Expression Profiling of Stationary and Migratory Intestinal Epithelial Cells after in vitro Wounding: Restitution is Accompanied by Cell Differentiation

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Key Words
Cell migration • Wound healing • Mucosal injury • Restitution • Intestinal repair • Regeneration • Cell differentiation • Epithelial-mesenchymal transition • Epithelial cells

Abstract
Rapid repair by cell migration, a process called "restitution", is essential for wound healing of mucous epithelia. Here, an established in vitro model for restitution, i.e., migration of the non-transformed intestinal epithelial cell line IEC-18 after scratch wounding, was investigated. This cell line is also known for its retained differentiation potential. The aim of this study was to test by expression profiling whether the differentiation state is altered during restitution in vitro. Using a sensitive RT-PCR method a systematic analysis of separated stationary and migratory cells was performed 48 h after in vitro wounding. Most characteristically, the differentiation state was changed in migratory cells when compared with stationary cells. For example, migratory cells lost markers of terminal differentiation and changed to a phenotype that assists the process of restitution by up-regulating the expression of genes such as plasminogen activator inhibitor-1, transforming growth factor α, heparin-binding EGF-like growth factor, α-smooth muscle actin, ornithine decarboxylase, and glyceraldehyde-3-phosphate dehydrogenase. However, there were no unequivocal signs of epithelial-mesenchymal transition (EMT) found in migratory cells.

Introduction
Mucous epithelia, such as the intestinal epithelium, represent a major barrier to the outside world and are vital for the complex communication with the environment [1]. Also, these surfaces are constantly exposed to a variety of noxious agents. Thus, a series of defensive lines have developed with major emphasis on two different regeneration and repair mechanisms that cover different time scales. One of the best studied systems thus far is the intestinal mucosa, which is self-renewed about every 5 days due to continuous regeneration from stem and precursor cells. Here, two types of intestinal stem cells in the crypts give rise to four different lineages, i.e., absorptive enterocytes (secreting also aminopeptidase N), mucus-producing goblet cells, Paneth cells (secreting...
antimicrobial peptides and lysozyme), and various types of enteroendocrine cells [2, 3].

There is also a second regeneration mechanism called „restitution“ [4] allowing for the rapid repair of superficial mucosal lesions via cell migration [5, 6]. This process starts within minutes following mucosal damage. Restitution is particularly well described to occur in the intestinal mucosa both in vivo [7] as well as in vitro model systems [8]. Enterocytes were identified as the migrating cells; whereas goblet cells do not actively contribute to the mechanism of restitution [7]. Mucosal restitution was shown to be stimulated by a number of regulatory peptides (i.e., motogens) including epidermal growth factor (EGF) and other EGF receptor ligands, trefoil factor family (TFF) peptides, transforming growth factor (TGF-β), and interleukin-8 (IL-8) [6, 9-11]. Furthermore, polyamines affect cell migration and repair of mucosal lesions significantly [12, 13]. Generally, epithelial cells undergoing restitution in vivo gradually interconvert between two types of cell polarity, i.e., they change from polarized epithelial cells (with apical-basal polarity) to polarized migrating cells (with planar polarity) [14]. Epithelial cells may also undergo an epithelial-mesenchymal transition (EMT) to become migratory [15-17]. The EMT involves trans-differentiation of epithelial cells to fibroblastoid, motile cells showing an altered, mesenchymal gene expression program [18-20].

Rapid repair of damaged mucous epithelia is essential for preventing inflammation, which is a critical component of cancer progression [21]. For example, some 90% of fatal malignancies in adult humans arise from epithelia. Furthermore, insufficient (resulting in abscesses and fistulae) as well as excessive wound healing (resulting in fibrosis due to EMT) cause major clinical problems, in particular in intestinal diseases [22, 23].

