

Basic Study

Association of hepatocyte-derived growth factor receptor/ caudal type homeobox 2 co-expression with mucosal regeneration in active ulcerative colitis

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Abstract

AIM: To characterize the regeneration-associated stem cell-related phenotype of hepatocyte-derived growth factor receptor (HGFR)-expressing cells in active ulcerative colitis (UC).

METHODS: On the whole 38 peripheral blood samples and 38 colonic biopsy samples from 18 patients with histologically proven active UC and 20 healthy control subjects were collected. After preparing tissue microarrays and blood smears HGFR, caudal type homeobox 2 (CDX2), prominin-1 (CD133) and Musashi-1 conventional and double fluorescent immunolabelings were performed. Immunostained samples were digitalized using high-resolution Mirax Desk instrument, and analyzed with the Mirax TMA Module software. For semiquantitative counting of immunopositive lamina propria (LP) cells 5 fields of view were counted at magnification $\times 200$ in each sample core, then mean \pm SD were determined. In case of peripheral blood smears, 30 fields of view with 100 μ m diameter were evaluated in every sample and the number of immunopositive cells (mean \pm SD) was determined. Using 337 nm UVA Laser MicroDissection system at least 5000 subepithelial cells from the lamina propria were collected. Gene expression analysis of HGFR, CDX2, CD133, leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), Musashi-1 and cytokeratin 20 (CK20) were performed in both laser-microdissected

samples and blood samples by using real time reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: By performing conventional and double fluorescent immunolabelings confirmed by RT-PCR, higher number of HGFR (blood: 6.7 ± 1.22 vs 38.5 ± 3.18 ; LP: 2.25 ± 0.85 vs 9.22 ± 0.65 ; $P < 0.05$), CDX2 (blood: 0 vs 0.94 ± 0.64 ; LP: 0.75 ± 0.55 vs 2.11 ± 0.75 ; $P < 0.05$), CD133 (blood: 1.1 ± 0.72 vs 8.3 ± 1.08 ; LP: 11.1 ± 0.85 vs 26.28 ± 1.71 ; $P < 0.05$) and Musashi-1 (blood and LP: 0 vs scattered) positive cells were detected in blood and lamina propria of UC samples as compared to controls. HGFR/CDX2 (blood: 0 vs 1 ± 0.59 ; LP: 0.8 ± 0.69 vs 2.06 ± 0.72 , $P < 0.05$) and Musashi-1/CDX2 (blood and LP: 0 vs scattered) co-expressions were found in blood and lamina propria of UC samples. HGFR/CD133 and CD133/CDX2 co-expressions appeared only in UC lamina propria samples. CDX2, Lgr5 and Musashi-1 expressions in UC blood samples were not accompanied by CK20 mRNA expression.

CONCLUSION: In active UC, a portion of circulating HGFR-expressing cells are committed to the epithelial lineage, and may participate in mucosal regeneration by undergoing mesenchymal-to-epithelial transition.

Key words: Hepatocyte-derived growth factor receptor; Caudal type homeobox 2; CD133; Musashi-1; Leucine-rich repeat-containing G-protein coupled receptor 5; Ulcerative colitis; Regeneration

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Core tip: HGFR⁺ cells in the circulation and lamina propria of active ulcerative colitis (UC) could also co-express caudal type homeobox 2 (CDX2), an epithelial stem cell marker, thus suggesting that HGFR⁺ cells have committed to the epithelial lineage. The presence of CD133/CDX2 and Musashi-1/CDX2 double positive cells in the subepithelial layer supports that mesenchymal-to-epithelial transition might be a crucial event in tissue regeneration of active UC.

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INTRODUCTION

The luminal border of the colonic wall is lined by an epithelial monolayer, which has several physiological functions including water- and electrolyte absorption, and barrier defence against luminal pathogens. Due

to the high turnover of shedding epithelial cells their continuous replacement is required from a local stem cell pool even in the healthy colon. Stem cells are located at the crypt base, and their progenies migrate towards the luminal surface where they undergo terminal differentiation to secretory (Paneth, enteroendocrine, and goblet cells) or absorptive (epithelial) cells^[1-3]. When tissue injury occurs, like in inflammatory bowel disease (IBD) or graft-versus host-disease, capacity of the local intestinal stem cell niche is not sufficient enough for a proper tissue repair^[1,4,5]. The classic concept of tissue repair holds that upon entering the damaged tissues inflammatory cells signal resident tissue-specific progenitor cells for mitosis^[1,4,6]. However, mesenchymal stem cells can also contribute to tissue repair after their mobilization, migration, and engraftment of the damaged area enhanced definitely by inflammation^[6,7]. In addition, circulating immature stem cells seem to participate in regeneration of several different tissues as well^[1,4-7].

Recently we have found that during severe inflammatory damage of the colon intraepithelial CD45⁺ marrow-derived cells within the colonic lymphoid aggregates (LA) contribute to epithelial regeneration^[8]. It has also been observed that LAs may determine the migration route of stem cells as well^[8]. Furthermore, in ulcerative colitis (UC) we have found hepatocyte-derived growth factor receptor (HGFR) positive subepithelial cells within the LA to be involved in the induction of mucosal repair^[9]. The presence of the homeobox gene CDX2 and cytokeratin (CK) positive cells detected in LAs suggested that mesenchymal-to-epithelial (MET) transition is located to LAs^[9]. Additionally, an elevated number of HGFR⁺ peripheral blood cells were observed in severely active UC^[9], however their importance and function have not yet been investigated.

