Epidermal growth factor receptor is involved in enterocyte anoikis through the dismantling of E-cadherin-mediated junctions

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Submitted 30 April 2008; accepted in final form 25 November 2008

Lugo-Martínez V, Petit CS, Fouquet S, Le Beyec J, Chambaz J, Pinçon-Raymond M, Cardot P, Thenet S. Epidermal growth factor receptor is involved in enterocyte anoikis through the dismantling of E-cadherin-mediated junctions. Am J Physiol Gastrointest Liver Physiol 296: G235–G244, 2009. First published December 4, 2008; doi:10.1152/ajpgi.90313.2008.—Enterocytes of the intestinal epithelium are continually regenerated. They arise from precursor cells in crypts, migrate along villi, and finally die, 3–4 days later, when they reach the villus apex. Their death is thought to occur by anoikis, a form of apoptosis induced by cell detachment, but the mechanism of this process remains poorly understood. We have previously shown that a key event in the onset of anoikis in normal enterocytes detached from the basal lamina is the disruption of adherens junctions mediated by E-cadherin (Fouquet S, Lugo-Martínez VH, Faussat AM, Renaud F, Cardot P, Chambaz J, Pinçon-Raymond M, Thenet S. J Biol Chem 279: 43061–43069, 2004). Here we have further investigated the mechanisms underlying this disassembly of the adherens junctions. We show that disruption of the junctions occurs through endocytosis of E-cadherin and that this process depends on the tyrosine-kinase activity of the epidermal growth factor receptor (EGFR). Activation of EGFR was detected in detached enterocytes before E-cadherin disappearance. Specific inhibition of EGFR by tyrphostin AG-1478 maintained E-cadherin and its cytoplasmic partners β- and α-catenin at cell-cell contacts and decreased anoikis. Finally, EGFR activation was evidenced in the intestinal epithelium in vivo, in rare individual cells, which were shown to lose their interactions with the basal lamina. We conclude that EGFR is activated as enterocytes become detached from the basal lamina, and that this mechanism contributes to the disruption of E-cadherin-dependent junctions leading to anoikis. This suggests that EGFR participates in the physiological elimination of the enterocytes.

intestinal epithelial cells; cell-cell junctions; anoikis; epidermal growth factor receptor; E-cadherin

CELL ADHESION AND CELL POLARIZATION in epithelial tissues are ensured by several types of junctional complexes, among which adherens junctions (AJ) are a major component. The assembly, maintenance, and disassembly of AJ are dynamic processes that are tightly regulated during tissue morphogenesis and homeostasis (9, 20). The epithelial lining of the mammalian intestine is renewed every 3–4 days, an exception to the rule that cell renewal is an extremely slow process that is formally proven (7, 58), neither have the signals been identified that would explain why enterocytes are highly sensitive to loss of anchorage and enter apoptosis within a few minutes following detachment from the basal lamina (13). Our laboratory has further demonstrated that loss of E-cadherin, the main transmembrane protein of epithelial AJ, is a key event for the induction of anoikis (13). These results suggest that E-cadherin-mediated adhesion could be the “guardian of survival” for enterocytes, consistent with the observation that apoptosis is increased in the intestinal epithelium of transgenic mice in which E-cadherin function has been abrogated (23).

E-cadherin has been shown to play a key role in AJ assembly. Ca2+-dependent homophilic ligation between cadherin molecules on adjacent cells initiates the assembly of a large multimolecular adhesion complex, which is connected to the actin cytoskeleton through proteins of the catenin family (49, 53, 59). Mechanisms that orchestrate AJ disassembly and lead
to the acquisition of a mesenchyme-like phenotype have been extensively studied, due to their implication in development and in malignant cell transformation (6, 9). In cellular models that recapitulate these processes, it has been shown that endocytosis of E-cadherin is a key event in AJ disassembly. Phosphorylation of E-cadherin, and of its catenin partners, by receptor tyrosine kinases (RTK) or non-RTK, has been shown to regulate endocytosis of the E-cadherin complex and AJ disruption (3, 36, 38).

Control of AJ disassembly during the physiological turnover of adult epithelia has been less well investigated. In the present study, we intended to decipher the mechanisms underlying the disruption of E-cadherin-mediated adhesion following loss of anchorage of enterocytes. We show, for the first time, that E-cadherin is internalized by endocytosis in enterocytes undergoing anoikis, and we present strong evidence that activation of the epidermal growth factor receptor (EGFR) contributes to E-cadherin endocytosis, loss of cell-cell adhesion, and subsequent apoptosis. Finally, we show that EGFR activation can be visualized in vivo, within the intestinal epithelial lining, in cells undergoing detachment from the basal lamina, suggesting that EGFR activation could be involved in the physiological shedding of enterocytes.