One of the best studied in vitro models for mucosal restitution are migrating IEC-6 and IEC-18 cells after scratch wounding [24]. Here, migration has been reported to depend on protein synthesis to different degrees [24]. Typical for such model systems is a collective cell migration mode [25]. IEC-6 and IEC-18 represent epitheloid non-transformed cell lines originating from the crypts of the small intestine of adult or newborn rats, respectively [26, 27]. They share features of undifferentiated crypt cells and they are thought to represent progenitor cells. However, both IEC-6 and IEC-18 cells retain the potential to differentiate into various cell lineages, including enterocytes, goblet, endocrine, and Paneth cells [28-30]. In the past, the motogenic effect of various peptides was investigated in IEC-6 and IEC-18 cells after in vitro wounding [31-35]. However, reports describing changes in gene expression for stationary and migratory cells are rather scarce [36, 37]. Thus, a sensitive RT-PCR method was applied allowing a systematic gene expression analysis of separated stationary and migratory IEC-18 cells 48 h after scratch wounding. The focus of the work presented here is on genes related to the differentiation state of the cells.

### Materials and Methods

**Culture of IEC-18 Cells and RNA Isolation from Stationary and Migratory Cells after Scratch Wounding**

The non-transformed IEC-18 cell line from juvenile rat ileal crypt cells (passages 37 to 50; [27]) was cultured as previously described [35]. The experiments were performed in plastic culture dishes (10 cm diameter; Greiner, Frickenhausen, Germany). First, the culture dishes were marked on the outer bottom surface to indicate the wound zones (4-5 rectangular zones with about 5 mm width; restricted to one half of the culture dishes).

Subconfluent IEC-18 cells were detached with trypsin/EDTA (0.05% trypsin and 0.53 mM EDTA, Gibco Invitrogen Corp., Karlsruhe, Germany) from the culture flasks for up to 5 min at 37°C, resuspended and seeded at a density of 300.000 cells per culture dish in fully supplemented growth medium (5% (v/v) FCS), and incubated at 37°C in a humidified atmosphere with 6.5% (v/v) CO₂ for 5 days. During this period, the cell culture medium was changed every other day. On day 5, the growth medium was replaced with 10 ml „starvation medium“, i.e., fully supplemented DMEM with a reduced serum content (0.2% v/v). After 2 days of starvation, the cell monolayers were scratched under sterile conditions using 5 mm fragments of double-edge stainless-steel razor blades (American Safety Razor Co., Staunton, VA, USA) fixed in a Barraquer razor blade holder (Deutschmann GmbH, Zittau, Germany). Routinely, 4-5 rectangular wound zones (~30-50 mm × 5 mm) were created according to the pattern marked on the outer bottom surface of the plate. The scratch wounded cell cultures were rinsed gently and repeatedly (up to 5 times) with starvation medium to remove residual cell debris and then incubated at 37°C with 10 ml of fresh starvation medium in 6.5%(v/v) CO₂ at 37°C.

After 48 h, the cells were washed with starvation medium and sorted for RT-PCR analysis by detaching first the „stationary cells“ with a plastic cell scraper. Only cells that did not have direct contact with the border zone (i.e., cells from that half of the culture dish which was not scratch wounded) were harvested in this way, and then aspirated/collected, and centrifuged (300 rpm, 20°C, 3 min) and 1 ml Trizol Reagent (Invitrogen, Karlsruhe, Germany) was added to the pellet.

Then, all remaining stationary cells including the border/start area at the scratch front were removed and discarded, so that only „migratory cells“ in the repopulated zone were left over (i.e., about 10 rows of cells directly behind the migratory...
These cells were rinsed gently and repeatedly with starvation medium. Then 1 ml Trizol Reagent (Invitrogen) was added directly to the culture dish and total RNA was isolated according to the manufacturer’s protocol. Alternatively, the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used for RNA isolation. All procedures of cell wounding and cell sorting were controlled with a microscope.

