids became identical. Scale bar = 200 µm.

Figure 4

Microscopic appearance of jejunal, ileal, and colonic organoids cultured in differentiation medium. The control organoids cultured in the maintenance medium formed spheroids, while those cultured in the differentiation medium formed enteroids. Jejunal organoids had budding structures, while ileal and colonial organoids formed thick-walled structures.
Changes in the expression of LGR5, BMI1, ATOH1, and HES1 in control organoids at 6, 12, 24, and 48 h after treatment with TNFα (30 ng/mL). Quantitative reverse transcription polymerase chain reaction was performed with control organoids (n = 3) in triplicate.
Organoid reconstitution assay in tumor necrosis factor-alpha (TNF-α) enriched condition. (A) Study flow diagram. (B) Organoid reconstitution assay of jejunal and ileal organoids derived from controls and CD patients (n=5 each) in TNF-α-enriched condition. Assays were conducted in triplicate. (C) Organoid reconstitution rate. Reconstituted organoid number is expressed as a percentage value, based on values of TNF-α-free jejunal organoids derived controls. Differences were evaluated using ANOVA with Bonferroni's multiple comparison test; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 7

MTT assay for organoid viability in TNF-α-enriched condition. MTT assay were performed in triplicate using jejunal and ileal organoids derived from controls and CD patients (n=3 each). The optical density (OD) of organoids containing well is expressed in ratio based on the values of TNFα-free control organoids containing well. Differences were evaluated using ANOVA with Bonferroni's multiple comparison test; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Figure 8

EdU assay for organoid proliferation in TNF-α-enriched condition EdU+ cell number was measured 2 h after EdU administration into 10 organoids from TNF-α-treated and -free control and CD patient-derived organoids (n=3 each). Differences were evaluated using ANOVA and the Bonferroni’s multiple comparison test; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 9
Wound healing assay. Non-healing wound areas in three different areas selected from TNFα-free and -treated organoids derived from controls and CD patients were measured (n=3 each). The area of the unhealed wound is expressed in %, based on the observed area of TNFα-free control organoids. Differences were evaluated using the two-way ANOVA test with Bonferroni’s multiple comparison test; ***p < 0.001, and ***p < 0.0001. Scale bar = 4 mm.

Figure 10

Gene expression profile of the endoscopic biopsy tissue samples from controls and patients with CD and organoids derived from these samples. (A) Clustering heatmap and (B) principal component analysis. The gene expression profile was clearly separated between the endoscopic biopsy tissue samples and the organoids derived from these samples. Among the gene expression profile of organoids, clear distinction was noticed between TNFα-free and -treated organoids. (C) Epithelial lineage-specific gene expression. The expression of differentiated cell markers was increased in endoscopic tissue biopsy samples. However, the expression enterocyte markers, such as VIL1, KLF5, and KRT5, and goblet cell markers, such as TFF3 and MUC13, were increased in the biopsy tissue samples as well as the TNFα-free organoids. In
the TNFα-treated control organoids, the expression of ISC markers, such as LGR5, OLFM4, and TNFRSF19, was increased compared with those of TNFα-treated CD patient-derived organoids.

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