In case of HGFR⁺ cells their possible origin and the route of migration in point of the blood stream and the lamina propria (LP) has not yet been elucidated. It is also unclear, whether these cells are committed or not to the epithelial lineage. For better understanding the role of HGFR⁺ cells in mucosal regeneration, in the present immunocytochemical study we assayed the injury-associated stem cell-related phenotype of HGFR⁺ cells in peripheral blood and colonic tissue of patients with active UC and compared to that of controls.

MATERIALS AND METHODS

Patients, subjects and samples

After informed consent, precolonoscopic blood samples and colonic biopsy samples were taken from Caucasian patients complaining on abdominal pain or cramps, frequent stool, bloody diarrhea, and fever. Two times 6 mL of peripheral blood were taken and collected into Vacutainer tubes containing EDTA (BD Bioscience) and 9 mL into Paxgene Blood RNA Tubes (Qiagen). The first 6 mL blood was discarded

to avoid skin epithelial cell contamination. The second 6 mL blood was stored at 4 °C, while the Paxgene tubes at -20 °C. Biopsy samples were collected for routine histological evaluations and for RNA isolation. On the whole 38 peripheral blood samples and 38 tissue samples from 18 patients with histologically proven active UC (male: 10; female: 8; average age: 30.5 years) and 20 healthy control subjects (male: 10; female: 10; average age 35.6 years) were investigated. The diagnosis of UC in an active stage was based on clinical symptoms (> 6 bloody stools per day, presence/absence of fever, tachycardia, abdominal pain), conventional laboratory abnormalities (elevated erythrocyte sedimentation rate and CRP, thrombocytosis, mild/moderate anemia, presence/absence of hypokalemia and/or hypomagnesemia, negative stool culture), and the result of colonoscopy. In active UC the average Mayo severity index score of patients was 11.61. None of the patients had been given corticosteroids, antibiotics or immunosuppressive treatment prior to taking samples. Control samples were collected from subjects with negative endoscopy and normal histology who underwent colonoscopy because of a positive fecal occult blood test (FOBT). The FOBT has been performed for screening purposes. In the healthy control group, apart from internal hemorrhoids, there were no pathological colonic alterations. The percentage of left/right-colon-sided biopsies was 90%/10%, respectively in normal, and 100%/0% in UC tissue samples. None of the patients participated in this study suffered from any other forms of inflammatory or tumorous diseases. For real-time RT-PCR validation, the biopsy samples collected were immediately snap frozen in Tissue-Tek OCT compound medium (Ted Pella Inc., CA, United States) at -80 °C.

Tissue microarray and blood smear preparation, immunolabelings and sample digitizing

Cores of 1 mm diameter were collected from selected areas of formalin fixed, paraffin embedded (FFPE) tissue blocks made from 18 UC and 20 normal colon samples of 38 patients by repeating each sample at least once, and were then placed into 80 samples recipient block. Tissue sections of 4 µm thickness were cut from the TMA blocks, mounted on adhesive glass slides and immunostained following endogenous hydrogen peroxidase blocking (0.5% H₂O₂-methanol) and heat induced epitope retrieval in 150 mL of pH 6.0 TRS buffer (Target Retrieval Solution, S1699; in case of anti-Met, anti-CDX2 clone ZC007, and anti-CD133 antibodies) or pH 8.0 1 mmol/L EDTA buffer (in case of anti-CDX2 clone AMT28 antibody) using a commercial microwave oven at 300 W power for 45 min.

Peripheral blood smears of all UC and control EDTA blood samples were performed. The blood smears were fixed in acetone at -20 °C for 5 min, and stored at -20 °C until immunostainings.

TMA slides and blood smears were incubated with rabbit polyclonal anti-Met culture supernatant antibody

(Santa Cruz Biotechnology Inc.; Clone: C-12; 1:100 dilution in PBS), monoclonal mouse anti-human CDX2 antibodies (Clone: AMT28; BioSystems; 1:100 dilution in PBS; Clone: ZC007; Invitrogen; 5 µg/mL working concentration, diluted in PBS) and monoclonal anti-human CD133/1-biotin antibody (Clone: AC133; Miltenyi; 1:100 dilution in PBS) at 37 °C for 60 min. After rinsing 3 times with PBS, samples incubated with anti-Met antibody were finally treated with an antirabbit EnVision polymerHRP conjugate kit (K4003, DAKO) for 40 min. Secondary immunodetection in the cases of samples incubated with anti-CDX2 and anti-CD133 antibodies was performed with EnVision System Labelled Polymer-HRP K4001 (Anti-Mouse 1/1; DAKO), as described in the manual. Signal conversion was carried out with Liquid DAB+Substrate Chromogen System (DAKO). After the final rinsing in PBS, hematoxylin co-staining was performed. Cores from normal human smooth muscle from colonic muscularis propria were used as negative controls.

TMA slides and blood smears were then digitalized using high-resolution MIRAX DESK instrument (Zeiss, Gottingen, Germany), and analyzed with the MIRAX TMA Module software (Zeiss).

Multiple immunofluorescent labelings

For the detection of co-expression of the assayed proteins HGFR/CDX2, HGFR/CD133, CD133/CDX2 and Musashi-1/CDX2 double immunofluorescent labelings were performed.