MATERIALS AND METHODS

Isolation of intestinal epithelial cells and induction of anoikis. Intestinal villus epithelium was isolated as entire epithelial linings by a method modified from Perreault and Beaulieu (39) and described previously in detail (13). Briefly, the small intestine from adult B6CBA mice was everted, washed, and cut into 5-mm pieces. Detachment of the epithelium from the mesenchyme was achieved by incubation for 2 h at 4°C in MatriSperse Cell Recovery Solution (BD Biosciences; Erembodegem, Belgium), a nonenzymatic solution initially designed to recover epithelial cells grown on EHS matrix. Detached epithelial linings were resuspended in ice-cold DMEM (Gibco, Invitrogen Life Technologies, Cergy Pontoise, France) containing 4.5 g/l glucose and supplemented with 20 mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin, 5 ng/ml recombinant human EGF (Gibco, Invitrogen Life Technologies), 0.2 IU/ml insulin (Novo Nordisk, Paris La Défense, France), and 5% fetal bovine serum (AbCys, Paris, France). Anoikis was induced by incubation at 37°C in suspension in the presence of 5% CO₂.

When indicated, the following drugs were added to MatriSperse and to the culture medium: the inhibitor of clathrin-dependent endocytosis chlorpromazine (100 μM, Sigma-Aldrich, Saint Quentin Fallavier, France), and the vacuolar proton pump inhibitor bafilomycin A1 (1 μM, Sigma-Aldrich). The following inhibitors were purchased from Calbiochem (VWR Internationals, Strasbourg, France): the broad-spectrum tyrosine kinase inhibitor genistein (200 μM), the inhibitor of the Src family tyrosine kinases PP2 (20 μM) and its negative control peptide PP3 (20 μM), the EGFR inhibitor tyrphostin AG-1478 (100 nM), and its negative control drug AG-9 (500nM), and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (50 μM). The Abl inhibitor STI571 (15 μM) was generously provided by Novartis Pharma (Rueil-Malmaison, France). For each drug, preliminary dose-effect experiments were performed.

Antibodies. The following antibodies were used: anti-E-cadherin (ECCD-2, TaKaRa Bio Europe, Gennep, France), anti-EEA1, anti-α-catenin (Abcam, Cambridge, UK), anti-β-catenin, anti-EGFR,
and anti-EGFR phosphorylated on tyrosine 845 or tyrosine 1173 (Cell Signaling Technology, Saint Quentin-en-Yvelines, France). Alexa-488 and Alexa-546-conjugated anti-IgG were used as secondary antibodies (Molecular Probes, Invitrogen Life Technologies).

**Immunofluorescence, imaging, and data processing.** Freshly removed small intestines or epithelial linings maintained in suspension for the indicated times were washed in ice-cold PBS, fixed in 4% paraformaldehyde, and embedded in Tissue Tek OCT (Shandon), and 7-μm cryosections were cut. Sections were sequentially incubated in permeabilizing buffer (1% wt/vol BSA, 0.1% vol/vol Triton X-100 in PBS) for 10 min at room temperature, and then with primary and secondary antibodies diluted in blocking buffer (anti-E-cadherin 1/500, all other primary antibodies 1/50, Alexa-conjugated secondary antibodies 1/400). Nuclei were stained with 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS.

Images were acquired on a Zeiss Axiophot epifluorescence microscope equipped with an Axiocam camera and Axiovision 4.5 software (Carl Zeiss, Le Pecq, France). The images presented in Figs. 1–6 were recorded with an oil immersion ×100 objective. For each condition, panels of at least 10 images were analyzed in Axiosvision 4.5, and the more representative one was chosen for presentation in the figure. Images were converted into TIFF files and assembled using Adobe Photoshop CS2 9.0 and Adobe Illustrator CS2 12.0.0. The percentage of cells displaying membrane E-cadherin was determined as follows: for each experiment (n ≥ 3) and each condition, at least three different sections were examined. Five images recorded with an oil immersion ×25 objective were necessary to cover the whole section. The percentage of cells displaying membrane E-cadherin was determined for each section, on the totality of the cells present on the five images. The results presented are means ± SD of three sections of one representative experiment. For some indicated experiments, quantifications were performed using the TissueQuest 2.2 software (TissueGnostics, Vienna, Austria), which allows a fluorescence analysis at the single-cell level, analogous to cytometry (11). This quantification was performed for each sample on 5–15 independent fields photographed at the ×25 objective (a minimum of 10,000 cells was analyzed for each sample). DAPI nuclear staining was used for cell identification.

