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Abstract

Complete loss of cell anchorage triggers apoptosis in primary human colonic epithelial cells (CEC), a phenomenon known as anoikis. Besides the induction of pro-apoptotic events, activation of survival pathways was observed in detached intestinal epithelial cell lines, providing a transient apoptosis protection. However, nothing is known about molecular mechanisms protecting primary CEC from anoikis. In this study intact CEC crypts were isolated and kept in suspension, a condition which leads to the loss of cell-cell anchorage and induces anoikis. To reconstitute cell-cell contacts, cells were centrifuged to form cell aggregates. Induction of apoptosis was assessed by caspase-3 activity assay; activation of survival pathways was analyzed by Western blot. Immediately after loss of cell anchorage a rapid activation of survival proteins was observed before active caspase-3 could be detected. Src hyperactivation significantly contributed to transient protection from anoikis in CEC because its inhibition reversed the protecting effect of re-establishment of cell contacts. Basal levels of active Src in CEC from patients with inflammatory bowel disease were markedly reduced compared to control patients. These results demonstrate that loss of cell anchorage activates survival pathways in primary human CEC providing transient anoikis protection. Src is an important mediator of this mechanism and therefore constitutes a key regulatory molecule coordinating survival signals mediated by cell adhesion in primary human CEC.
Src is a sensor for loss of cell contact and mediates short-
term survival of primary human colonic epithelial cells

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Abstract

Background & Aims: Complete loss of cell anchorage triggers apoptosis in primary human colonic epithelial cells (CEC), a phenomenon known as anoikis. Besides the induction of pro-apoptotic events, activation of survival pathways was observed in detached intestinal epithelial cell lines, providing a transient protection against apoptosis. However, nothing is known about molecular mechanisms protecting primary CEC from anoikis. Methods: Intact CEC crypts were isolated and kept in suspension to induce anoikis. To reconstitute cell-cell contacts, cells were centrifuged to form a cell aggregate. Induction of apoptosis was assessed by caspase-3 Western blot and a colorimetric assay; activation of survival pathways was analyzed by Western Blot ((p)Akt, (p)Erk, (p)EGFR, (p)Src) and ELISA (NF-κB, EGF, TGF-α). The role of Src signaling was investigated using a specific inhibitor. Results: Immediately after loss of cell anchorage a rapid activation of all tested survival proteins was observed before active caspase-3 could be detected. Furthermore, CEC undergoing anoikis released large amounts of EGF and TGF-α. Src hyperactivation contributed to transient protection from anoikis in CEC because its inhibition reversed the protecting effect of re-establishment of cell contacts. Basal levels of active Src in CEC from patients with Crohn’s disease and ulcerative colitis were markedly reduced compared to control patients. Conclusions: Loss of cell anchorage activates distinct survival pathways in primary human CEC providing transient protection against anoikis. Src contributes to this mechanism and therefore constitutes a key regulatory molecule coordinating survival signals mediated by cell adhesion.
Introduction

Within the intact mucosa colonic epithelial cells (CEC) are tightly bound to the underlying extracellular matrix and form stable intercellular contacts with their adjacent cells. The cell anchorage-mediating structures – focal adhesions as well as tight and adherens junctions - serve a mechanical function and provide important outside-in survival signals to the cell. Loss of cell anchorage and the concomitant loss of such survival signals leads to the induction of apoptosis of most adherent cells, a phenomenon termed anoikis (1-3). Physiologically, anoikis is of importance in the life cycle of intestinal epithelial cells, which are shed from villus tips in the small intestine and from the inter-crypt epithelium of the colon, respectively, and die by anoikis (4; 5). Pathophysiologically, increased rates of epithelial apoptosis are associated with intestinal inflammation, especially inflammatory bowel disease (IBD). Mucosal tissues from IBD patients display significantly elevated rates of CEC apoptosis (6-10), resulting in disruption of epithelial barrier function and therefore aggravating inflammatory processes.