HGFR/CDX2, HGFR/CD133, Musashi-1/CDX2 labelings:

TMA slides and peripheral blood smears were covered with rabbit polyclonal anti-Met culture supernatant antibody (Santa Cruz Biotechnology Inc.; Clone: C-12; 1:100 dilution in PBS) or anti-Musashi-1 monoclonal antibody (EP1302, Abcam, 1:100 dilution in PBS) at 37 °C for 60 min. After rinsing them thrice in PBS, diluted goat polyclonal anti-Rabbit IgG Antibody, biotin-SP conjugate (Millipore Merck; Clone: AP132B; 1:500 dilution in PBS) was added to each sample, and they were incubated at 37 °C for 30 min. After washing them thrice in PBS, samples were covered with diluted Texas Red Streptavidin (1 µL streptavidin in 100 µL of PBS; Jackson Immuno Research Laboratories Inc.) and were also incubated at 37 °C for 30 min. Then samples were incubated with monoclonal mouse anti-human CDX2 antibody (Clone: AMT28; BioSystems; 1:100 dilution in PBS) or monoclonal anti-human CD133/1-biotin antibody (Clone: AC133; Miltenyi; 1:100 dilution in PBS) at 37 °C for 60 min. After rinsing them thrice in PBS, samples were covered with 100x diluted FITC-labeled anti-mouse IgG antibody (Sigma-Aldrich), and were incubated at 37 °C for 30 min. After PBS rinsing, samples were covered with antifading VectaShield (Vector Laboratories Inc.) and coverslips.

CD133/CDX2 labeling: TMA slides and peripheral blood smears were covered with monoclonal anti-human CD133/1-biotin antibody (Clone: AC133;

Miltenyi; 1:100 dilution in PBS) at 37 °C for 60 min. After rinsing them thrice in PBS, diluted Biotin-SP-Conjugated Affinipure Goat anti-mouse IgG (1 µL biotin in 100 µL of PBS; Jackson Immuno Research Laboratories Inc.) was added to each sample, and they were incubated at 37 °C for 30 min. After rinsing them thrice in PBS, samples were covered with diluted Texas Red Streptavidin (1 µL streptavidin in 100 µL of PBS; Jackson Immuno Research Laboratories Inc.), and were incubated at 37 °C for 30 min. After washing them thrice in PBS, samples were incubated with monoclonal mouse anti-human CDX2 antibody (Clone: AMT28; BioSystems; 1:100 dilution in PBS) at 37 °C for 60 min. After rinsing them thrice in PBS, samples were covered with 100x diluted FITC-labeled anti-mouse IgG antibody (Sigma-Aldrich) and were incubated at 37 °C for 30 min. After PBS rinsing, samples were covered with antifading VectaShield (Vector Laboratories Inc.) and coverslips.

Finally, immunofluorescently labelled TMA slides and blood smears were digitalized as described previously.

Digital microscopic evaluation

In case of HGFR and CD133 immunolabelings, cells with diffuse, moderate-strong cytoplasmic and/or membrane staining were encountered. In case of CDX2 and Musashi-1, cells showing strong nuclear immunoreactivity were encountered. In normal samples all lymphoid aggregates, in UC samples at least 2 lymphoid aggregates within the lamina propria were examined. The percentage of immunoreactive cells was determined, except in cases where immunopositive cells were found only scattered in the observed area. For semiquantitative counting of immunopositive lamina proprial cells 5 fields of view were counted at magnification $\times 200$ in each sample core, then mean \pm SD were determined. In case of peripheral blood smears, 30 fields of view with 100 µm diameter were evaluated in every sample and the number of immunopositive cells (mean \pm SD) was determined.

Laser microdissection

From the biopsy samples stored in Tissue-Tek, 4 µm thick cryostat sections were cut, and placed onto 180 °C heat pre-treated (RNase free) glass slides. Sections were stained with methylene blue dissolved in diethyl-pyrocabonate-treated RNase free water. From one biopsy sample, at least 5000 subepithelial cells (from 2 to 4 cryostat section/sample) from the lymphoid aggregates were then laser capture microdissected by using a 337 nm UVA Laser microdissection system (PALM, Carl Zeiss MicroImaging GmbH, Germany).

RNA isolation and reverse transcription polymerase chain reaction analyses

Total RNA was extracted using the RNeasy Mini

Kit (Qiagen) for biopsy samples and the Paxgene Blood RNA Kit (Qiagen) for peripheral blood samples according to the manufacturer's instructions. After quantitative (Nanodrop) and qualitative analysis (Bioanalyzer Pico 600 chip kit RNA program; RIN > 8 in all cases), reverse transcription was performed by using 1 µg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, United States).

HGFR (ID: Hs.00179845_m1), CDX2 (Hs.174249) and CD133 (PROM1) (ID: Hs.614737) triplicated Taqman real-time polymerase chain reactions were used to measure mRNA expression of the observed parameters using an Applied Biosystems Micro Fluidic Card System. The measurements were performed using an ABI PRISM 7900HT Sequence Detection System as described in the product's User Guide (<http://www.appliedbiosystems.com>, California; United States).

For the examination of leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) (F: TGCTCT-TCACCAACTGCATC; R: CTCAGGCTCACCAGATCCTC), Musashi-1 (F: TACGCCAGCCGGAGTTATAC; R: ATTGGTCCGTAGGCAGTGAG) and cytokeratin (CK-)20 (F: CTGATGCAGATTCGGAGTAACA; R: TCTCTCTCCAGGGTGCTTAAC) gene expression changes triplicated quantitative real-time (qRT) PCR was performed using Probes Master and SYBR green (Roche GmbH, Germany). Gene expression levels for each individual sample were normalized to GAPDH expression. Mean relative gene expression was determined and differences were calculated using the $2^{-\Delta C_t}$ method. The whole cycle number was 45.

Regarding CK20 RT-PCR, SW480 colon carcinoma cells (90 cells/9 mL blood in Paxgene tube) and crypt epithelial cells (90 cells/5000 laser microdissected subepithelial cells) were used as positive controls.

Ethical consideration

All routine colonic biopsy specimens and blood samples from the patients were taken after informed consent and ethical permission was obtained for participation in the study.

Statistical analysis

The data were expressed as the mean \pm SD. For the statistics, Student's *t*-test was used. $P < 0.05$ was considered as statistically significant.