**RT-PCR Analysis**

The extracted RNA was digested with RNAse-free DNase (Fermentas, St. Leon-Rot, Germany) at 37°C for 30 min using 2 units/0.5 µg RNA. The concentration and purity of the RNA was estimated with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Peqlab Erlangen, Germany). cDNA synthesis was performed with 0.5 µg RNA primed with oligo(dT)12-18 using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. For RT-PCR analysis HotStarTaq Plus DNA polymerase (Qiagen) was used. The cDNA was checked for contaminating chromosomal DNA by amplification of both an intron-specific sequence (oligodeoxynucleotides MB822 and MB823; see Table 1) and a promoter sequence from the β-actin gene (oligodeoxynucleotides MB1510 and MB1511; see Table 1). The relative expression level of 16 genes was monitored using specific primer pairs as listed in Table 1. As a control for the integrity of the cDNA preparations, transcripts for β-actin were amplified in parallel reactions.

**Western Blot Analysis**

Alternatively, proteins were isolated from stationary or migratory cells using 1 ml Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Protein concentrations of the samples loaded onto the gel (8 µg per lane) were monitored by the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany). Western blot analysis was performed as reported previously using 15% SDS-PAGE and the ECL detection system [38]. Bands were recorded with GeneGnome (Syngene, Cambridge, UK). The following antisera were used: polyclonal rabbit antiserum against mouse stromal cell-derived factor-1β (SDF-1/Cxcl12; dilution 1:1000; Torrey Pines Biolabs).
Results

Fig. 1 represents the results of the RT-PCR analysis of 16 selected genes which showed characteristic and highly reproducible expression patterns when stationary and migratory cells were compared. Generally, 3 different gene expression patterns were observed: (i) expression in stationary cells is stronger than in migratory cells, (ii) expression in stationary and migratory cells is about the same, and (iii) expression in stationary cells is weaker than in migratory cells. The first group comprises the following genes: lysozyme, aminopeptidase N, tenascin C, SDF-1/Cxcl12, Musashi-1, and Gata5. Members of the second group are Gata6, Snai2 (previously: Slug), and Twist. The third group consists of plasminogen activator inhibitor-1 (PAI-1/serpin E1), transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), α-smooth muscle actin (α-SMA/Acta2), ornithine decarboxylase (ODC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Transcripts for β-actin were amplified as a control for the amount and integrity of the cDNAs. The results of a typical experimental series consisting of two parallel experiments are shown. Both the stationary cells as well as the migratory cells were isolated and analyzed separately.

Furthermore, Western blot analysis concerning SDF-1 was performed in order to test whether the protein expression was different in stationary and migratory cells (Fig. 2). As a control, actin expression was analyzed. Clearly, the SDF-1 level decreased in migratory cells when compared with stationary cells; whereas actin levels were comparable in stationary and migratory cells.

Discussion

A sensitive and highly reproducible method is described allowing the systematic analysis of gene

Inc., antibodies-online GmbH, Aachen, Germany), polyclonal rabbit antiserum against the C-terminal region of actin (affinity purified, dilution 1:1000; Sigma-Aldrich Chemie, Taukirchen, Germany), and peroxidase-labeled anti-rabbit IgG (H+L) PI-1000 (1:4000 dilution; Vector Laboratories Inc., Alexis Biochemicals/Enzo Life Sciences, Lörrach, Germany).
Expression patterns of separated stationary and migratory cells. Comparable RT-PCR results with those presented in Fig. 1 were also obtained when totally scratched or unscratched dishes were compared as controls (data not illustrated). However, the differences were not that pronounced as in Fig. 1 because the totally scratched dished always contained a mixture of both stationary and migratory cells. Further on, when stationary cells were isolated quickly avoiding the time consuming detachment and washing procedure, RT-PCR results were again comparable with those presented in Fig. 1 (data not illustrated). IEC-18 cells were also treated with mitomycin C (2 µg/ml) to inhibit cell proliferation. Even these cells showed the same decreased SDF-1, lysozyme and Gata5 expression in migratory cells as in Fig. 1 (data not illustrated). Finally, the expression patterns of stationary and migratory cells were analogous to that of Fig. 1 also at the protein level as shown for SDF-1 and actin (Fig. 2). We also tested whether migratory cells re-expressed the RT-PCR profiles of the stationary cells after they regained confluence after 20 days. Only minimal differences between stationary cells and cells from the re-populated area were observed indicating re-differentiation of the migratory cells (data not illustrated).