While loss of cell anchorage leads to the rapid induction of apoptosis in normal intestinal epithelial cells, carcinoma cells are largely resistant against anoikis. Several lines of evidence indicate that the acquisition of such anoikis resistance constitutes an essential prerequisite for tumor progression and metastases in most cancers of epithelial origin (11; 12): Firstly, tumor-derived cells tend to be anoikis-resistant and – in contrast to their non-malignant counterparts – frequently display the ability to survive and proliferate in the absence of cell adhesion as colonies in soft agar (13). Secondly, overexpression of oncogenes, such as Ras (14-17), β-catenin (18), and EGFR (19) in non-malignant epithelial cells renders these cells anoikis-resistant. Thirdly, in contrast, loss of oncogenes, such as Ras, in carcinoma cells
blocked their in vivo tumorigenicity and anoikis-resistance (17; 20). Anoikis-resistance of carcinoma cells might therefore serve as a novel tumor-specific therapeutic target. For these reasons studies of anoikis have gained importance during recent years as the characterization of mechanisms mediating sensitivity to anoikis is expected to provide important insights into epithelial homeostasis and carcinogenesis.

However, the exact molecular mechanisms that control anoikis-susceptibility are still largely unknown. Recent studies showed that immediately after detachment intestinal epithelial cell line cells activate a set of important survival kinases such as Erk1/2 and Src as well as NF-κB. By this mechanism they seem to be transiently protected from anoikis (21; 22).

All relevant recent studies were conducted with the IEC-18 and RIE-1 cell lines from normal rat intestinal epithelium but were not confirmed in primary epithelial cells. We therefore sought to investigate the regulation of NF-κB and distinct kinases known to play a role in survival of primary human CEC.
Materials and methods

CEC isolation, induction of CEC apoptosis, and reconstitution of cell-cell contacts

CEC were isolated as previously described (23). Briefly, normal human colonic mucosa from surgical specimens obtained from patients undergoing surgery for large bowel neoplasia (> 10 cm distance from the tumor) were cut into small strips. Mucus was removed by incubation for 30 minutes at room temperature in 1 mM DTT (Sigma, Taufkirchen, Germany) in 50 ml Hanks’ balanced salt solution (HBSS, PAA, Linz, Austria). Mucosal strips were incubated in 1 mM EDTA (Sigma, Taufkirchen, Germany) for 10 min at 37°C, briefly rinsed in HBSS, and transferred to tubes containing fresh HBSS at room temperature. Tubes were shaken vigorously 5 to 10 times. Mucosal strips were removed by passing the slurry over a coarse mesh (400 µm, Carl Roth GmbH, Karlsruhe, Germany). The suspension containing the detached CEC crypts was passed over a mesh filter (80 µm pore size, Sefar, Kansas City, MO), and intact CEC crypts were eluted by inverting the filter in serum-free culture medium (keratinocyte serum-free medium; GIBCO-BRL, Eggenstein, Germany). Using this method, CEC were purified as intact crypts. Thus cell-cell contacts within the CEC crypts were preserved while cell-matrix contact was lost. CEC were liberated from isolated crypts by suspension in a polypropylene tube on a whip-shaker, inducing apoptosis as described previously (4).

To reconstitute cell-cell contacts between CEC, cell aliquots were harvested after 0, 30, and 60 min and gently centrifuged to form a cell aggregate analogous to our pellet model introduced before (24). Each pellet was generated from 2 ml aliquots of the suspension by centrifugation at 100 x g and 4°C for 5 minutes. Pellets were subsequently incubated for 1h at 37°C and assessed for caspase-3 activation. As
controls cytosolic extracts from CEC before and after one additional hour of suspension culture were analyzed.