RESULTS

HGFR, CD133, CDX2 and Musashi-1 expressions in peripheral blood and lamina propria

The number of HGFR⁺ and CD133⁺ cells in the peripheral blood samples and colonic biopsies of patients with active UC was significantly higher comparing to healthy controls. CDX2⁺ cells were found only in the blood samples of active UC patients, *i.e.*, no CDX2 immunoreactivity was detected in the peripheral blood of healthy controls. In the LP, CDX2

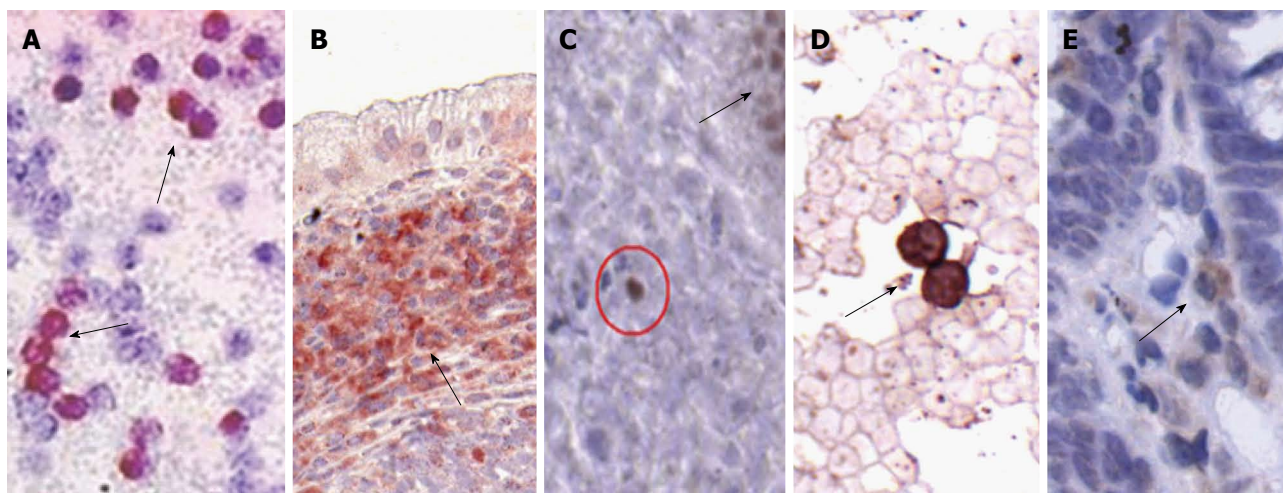


Figure 1 Immunopositive cells in peripheral blood and lamina propria of patients with ulcerative colitis. A: Hepatocyte-derived growth factor receptor (HGFR)⁺ cells in peripheral blood (black arrows indicate immunoreactive cells; magnification × 400); B: HGFR⁺ cells in lamina propria (black arrow indicates a group of immunoreactive cells underneath the epithelium; magnification × 200); C: CDX2⁺ (clone: ATM28) cell (red circle) in lamina propria (black arrow indicates a crypt base with CDX2⁺ epithelial cells; magnification × 400); D: CD133⁺ cells in peripheral blood (black arrow indicates immunoreactive cells; magnification × 600); E: CD133⁺ cell (black arrow) in lamina propria underneath the epithelium (magnification × 300). In all cases hematoxylin co-staining was performed.

immunoreactivity displayed high specificity to epithelial cell nuclei, however we observed some sporadic CDX2⁺ cells in the stroma involving infrequently LAs, and without connection to the crypts. In UC tissue samples the number of CDX2⁺ cells was significantly higher than in controls. In consideration of the unexpected presence of CDX2⁺ cells in the LP we repeated the anti-CDX2 immunostaining with another antibody (clone ZC007, Invitrogen) to avoid unspecific immunoreactions, and moreover, we performed Musashi-1 immunohistochemistry as well. After analyzing the results of the distinct CDX2 immunoreactions no significant differences were found between the numbers of CDX2-ZC007 and CDX2-AMT28 immunoreactive cells. Some stromal cells showing only Musashi-1 positivity with cytoplasmic and/or nuclear localisation were also detected.

In blood samples of UC patients Musashi-1 positive cells were found only sporadically. Immunopositive cells are presented in Figure 1.

Using double immunofluorescent labeling HGFR/CDX2 double immunopositive cells were found only in blood samples of UC patients, however they were detectable in tissue samples of both controls and UC patients. The number of HGFR/CDX2 double immunopositive cells was significantly higher in LP of active UC patients as compared to healthy controls. In UC samples some stromal CDX2⁺ cells also showed weak-to-moderate Musashi-1 positivity. We detected no HGFR/CDX2⁺ cells in blood or colonic tissue of patients with UC, which indicates that in UC all CDX2⁺ cells in peripheral blood and lamina propria expressed HGFR simultaneously. Regarding co-expression of Musashi-1/CDX2 in UC blood smears only few numbers of double immunopositive cells were found. HGFR/CD133, and CD133/CDX2 double immunoreactive cells were detected sporadically in the LP of active UC.

Double immunoreactive cells are presented in Figure 2. The number of immunopositive cells is indicated in Table 1.

RT-PCR validation

Real-time RT-PCRs for evaluating HGFR, CD133, CDX2, Musashi-1, Lgr5 and CK20 gene expressions were also performed. The expression values in active UC blood samples and laser-microdissected LP tissues were compared with the values of healthy subjects.

According to the $2^{-\Delta\Delta CT}$ method, foldchanges of HGFR, CD133 and CDX2 expressions were significantly higher in all UC samples ($P < 0.005$) than in healthy controls with the exception of CDX2 in peripheral blood, where it displayed an increasing tendency in UC as compared to controls. $\Delta\Delta CT$ s represent the difference between the average threshold cycle differences (ΔCT) of normal and UC samples.