As one hallmark, migratory IEC-18 cells show decreased expression of genes encoding secretory proteins characteristic of the differentiation state, such as lysozyme and aminopeptidase N, when using β-actin expression as a standard. Typically, the decrease in lysozyme expression was a highly pronounced change in all experiments. Lysozyme is a characteristic secretory product of Paneth cells which are exclusively located at intestinal crypts. Thus, the drastically reduced level of lysozyme expression is an indication that stationary IEC-18 cells have adopted some characteristics of Paneth cells and that migratory cells hardly show this differentiation. Furthermore, IEC-18 cells show some sign of enterocyte differentiation (expression of aminopeptidase N) which is also decreased during migration. A similar situation has been reported for migrating intestinal Caco-2 cells which lost the enterocyte marker sucrase-isomaltase when compared with stationary cells [37].

The view that migratory cells show a changed differentiation state is particularly in line with the down-regulation of the transcription factor Gata5. In the mouse, Gata5 mRNA levels are highest in the ileum (i.e., the intestinal region where also IEC-18 cells originate from); whereas Gata6 transcript levels are elevated in the duodenum and proximal jejunum [39]. High level Gata5 expression was found in Paneth cells, and lower levels also in goblet and enteroendocrine cells, but not in enterocytes; whereas Gata6 was restricted to the enteroendocrine lineage [40]. This points to a function of Gata5 for terminal differentiation of intestinal cells, particularly Paneth cells. The view of Gata5 as a terminal differentiation marker is also supported by differentiation experiments with HT-29 cells [41]. Thus, the decrease of Gata5 in migratory IEC-18 cells is a further indication that at least part of the terminal differentiation program is not active any more.

The extracellular matrix protein tenascin C plays a crucial role during wound healing and metastasis [42, 43]. This mainly mesenchymal protein is known to be induced by the EMT [44]. Thus, the decreased level of tenascin C expression in migratory cells is an indication that migratory IEC-18 cells do not undergo EMT under the conditions investigated here.

Of note, expression of SDF-1 is also strongly reduced in migratory IEC-18 cells. This secretory α-chemokine is constitutively expressed mainly in normal intestinal villus epithelium [45] and is also important for the active recruitment of stem cells during repair of damaged tissue [46]. SDF-1 may also be secreted by stem/progenitor cells and be involved in autocrine/paracrine regulation mechanisms. The expression of SDF-1 in IEC-18 cells is comparable with its synthesis in IEC-6 cells where SDF-1 promotes restitution [47]. Furthermore, IEC-18 cells express the stem cell marker Musashi-1 similar to IEC-6 cells [48]. Musashi-1 inhibits transcription of mNumb, a Notch signaling pathway inhibitor, and is found at the +4 label retaining cells in intestinal crypts [49]. Thus, the decreased expression of Musashi-1 in migratory cells is not a sign for increased stem cell properties of these cells. This is also a further indication that migratory cells do not undergo EMT because EMT has been reported to generate cells with properties of stem cells [50].

Transcription factors belonging to the Snail (such as Snaï2) and basic helix-loop-helix families (such as Twist) are well known inducers of EMT by repressing E-cadherin expression and they are up-regulated during EMT [51]. Thus, expression levels of Snaï2 and Twist were monitored in both stationary and migratory cells. The fact that Snaï2 as well as Twist show nearly equal expression levels in both stationary and migratory IEC-18 cells argues against EMT occuring in migratory IEC-18 cells. This view is supported by the expression profiles of the classical EMT markers vimentin and E-cadherin [44], which did not show unequivocally the expected up- and down-regulation, respectively, in migratory IEC-18 cells (data not shown). There was only a tendency indicating
particularly down-regulation of E-cadherin expression in migratory IEC-18 cells. The results concerning EMT presented here are in contrast to a previous study which showed typical signs of full EMT in migratory Caco-2 cells, i.e., down-regulation of E-cadherin and up-regulation of N-cadherin, Snai1, and Snai2 expression [37]. One reason for the discrepancy could be that IEC-18 cells represent a non-transformed cell line; whereas Caco-2 cells are derived from a colon carcinoma.