**NF-κB activity assay**

For the preparation of nuclear extracts CEC were washed with 5 ml ice-cold phosphatase inhibitor buffer (6.25 mM NaF, 12.5 mM β-glycerophosphate, 12.5 mM PNPP, 1.25 mM Na₃VO₄ in phosphate buffered saline) and centrifuged at 100 x g and 4°C for 5 min. Pellets were resuspended in 1 ml ice-cold hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM NaF, 10 µM Na₂MoO₄, 100 µM EDTA) and incubated for 15 min at 4°C. 50 µl Nonidet P-40 (Boehringer, Mannheim, Germany) were added and cells were centrifuged at 1000 x g and 4°C for 1 min. Pellets were resuspended in 50 µl of *complete lysis buffer* of the TransAM NF-κB assay kit (Active Motif, Carlsbad, CA), incubated on ice for 30 min under gentle agitation and centrifuged at 10,000 x g and 4°C for 10 min. Supernatants containing the nuclear extracts of CEC were stored at -80°C. Activation of NF-κB was quantified using the TransAM NF-κB assay kit according to the producer's instructions.

**Western blotting**

CEC cytosol was extracted as described (25) at the indicated times after incubation. Proteins were separated by sodium dodecyl-sulfate polyacrylamide electrophoresis and transferred to nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween20 phosphate buffered saline (blocking buffer), followed by 1h of incubation with the appropriate primary antibody diluted in blocking buffer. The following primary antibodies were used: anti-caspase-3 (1:3,000; Transduction Laboratories, Lexington, KY), anti-Akt anti-pAkt (Ser473), anti-pEGFR, anti-EGFR, anti-pErk1/2,
anti-Erk1/2, anti-pSrc (Y416), and anti-Src (all 1:1,000; Cell Signaling, Beverly, MA). Equal loading of the cytosolic samples was demonstrated by reprobing membranes with an anti-β-actin antibody (1:10,000; Chemicon, Hofheim, Germany). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies.

**Caspase activity assay**

Caspase-3-like activity in CEC cytosolic extracts was determined using the synthetic substrate N-acetyl-Asp-Glu-Val-Asp-para-nitroanilide (Ac-DEVD-pNA; Biomol, Hamburg, Germany). Cytosolic extracts (30 µg protein) were incubated with Ac-DEVD-pNA (200 µM) for 2h at 30°C. Caspase-like activity was quantified by absorption at 405 nm. As a positive control for caspase-3-like activity, extracts from CEC undergoing anoikis for 2h was used. BSA solution served as a negative control.

**ELISA of EGF and TGF-α**

Secretion of EGF (Biosource, Camarillo, CA) and TGF-α (Calbiochem, Darmstadt, Germany) was measured by ELISA according to the producers’ instructions. The detection limit was 1 pg/ml for EGF and 2.1 pg/ml for TGF-α.

**Inhibition of Src signaling**

To block Src signaling, CEC were incubated in the presence of 50 µM of the Src-specific inhibitor PP1 (Biomol, Hamburg, Germany), as this dose turned out to be the most effective in previous experiments (24).
Statistical analysis

Caspase-3-like activity data are expressed as median ± 5th/95th percentile. Statistical analysis was performed using the students T-test. Mean values were considered significantly different with a P-value of < 0.05. Linear regression of data obtained from EGF- and TGF-α ELISAs and NF-κB activity assays was performed and Spearman correlation was used for statistical analysis.
Results

Loss of cell anchorage induces activation of distinct survival pathways in primary human CEC

As reported previously, complete loss of cell anchorage results in a rapid induction of anoikis in primary CEC (4; 24-26), indicated by the rapid activation of caspases. However, recent studies indicate that besides the induction of pro-apoptotic signaling activation of pro-survival pathways is initiated in the rat intestinal epithelial cell line IEC-18. We therefore sought to assess whether this phenomenon also occurs in primary human CEC. To this aim, freshly isolated CEC were incubated as suspension culture. Aliquots were taken every 30 min and analyzed for the activation state of several important survival signaling molecules. Akt phosphorylation increased during the initial 60 min of incubation, but strongly and continuously decreased at the later time points (Fig. 1A). In contrast, phosphorylation of EGFR, Erk1/2 and Src strongly increased and maximum levels of phosphorylated EGFR and Erk1/2 were detected after 60 min of suspension culture, while phosphorylation of Src was still increasing thereafter (Fig. 1A).