Though CDX2 is a known crypt epithelial stem cell marker, to exclude contamination with epithelial cells Musashi-1, Lgr5, and CK20 mRNA expressions were also analyzed in blood and LP samples. Similarly to CDX2, foldchanges of Musashi-1 and Lgr5 expressions were significantly higher in the LP of UC patients than in controls ($P < 0.005$ in all cases). Musashi-1 and Lgr5 gene expressions showed an increasing tendency in UC blood samples compared to controls. No detectable CK20 gene expression was present in UC and normal samples. Foldchanges are visualized in Figures 3 and 4.

DISCUSSION

The concept of mucosal repair means that inflammatory cells enter the damaged area and signal local progenitor cells for mitosis. In addition, upon damage signals multipotent mesenchymal stem cells may also

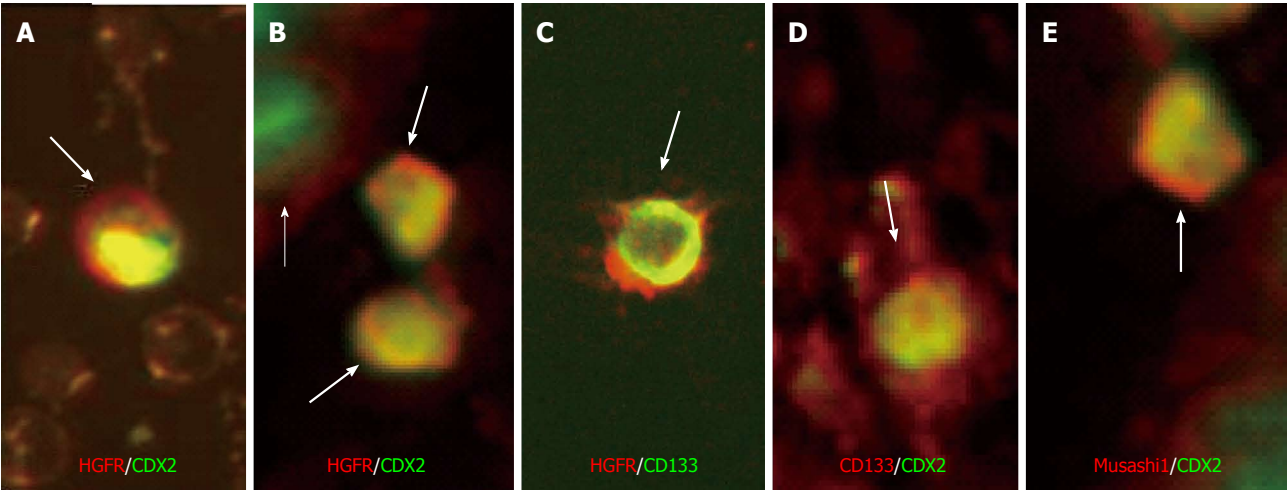


Figure 2 Double fluorescent immunoreactive cells in peripheral blood and lamina propria of patients with active ulcerative colitis. A: Hepatocyte-derived growth factor receptor (HGFR)/CDX2⁺ cell in peripheral blood (white arrow indicates immunoreactive cell; magnification × 600); B: HGFR⁺/CDX2⁺ cells in lamina propria (white arrows indicate immunoreactive cells underneath the epithelium; white thin arrow indicates a crypt base with CDX2⁺ epithelial cell; magnification × 600); C: HGFR⁺/CD133⁺ cell in lamina propria (white arrow; magnification × 600); D: CD133⁺/CDX2⁺ cell in lamina propria (white arrow; magnification × 600); E: Musashi-1⁺/CDX2⁺ cell in the lamina propria (white arrow; magnification × 600). Red fluorescent labeling: Texas-Red; Green fluorescent labeling: FITC.

Table 1 Number of immunopositive cells detected in peripheral blood and colonic biopsy samples		
Average number of cells	Peripheral blood (normal/UC)	Lamina propria (normal/UC)
HGFR ⁺ CDX2 ⁺ (Clone: AMT28) ³	6.7 ± 1.22/38.5 ± 3.18 ¹ 0/0.94 ± 0.64 ^{1,2}	2.25 ± 0.85/9.22 ± 0.65 ¹ 0.75 ± 0.55/2.11 ± 0.75 ^{1,2}
CDX2 ⁺ (Clone: ZC007) ³ CD133 ⁺	0/1.0 ± 0.59 ^{1,2} 1.1 ± 0.72/8.3 ± 1.08 ¹	0.8 ± 0.52/2.16 ± 0.71 ^{1,2} 11.1 ± 0.85/26.28 ± 1.71 ¹
Musashi-1 ⁺ HGFR ⁺ /CDX2 ⁺ (Clone: AMT28)	0/scattered 0/1.0 ± 0.59 ¹	0/scattered 0.8 ± 0.69/2.06 ± 0.72 ¹
HGFR ⁺ /CD133 ⁺ Musashi-1 ⁺ /CDX2 ⁺ (Clone: AMT28)	0 0/scattered	0/scattered 0/scattered
CD133 ⁺ /CDX2 ⁺ (Clone: AMT28)	0	0/scattered

¹Indicates significant differences between normal *vs* UC samples (*P* < 0.05); ²Indicates that all CDX2⁺ cells in peripheral blood and lamina propria expressed HGFR as well; ³Indicates that no significant differences between the numbers of CDX2-AMT28 and CDX2-ZC007 immunoreactive cells were detected. Data are expressed as mean ± SD. UC: Ulcerative colitis.