A typically up-regulated gene in migratory IEC-18 cells is the serpin PAI-1. This result is reminiscent to increased PAI-1 expression in epidermal keratinocytes and renal epithelial cells after scratch wounding [52, 53]. Prolonged PAI-1 expression has been shown to be essential for epidermal wound repair [54]. Increased PAI-1 expression has also been reported to facilitate the epithelial invasive potential by affecting matrix remodeling with major inducers of PAI-1 expression being the EGF receptor ligands HB-EGF and TGFα [55]. Of note, the expression of HB-EGF and TGFα were also up-regulated in migratory IEC-18 cells. This is in line with a report on increased expression of HB-EGF after wounding of intestinal epithelial cell monolayers [56]. HB-EGF enhances intestinal restitution [57] and it is cytoprotective for IEC-18 cells during hypoxia [58]. Furthermore, also TGFα expression has been demonstrated in the intestine and in IEC-6 cells [59]. TGFα enhances restitution of IEC-6 cells [31] and also leads to an autocrine stimulation of TGFα expression in these cells [60].

Another striking feature of migratory IEC-18 cells is transcriptional up-regulation of α-SMA expression. α-SMA is a hallmark of myofibroblasts which can differentiate from fibroblasts via mechanical tension and activation by TGFβ1 [61]. This process is essential for connective tissue remodeling during wound healing. Furthermore, the myofibroblast phenotype can originate also from a TGFβ1-triggered epithelial-myofibroblast transdifferentiation, which is a typical EMT process, and is important for disease states such as the various forms of tissue fibrosis, e.g., in the intestine and lung [22, 62]. Myofibroblasts are specialized contractile fibroblasts and α-SMA expression is associated with the generation of increased contractile forces and stress fiber formation. α-SMA expression is regulated by TGFβ1 in a complex manner [63]. Force-induced regulation of α-SMA expression is also associated with p38 activation in a feed-forward amplification loop [64].

ODC is the initial rate-limiting enzyme involved in polyamine biosynthesis. Inhibition of this enzyme with α-difluoromethylornithine (DFMO) resulted in severe defects of wound healing of the gastrointestinal tract and also in the migration of IEC-6 cells [12, 13, 65]. The up-regulation of ODC in migratory IEC-18 cells is comparable with increased ODC expression in epidermal keratinocytes after scratch wounding [52] and elevated ODC levels might support restitution of IEC-18 cells as well as proliferation [66]. Based on a previous study with IEC-6 cells [67], the elevated ODC expression rate could be a result of the increased expression of TGFα and HB-EGF in migratory IEC-18 cells.

Furthermore, the expression of GAPDH is increased in migratory cells. In contrast, the β-actin control did not show this variation to such an extent. GAPDH represents a key glycolytic enzyme which has long been considered as an invariant control in studies of gene expression. However, there are multiple reports that GAPDH is a functionally quite diverse protein whose gene expression varies with the extent of cell proliferation and differentiation [68, 69], and in particular also with cell motility [70].

Taken together, the results show that IEC-18 cells lose signs of terminal differentiation when they become migratory and also adopt a phenotype with increased expression of PAI-1, TGFα, HB-EGF, α-SMA, ODC and GAPDH. These changes are probably reversible upon the cells having reached confluence again. Similar alterations concerning PAI-1, TGFα, HB-EGF, and ODC were observed in epidermal keratinocytes after scratch wounding [52]. The genes induced in migratory cells are well suited to support the newly adopted function as they assist the process of restitution by enhancing motility and matrix remodeling and by adjusting to different energy requirements. However, there were no definite signs of EMT detectable in migratory cells. This situation is also comparable with epidermal wound healing [71].

For the future, the sensitive method described here will allow systematic gene expression studies in stationary and migratory cells particularly facilitating the molecular characterization of mucosal restitution processes in vitro. Generally, a broad medical impact can be expected from such studies. For example, de-differentiation has been shown to play an important role also for recovery of the renal epithelium after an ischemic insult [72].

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