In addition, activation of the transcription factor NF-κB was assessed. Nuclear extracts were prepared from CEC cultured in suspension and analyzed for their content of NF-κB p65. Immediately after loss of cell anchorage a time-dependent increase of NF-κB activity was observed (Fig. 1B). Within the first 30 min of suspension culture the amount of NF-κB p65 increased by 60% and was 2.5-fold increased after 120 min.

Noteworthy, the initial activation of the tested kinases and NF-κB was observed before caspase-3 was fully activated, because no relevant increase of caspase-3-like activity was detected before 60 min (Fig 1C). Taken together, these data provide evidence that loss of cell anchorage does not only induce pro-apoptotic
effects such as the activation of caspases, but also triggers several survival pathways in CEC.

**Loss of cell anchorage results in increased secretion of growth factors**

As demonstrated in Fig 1A, suspension culture of CEC induced phosphorylation of EGFR. We suspected that this activation might be due to an increased secretion of EGFR ligands such as EGF and TGF-α. To test this hypothesis, supernatants of CEC suspension cultures were assessed for their amounts of EGF and TGF-α. Fig. 2 shows that 90 min after induction of anoikis, strongly elevated levels of EGF were detected and a 2.4-fold increase of EGF concentration was observed after 120 min. High amounts of TGF-α were released from CEC immediately after loss of cell anchorage, resulting in a 40-fold increase of TGF-α within the initial 30 min of suspension culture and a 120-fold increase after 120 min.

Therefore, induction of anoikis triggers enhanced secretion of growth factors. The observed activation of EGFR might therefore be a result of elevated concentrations of stimulating ligands in the culture medium.

**Reconstitution of cell-cell contacts blocks CEC apoptosis**

We suspected that the activation of survival pathways might serve as a transient protection mechanism against an irreversible entry into anoikis as it was observed for IEC-18 and RIE-1 cells (21; 22; 27). We recently demonstrated that intact cell-cell contacts provide important survival signals to CEC and protect from anoikis (24). Therefore an experimental setting was established to test whether CEC
can be protected from anoikis by the observed cell-cell contact-mediated activation of survival pathways: CEC were incubated as suspension culture. Cell aliquots were harvested after 0, 30, and 60 min and gently centrifuged to form a cell aggregate. Cell aggregates were then incubated for another 60 min and assessed for caspase-3 activation. As controls, cytosolic extracts from CEC before and after one additional hour of suspension culture were analyzed. As shown in Fig. 3A progress of anoikis was effectively blocked when CEC were rescued by reconstitution of cell-cell contacts. Caspase-3-like activities in aggregated CEC were significantly reduced compared to CEC incubated as suspension culture. Moreover, the increased levels of Src phosphorylation observed after loss of cell anchorage (Fig. 1A) also strongly declined when cell-cell contacts were reconstituted (Fig. 3B).

These data indicate that immediately after loss of cell anchorage CEC are transiently protected against an irreversible entry into apoptosis.

**Inhibition of Src abolishes the anoikis-blocking effect of cell-cell contacts**

As a correlation between Src phosphorylation and rates of apoptosis was observed (Fig. 3), we asked whether and to which extent Src activity contributed to the observed protection from anoikis. If the increase in Src activation confers transient protection against anoikis, inhibition of Src activity should counteract anti-apoptotic effects of reconstituted cell-cell contacts. To answer this question, the rescue experiment shown in Fig. 3A was performed in the presence of the Src-specific inhibitor PP1. Inhibition of Src completely abolished the protection conferred by reconstituted cell-cell contacts in contrast to suspension cultures. No significant differences in caspase-3-like activities were detected between aggregated cells and CEC incubated in suspension culture (Fig. 4).
These data establish the essential role of Src for transient protection from apoptosis immediately after loss of CEC cell anchorage.