contribute to tissue repair after their mobilization, migration, and engraftment of the inflamed mucosa^[6]. Furthermore, circulating immature cells with a potential of stem cell capacity are also likely to participate in colonic mucosal regeneration^[10]. In UC, following inflammatory mucosal damage successful epithelial regeneration demands the complex interaction and participation of a local and marrow-derived stem cell pool^[11]. The course and regulation of mucosal repair sorely depend on the balance between pro- and anti-inflammatory cytokines influenced mainly by LAs and ILFs^[12]. These lymphoid elements are thought to be involved in controlling and organizing of homing, development, and differentiation of stem cells, as

well^[13]. In the present study we assayed the regeneration-associated stem cell-related phenotype of HGFR⁺ cells both in peripheral blood and colonic tissue samples of patients with clinically active UC. The proto-oncogene *Met* is known to encode the high-affinity transmembrane tyrosine kinase receptor for hepatocyte growth factor (HGF). C-Met/HGFR and its family members promote mainly migration and invasion of cancer cells. Signaling within and beyond this pathway seems to be an important factor regarding systemic spread of metastases through induction of epithelial-to-mesenchymal transition (EMT)^[14]. HGFR and its ligand, HGF serve as potential mitogenic factors for epithelial cells, enhancing cell motility, with morphogenetic effects, and a crucial role in wound healing within the digestive tract^[15-17]. In experimental colitis of rats the HGF/HGFR system was found to be a potential therapeutic target to facilitate intestinal repair^[18]. Though HGFR is predominantly expressed on epithelial cells, it is also implicated in hematopoiesis^[19]. Expression of HGFR by the hematopoietic compartment was detected in progenitor cells, macrophages, B cells, and dendritic cells. HGFR signaling has been proposed to take part in the development of monocytes-macrophages, and in homing of B cells to lymphoid tissues^[20,21]. Basically the HGF/HGFR system is indispensable during embryonic development. HGF is a pleiotropic factor that promotes several cellular functions, including survival, tissue protection, regeneration, and exerts anti-inflammatory activities^[16]. Moreover, HGF was found to regulate various immune functions, like cytokine production, cellular migration, and adhesion^[22-27]. Nonetheless, under normal conditions the HGF/HGFR signaling seems to be dispensable, since previous studies demonstrated no structural and

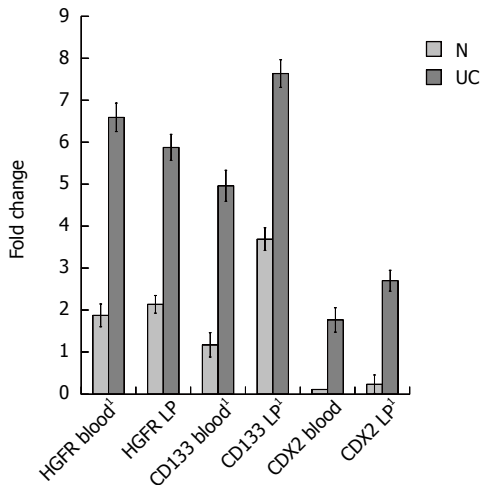


Figure 3 Foldchange alterations of the assayed genes in peripheral blood and lamina propria samples. ¹Significant ($P < 0.005$) gene expression alterations between ulcerative colitis (UC) and healthy control (N) samples. LP: Lamina propria; HGFR: Hepatocyte-derived growth factor receptor.

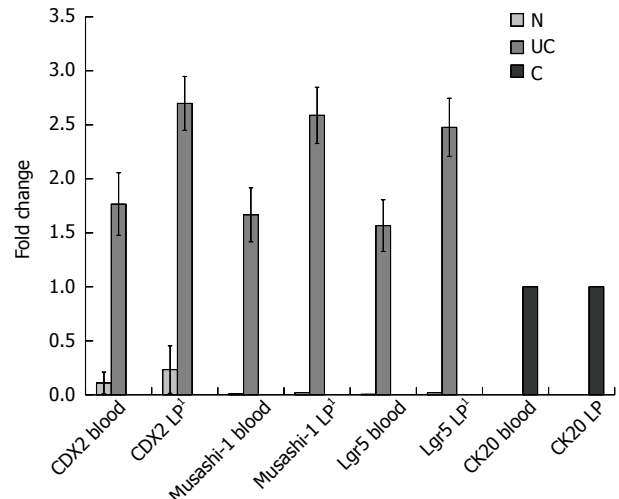


Figure 4 Foldchange alterations of the assayed epithelial genes in peripheral blood and lamina propria samples. ¹Significant ($P < 0.005$) gene expression alterations between ulcerative colitis (UC) and healthy control (N) samples. C: Control samples (SW480 cells in blood, crypt epithelial cells in lamina propria (LP) laser-microdissected samples).

functional abnormalities in different c-met-knockout cell types^[28-31].

HGF is essential in paracrine signaling of mesenchymal and epithelial cells, particularly during embryogenesis, regeneration, and also in carcinogenesis^[32]. In adults, HGFR signaling might be involved both in tissue repair and invasive tumor growth, depending mainly on the type of tissues.