**Src phosphorylation is reduced in CEC of patients with IBD**

While increased Src expression and activity are described for colonic carcinoma cells (28), nothing is known about protein and activation levels of Src in the colonic epithelium of patients with IBD. To answer this question, cytosolic extracts of CEC were generated from controls (C) and patients with Crohn's disease (CD) and ulcerative colitis (UC) and assessed for their levels of pSrc and total Src (Fig. 5). CEC of IBD patients displayed similar amounts of total Src compared to controls. However, Src phosphorylation was reduced in four, markedly reduced in three and totally absent in three of twelve analyzed IBD CEC samples. It is important to note that all analyzed CEC were isolated from IBD patients who were not under immunosuppressive therapy. A reduction of Src phosphorylation caused by these drugs can therefore be excluded.

These results clearly demonstrate that basal Src activation is considerably decreased in the IBD mucosa.
Discussion

The current study provides evidence that loss of cell anchorage leads to a transient increase in the activity of EGFR, Erk1/2, Src and NF-κB and to secretion of EGF and TGF-α in primary human CEC. By blocking experiments we further identified activated Src as essential contributor to this transient protection against anoikis.

Several models exist for the investigation of apoptosis after loss of cell anchorage. Usually, non-transformed, immortalized cell lines, for instance IEC-18, RIE-1 (rat intestinal epithelial cell lines) (12; 21; 27), MDCK (1; 29) or carcinoma cell lines (28; 30) are utilized. In contrast, only few studies with primary cells are available (24; 26; 31-33). However, apoptosis in intestinal epithelial cell lines, such as IEC-18, can not be detected before several hours after loss of cell anchorage (12; 17) and detachment of colon carcinoma cell lines like HT-29 and CaCo-2 does not result in apoptosis at all if detachment is not coupled with serum depletion (34). In contrast, primary human CEC almost completely undergo anoikis within three hours after loss of cell anchorage (4; 24; 25). A similar rapid activation of the apoptotic cascade was only detected in mouse primary intestinal epithelial cells (32).

The molecular mechanisms controlling anoikis of normal and cancer cells are only incompletely understood. Their elucidation is expected to provide important insights into epithelial homeostasis and carcinogenesis (27). A central aspect in this context is the question concerning the “point-of-no-return”, referring to the condition of a cell which irreversibly leads to apoptosis (35). Recent data indicate that the activation of the caspase cascade is not an irrevocable process as assumed before. Instead, in addition to the induction of pro-apoptotic events, loss of cell anchorage also triggers several anti-apoptotic pathways and the ratio of these pro- and anti-
apoptotic signals seems to be important for the regulation of the apoptotic behaviour of epithelial cells (11; 21; 22; 27; 36).

Immediately after loss of cell anchorage of IEC-18 cells a transient increase of Src activity (21) as well as an induction of NF-κB activity were detected (22). Anoikis-induced NF-κB activation in turn led to an increased production of anti-apoptotic proteins such as Bcl-2 and IAP-1 (11; 22; 27). Due to these mechanisms, IEC-18 cells are transiently protected from anoikis.

The present data now for the first time provide evidence that the activation of survival pathways after loss of cell anchorage is not only a cell line-specific phenomenon but also occurs in primary human CEC. Besides the induction of Src and NF-κB (11; 21; 22; 27), activation of EGFR, Erk1/2, and increased secretion of EGF and TGF-α were also observed. All tested kinases already were phosphorylated to some degree immediately after isolation. It could not be assessed to which extent the isolation process alone influenced the activation state of each molecule. However, one could speculate that the mechanical strain during the isolation procedure could lead to a moderate induction of several stress-associated signaling pathways. Therefore, the observed phosphorylation levels at time point zero might be slightly elevated compared to CEC within the intact mucosa. Nevertheless incubation in suspension culture resulted in further strong activation of the analyzed proteins. It is important to note that in suspended CEC the initial activation of the tested survival pathways preceded the complete processing of caspase-3. The most rapid induction was observed for EGFR and Erk1/2, resulting in peak phosphorylation after 60 min and decreasing thereafter. In contrast, Src phosphorylation continuously increased during the monitored time interval. Kinetics of Src activation in CEC differ from previously published data derived from experiments with detached IEC-18 cells: While in primary CEC a continuous increase in Src phosphorylation occurred for at
least 120 min, IEC-18 cells displayed a decrease of activation already after 45 min (21) and after 4h no more phosphorylation was detectable (12).

Rosen et al. showed that Src kinase activity in detached IEC-18 cells can be triggered by exogenous TGF-α and that cells are protected from anoikis by this mechanism (12). In our CEC suspension cultures we observed an association between levels of Src phosphorylation and TGF-α concentration in the supernatant, suggesting an autocrine loop where TGF-α secreted by CEC contributes to the activation of endogenous Src kinases. However, TGF-α levels detected in CEC supernatants were 500- to 1000-fold lower than amounts of TGF-α added by Rosen (12) and, in our hands, blocking TGF-α effects with a neutralizing antibody did not significantly inhibit increased rates of apoptosis in the rescue model (data not shown). Therefore, TGF-α-induced activation of Src is unlikely the critical factor for the observed Src-dependent protection from anoikis.

Activities of EGFR, Erk1/2, NF-κB, and Src are associated with pro-survival features of the cell. Rapid activation of NF-κB and Src even seems to be essential for short-term survival of IEC-18 cells immediately after detachment, as inhibition of either molecule strongly accelerated anoikis (22; 27). Moreover, inhibition of NF-κB activation through conditional ablation of NEMO led to apoptosis of primary mouse CEC (37). Expression and activity of Src also strongly correlated with resistance to anoikis and were increased in more than 80% of human colon cancers compared to the normal colonic epithelium (28). Src is associated with cell-matrix contact-mediating focal adhesions (38) and cell-cell contact-mediating adherens junctions (39; 40) and was recently shown to be implicated in cell-anchorage-dependent signaling (24). It is therefore a potential key molecule in sensing the loss of cell anchorage.
Our data indicate that the transient activation of distinct survival pathways, especially of Src kinase, plays a similar important role for transient protection from anoikis in primary human CEC, because inhibition of Src annihilated the protective effect caused by re-establishing cell-cell contacts. This observation can be explained by two possible mechanisms: (1) Src hyperactivation and its effects on cellular survival are blocked by the inhibitor. Lacking Src-mediated signals, the apoptotic process might advance to pass the hypothesized “point-of-no-return” before cells were rescued from apoptosis by reconstitution of cell contacts. (2) Inhibition of Src abolished cell-cell contact-mediated survival signalling (24) and the apoptotic cascade is allowed to proceed after reconstitution of cell contacts.

It is important to note that directly after isolation (0 min) blockade of anoikis was observed even in the presence of the Src inhibitor. In the present study CEC were aggregated immediately after addition of the inhibitor. However, in previous experiments a 10 min preincubation with PP1 abolished the protective effect of cell aggregation (24). These results indicate that a pretreatment interval of up to 10 min is necessary to confer efficient Src inhibition and concomitantly highlights the importance of Src-dependent signalling for cell-cell contact-mediated survival of primary human CEC.

Taken together our results indicate that Src is a key regulatory molecule coordinating survival signals mediated by cell-matrix and cell-cell adhesion and that anti-apoptotic mechanisms induced by loss of cell anchorage delay the irreversible entry of CEC into apoptosis (Figure 6). Such transient protection from anoikis permits cellular survival if cellular anchorage can be reconstituted within a short interval (36; 41). This mechanism might be of pivotal importance for several physiological processes, e.g. cytokinesis of dividing cells or wound healing processes (21; 22).
Interestingly, CEC of IBD patients display markedly decreased levels of basal Src phosphorylation and, according to our findings, might be responsible for the elevated rates of epithelial apoptosis and for the impaired wound healing in the IBD epithelium. The analyzed CEC were isolated from IBD patients who were not under steroid or salicylate therapy, indicating that the observed reduction of Src phosphorylation is not due to drug therapy. So far, it is not known how IBD therapeutics influence activation potentials of signaling molecules. However, this question might be important as certain drugs used for IBD therapy might be able to restore activities of survival proteins such as Src and therefore reduce CEC apoptosis. This subject is part of our ongoing investigations.