Both chronic fibrosis and tumorigenesis can be originated from a sustained healing response due to chronic tissue injury. In fact, chronic fibrosis may directly predispose to cancer^[33]. In UC, there is an active and persistent immune-mediated inflammation, therefore the cycle of continuous mucosal injury and repair may lead to an increased risk of colonic wall fibrosis and thus, to the development of cancer. HGF and HGFR expressions are both elevated in the inflamed mucosa of UC patients^[34], and additionally, increased plasma HGF levels were found in human UC, and in colitic mice^[34,35]. After inducing tissue injury and/or inflammatory cytokines stromal fibroblasts, vascular endothelial and smooth muscle cells, neutrophils, and mast cells represent the major sources of HGF^[36]. Fibroblast-like stromal cells from human lymphoid tissues constitutively produce HGF, which is affected by activated T cells^[37]. Emerging evidences indicate that HGF possesses potent anti-apoptotic capacity, as well^[38-40]. Within the mucosa the HGF-HGFR signaling facilitates colonic mucosal remodeling and significantly lessens inflammation^[9,18], breaking the cycle of injury and repair. Conversely, HGFR is overexpressed in UC-associated colorectal cancer^[34], suggesting that the HGF/HGFR system is unequivocally involved in carcinogenesis. According to these data, it is likely that the pro- or anti-carcinogenic effects of the HGF/HGFR system is context-dependent influenced mainly by the duration of inflammation, *i.e.*, acute or chronic. During active inflammation of the colonic mucosa the

HGF/HGFR system usually favors mucosal healing. The high number of HGFR⁺ subepithelial and circulating cells found in our study may be the consequence of an increased demand of cells with extensive mucosal reparative capacity.

Although in DSS-colitic rats both an increase in serum HGF level and expression of colonic HGF mRNA were observed, the HGF protein level in colitic mucosa was still reduced^[41]. HGF also interacts with heparin-like low-affinity receptors, like the heparin sulfate proteoglycans, which are expressed on the cell surface and within extracellular matrix that retain HGF in tissues^[42-44]. In case of severe mucosal damage, when loss of the epithelial layer and the surrounding extracellular matrix can be detected, the local HGF concentration in the injured colonic tissue might be reduced.

Considering our results, the elevated local expression of both mRNA and protein of the high-affinity HGFR may function to retain and bind all the available local HGF for reducing the inflammation, and to promote mucosal regeneration. The mechanism underlying the protective mucosal actions of HGFR signaling in UC is likely attributable to its ability to inhibit apoptosis and inflammation.

In the intestine, CDX2, an intestine-specific transcription factor essential for the regulation of genes related to epithelial functions, is involved in maintaining epithelial homeostasis^[45,46], and controlling the expression of a number of downstream genes, some of which fundamentally contribute to inflammation^[47]. Further, CDX2 was shown to inhibit cell growth and migration *in vitro*, as well as dissemination of colon tumor cells *in vivo*^[48]. Until now only limited attentions have been paid for the investigation of the relation of CDX2 to intestinal inflammation. In a recent study diminished epithelial CDX2 expression was described

in UC^[49]. Moreover, TNF- α was suggested to down-regulate the expression of CDX2 in the inflamed mucosa^[50]. On the contrary, however, we found that the number of CDX2⁺ submucosal cells in active UC was slightly elevated. Although CDX2 expression may be influenced by the hypoxia inducible factor 1 (HIF1) expressed during inflammation^[51,52], it remains unclear how the expression of CDX2 is regulated exactly within the affected tissues.

Mesenchymal myofibroblasts are involved in mucosal healing process, and epithelial regeneration (re-epithelialization). Upon expression of Wnt proteins, and secretion of growth factors, like epidermal growth factor, HGF, and bone morphogenic factor-4 myofibroblasts considerably promote the induction of CDX2^[53]. Furthermore, myofibroblasts support the local stem cell niche *via* multiple mechanisms, including interactions *via* the Wnt/ β -catenin and Notch pathways to regulate stem cell behavior^[54,55].

In case of our UC patients the parallel presence of HGFR⁺/CDX2⁺ double-positive cells in circulation and colonic subepithelium could be indicative for the involvement of HGFR⁺ cells in regeneration of the damaged mucosa.

Since detection of CDX2⁺ cells in the LP and blood samples of UC patients was an unexpected result, we performed Musashi-1 immunolabeling, and Musashi-1 and Lgr5 RT-PCR assays. Both Musashi-1 and Lgr5 are considered as markers of intestinal epithelial stem cells^[56-58], and Lgr5 is a target of the Wnt pathway, as well^[58]. Musashi-1 is co-localizes with Notch genes and augments Notch signaling, essential for maintaining cellular progenitor state^[59]. On the other hand, Musashi-1⁺ blood cells might even represent circulating smooth muscle cell precursors^[60]. Lgr5⁺ cells are mainly intestinal stem cells or crypt basal cells (CBC) being able to give rise to all intestinal epithelial lineages, indicating a self-renewing population of stem cells^[59]. Moreover, Lgr5 might also be implicated in cancer stemness^[61].

The parallel presence of CDX2, Musashi-1 and Lgr5 in UC blood and LP samples along with the absence of CK20 expression may indicate that a portion of circulating HGFR⁺ cells has already been committed to the epithelial lineage. However, further experiments are required to understand the role of CDX2 in the regenerative phase of UC, and the exact origin of CDX2⁺ (Musashi-1⁺ and Lgr5⁺) cells.

CD133 (Prominin-1), a 5-transmembrane domain glycoprotein expressed by hematopoietic and mesenchymal stem cells, other organ-specific stem cells and tumor initiating cells, is currently regarded as one of the most significant markers of colonic cancer stem cells, as well^[62-64]. The molecule is designated for its prominent location on protrusions of cell membranes^[65,66]. Additionally, CD133 can also be expressed by epithelial stem cells^[67], and in this respect represents another target for Wnt signaling pathways. Therefore, CD133 is of importance yet in

intestinal regeneration, and, further, in decreasing inflammation^[68].