Further studies will identify potential checkpoints of CEC apoptosis. Knowledge of these checkpoints might lead to therapeutic strategies reversing resistance to anoikis in precancerous stages of colorectal cancer.
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**Figure legends**

**Figure 1**

Induction of survival pathways after detachment of CEC.

CEC were incubated in suspension culture and activation of signaling molecules was determined. (A) Phosphorylation states and total levels of Akt, EGFR, Erk1/2 and Src were assessed within an interval of 120 min. (B) Nuclear cell extracts generated from CEC undergoing anoikis were analyzed for levels of NF-κB p65. n=18; r=0.84; p<0.0001. (C) Induction of apoptosis was confirmed by detection of caspase-3 activation. Western blot analysis was performed with an antibody detecting both the pro-enzyme (32 kDa) and the activated form (17 kDa) of caspase-3. For functional analysis of caspase-3-like activity the synthetic substrate Ac-DEVD-pNA was used. n=9, *p<0.05, **p<0.001.

**Figure 2**

The onset of CEC anoikis induces secretion of EGF and TGF-α.

Supernatants of CEC suspension cultures were analyzed for their content of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). EGF: n=25; r=0.727; p<0.0001. TGF-α: n=23; r=0.811; p<0.0001.

**Figure 3**

Blockade of anoikis by reconstitution of cell-cell contacts.

(A) CEC were incubated in suspension culture. At the indicated time points cell aliquots were harvested and gently centrifuged to form a cell aggregate and to reconstitute physiological cell-cell contacts. These aggregates were then incubated for another 60 min. As controls cytosolic extracts from CEC before aggregation and after one additional hour of suspension culture were collected and all samples were
assessed for caspase-3-like activities (n=10). (B) Cytosolic extracts generated in A from CEC with or without reconstituted cell-cell contacts were analyzed for Src phosphorylation.

Figure 4
Inhibition of Src abolishes the anoikis-blocking effect of cell-cell contact reconstitution.

CEC were incubated as suspension culture in the presence of PP1 (50 µM). At the indicated time points cell aliquots were aggregated to form pellets, which were then incubated for another 60 min. As controls CEC were collected before aggregation and after one additional hour in suspension culture. All samples were assessed for caspase-3-like activities (n=10).

Figure 5
Src activation is reduced in CEC of IBD patients.

Cellular extracts of CEC generated from controls and patients with CD and UC were assessed for their levels of pSrc and total Src.

Figure 6
Role of Src kinase for cell contact-mediated survival.

Within the intact mucosa cell-matrix and cell-cell contact-dependent survival signals are mediated via Src. Loss of cell-matrix-anchorage can be compensated by cell-cell contact-mediated signaling via Src. Loss of both cell-matrix and cell-cell anchorage results in Src hyperactivation, which contributes to transient protection from anoikis. During this interval of transient protection CEC can be rescued from anoikis by reconstitution of physiological cell contacts.
Figure 1

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Figure 2
Figure 3

A

![Box plot showing caspase-3-like activity OD values](image)

- **before aggregation**
- **aggregation + 1h pellet incubation**
- **+ 1h suspension culture**

- **p=0.002**
- **p=0.003**
- **p=0.006**

- **0 min**
- **30 min**
- **60 min**

**Suspension culture**

B

![Western blots showing pSrc and Src](image)

- **pSrc**
- **Src**

- **-**: before aggregation
- **+**: aggregation + 1h pellet incubation
Figure 4

The figure shows a box plot representing caspase-3-like activity (OD_{405}) over time in suspension culture (50 μM PP1). The x-axis represents time points: 0 min, 30 min, and 60 min. The y-axis represents the caspase-3-like activity. Different conditions are indicated by different patterns:

- White bars represent before aggregation.
- Diagonal striped bars represent aggregation + 1h pellet incubation.
- Solid bars represent + 1h suspension culture.

Significance levels are indicated as follows:

- p=0.020 (statistically significant).
- n.s. (not statistically significant).

The data shows a significant difference at 30 min compared to 0 min (p=0.020), but no significant difference between 30 min and 60 min.
Figure 5

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Figure 6
Reference List