The expression of CD133 within the inflamed colonic epithelium is significantly lower in UC patients with a longer duration of the disease^[69]. The risk of developing colorectal cancer is considerably higher in patients suffering from UC, especially in those with long-standing disease, *i.e.*, over 10 years^[70]. Thus, the decreased CD133 level in the inflamed mucosa could be associated with the development of UC-related colorectal cancer. In our study the number of CD133⁺ cells were significantly higher in the blood and subepithelium of patients with newly diagnosed UC in an active phase of the disease. The fact that HGFR⁺/CD133⁺ cells were detected only in the lamina propria suggests that a portion of HGFR⁺ cells in LAs may be originated from a local stem cell pool rather than being immigrating cells. CD133 may also influence cellular polarity, migration, and the interaction of stem cells with their neighboring ones and the extracellular matrix^[71], and thus promote mucosal healing.

Some Lgr5⁺ cells co-express CD133, and these CD133⁺ cells can generate the entire intestinal epithelium^[59]. In addition to Lgr5 and CD133, other potential intestinal stem cell markers have been identified, like Musashi-1, expressed by both quiescent label-retaining cells and actively cycling CBCs^[59]. In general, during tissue repair stem cells might display a great transient plasticity, so upon a dynamic interplay they can change their current phenotype to ensure successful tissue regeneration^[72].

The co-expression of HGFR and CD133 on the surface of colonic subepithelial cells may further indicate the potential involvement of HGFR⁺ cells in UC-associated carcinogenesis. In the colonic mucosa LAs and ILFs are supposed to have a special organizer role in epithelial repair^[12]. Our finding, that CD133 and CDX2 are co-expressed in cells of LAs also supports that MET is primarily localized to the area of these lymphoid structures. It is still questionable whether the complex mucosal healing is related to the recruitment of a quiescent local stem cell population or requires renewal by bone marrow cells, and hence, remain to be determined. One cannot exclude the possibility that stromal cell-derived factor 1 or vascular endothelial growth factor have a role in the chemoattraction of marrow derived cells to the lamina propria. In our study, we did not study these factors. However, HGF alone may also serve as a chemoattractant of circulating HGFR⁺ cells since it has been reported that HGF level is elevated in the inflamed colonic mucosa^[34]. Although it is apparent that the intestinal epithelium responds to inflammation and mucosal injury by initiating a repair response, the specific effects on the turnover of epithelial stem- or progenitor cells, and the exact mechanism how the inflammatory milieu may perturb epithelial differentiation and/or function, are still obscure. Furthermore, the bidirectional interactions between stem cells and their niche are of special

importance to determine stem cell behavior, and thus leading toward self-renewal or differentiation.

In conclusion, based on our present results the elevated number of HGFR⁺ subepithelial and circulating cells within the inflammatory colon may be an indicator of the increased demand of cells with high mucosal regenerative capacity. The presence of HGFR/CDX2 double immunoreactive cells along with the detectable expression of Musashi-1 and Lgr5 in the peripheral blood and LP of patients with UC highlight on the potential involvement of HGFR⁺ cells in colonic mucosal healing related to severe inflammatory injury. Moreover, the data indicate that HGFR⁺ cells in the circulation are already committed to the epithelial lineage. The presence of HGFR/CD133 double immunopositive cells in the LP suggests that a portion of HGFR⁺ cells may be originated from a local stem cell pool, and besides repair functions, they could potentially be involved in UC-associated carcinogenesis as well. In addition, CD133 could also affect the regulation of cell motility, another crucial element of wound healing. The detection of CD133⁺/CDX2⁺ cells along with the presence of Musashi-1 in subepithelial lymphoid aggregates support that mesenchymal-to-epithelial transition, an essential event in epithelial regeneration is primarily located to those lymphoid structures. In conclusion, both HGFR and CDX2 seem to be definitely involved in colonic mucosal regeneration of clinically active UC patients, however further investigations are needed to determine their definite role and function, and relation to local and bone marrow stem cell pools.

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COMMENTS

Background

In ulcerative colitis hepatocyte-derived growth factor receptor (HGFR) positive subepithelial cells within lymphoid aggregates are supposed to be involved in the induction of mucosal repair. An elevated number of HGFR⁺ peripheral blood cells were observed in severely active ulcerative colitis, however their importance and function have not yet been investigated. In case of HGFR⁺ cells their possible origin, the route of migration in point of the blood stream and the lamina propria, and whether these cells are committed or not to the epithelial lineage have not yet been elucidated.

Research frontiers

Previous chromosomal chimerism experiments have already proved that part of epithelial stem cells are originated from a local stem cell pool, while another part of them is of marrow origin.

Innovations and breakthroughs

This is the first study evaluating the injury-associated stem cell-related phenotype of HGFR⁺ cells in peripheral blood and colonic tissue of patients with active ulcerative colitis and compared to that of controls.

Applications

The presence of HGFR/CDX2 double immunoreactive cells in the peripheral blood and lamina propria of ulcerative colitis patients highlight on the potential involvement of HGFR⁺ cells in colonic mucosal healing related to severe inflammatory injury. The data also indicate that HGFR⁺ cells in the circulation are committed to the epithelial lineage. The presence of HGFR/CD133 double immunopositive cells in the lamina propria suggests that a portion of HGFR⁺ cells may be originated from a local stem cell pool.

Terminology

Mesenchymal-to-epithelial transition (MET), a crucial physiologic event converts motile mesenchymal cells to polarized epithelial cells, thus favors -among others- epithelial regeneration. Within pathological circumstances, increasing evidence indicates that MET may regulate epithelial carcinogenesis, as well.

Peer-review

Authors demonstrated that higher number of HGFR, CDX2, CD133 and Musashi-1 positive cells were detected, and that HGFR/CDX2 and Musashi-1/CDX2 co-expressions were found in blood and lamina propria of UC samples. These results are interesting. Previous studies have established that HGFR⁺ cells may have a role in mucosal healing in ulcerative colitis. However, as the authors point out, the migration pathway of HGFR⁺ cells from the blood stream to the lamina propria has not been elucidated.

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