Analysis of colocalization of internalized E-cadherin and EE1A was performed on a Zeiss LSM510 confocal laser-scanning microscope with a planapochromatic ×63 1.4 numerical aperture objective.

**Determination of apoptosis.** Apoptosis was quantified by counting apoptotic nuclei on cryosections after staining with DAPI (100 ng/ml, Research Organics). A minimum of 1,000 nuclei/section was counted for each determination. The results are the means ± SD of three sections of one representative experiment.

**Correlative light electron microscopy.** The correlative light electron microscopy method was adapted from Powell et al. (42). Briefly, cryosections of intestine were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde and labeled with anti-EGFR phosphorylated on tyrosine 845, as described above, except that the secondary antibody was an Alexa fluor-488-fluororhodamine-conjugated anti-rabbit Fab′ (Nanoprobes, Argene, Varilhes, France). EGFR labeled cells were located on sections using the Zeiss Axiophot epifluorescence microscope. Then sections were postfixed in 2.5% glutaraldehyde, and HQ Silver (Nanoprobes) was used to enhance the gold probe during 5 min in a dark room, following the manufacturer’s instructions. Samples were then dehydrated in graded alcohol and embedded in Epon resin (Poly/Bed 812, Polysciences Warrington, PA). Ultrathin sections of ~65 nm were counterstained with uranyl acetate (30 min, 40°C) and lead citrate (10 min, 25°C) using an LKB 2168 electrostainer. Observations were performed on a Hitachi 7700 microscope equipped with an Axiocam camera and Axiovision 4.5 software.

**Fig. 2.** Tyrosine kinase activity is involved in the disassembly of E-cadherin-mediated junctions of enterocytes undergoing anoikis. A: immunolabeling of E-cadherin was performed on detached intestinal epithelial cells after a 30-min incubation in suspension in the presence of vehicle alone (control), 200 μM genistein (wide spectrum tyrosine kinase inhibitor), 20 μM PP2 (src inhibitor), 20 μM PP3 (control peptide), 10 μM AG-957, or 15 μM STI571 (Abl inhibitors). Nuclei were stained by DAPI. Dotted lines delineate zones of cells that have lost junctional E-cadherin staining. E-cadherin was maintained at cell-cell junctions, and cells remained attached as coherent sheets only in the presence of genistein or AG-957. Bar: 10 μm. **B:** percentages of cells exhibiting membrane E-cadherin labeling were determined using the TissueQuest software. The results are expressed as the fold increase related to control cells (treated with the vehicle alone) and are the means ± SD of three independent experiments. *P ≤ 0.05 related to control cells as determined by Student’s t-test.
vations were made using a JEOL CX100 equipped with a Gatan Digital camera (3.11.0), and the micrographs were processed with softwares Gatan and Adobe Photoshop CS2 9.0 and assembled with Adobe Illustrator CS2 12.0.0.

RESULTS

Disassembly of E-cadherin-mediated junctions and E-cadherin endocytosis following detachment of intestinal epithelial linings. The fate of E-cadherin was studied in a model of enterocyte anoikis, which we developed previously (13). In this model, normal intestinal epithelial cells are detached from the underlying basal lamina as entire epithelial linings and cultured in suspension (Fig. 1A). As our laboratory previously showed (13), E-cadherin was initially present all along the basolateral membrane of the well-organized and still polarized epithelial linings, but was progressively lost from cell-cell contacts during incubation in suspension (Fig. 1A; see also the control in Fig. 1, B and C). In the presence of chlorpromazine, an inhibitor of clathrin-dependent endocytosis (56), detached enterocytes maintained E-cadherin at their surface and remained organized as epithelial sheets (Fig. 1B), suggesting that loss of anchorage triggers E-cadherin internalization by a clathrin-dependent endocytic pathway. Treatment of epithelial sheets with the vacuolar proton pump inhibitor bafilomycin A1, known to slow down the traffic from sorting endosomes to late endosomes and then to lysosomes (55), enhanced the accumulation of E-cadherin into intracellular vesicles (Fig. 1C), which exhibited partial colocalization with the early endosome marker EE1A (Fig. 1D). Altogether, these results show that, in response to detachment, enterocytes undergo disassembly of E-cadherin-mediated cell-cell interactions and clathrin-dependent endocytosis of E-cadherin.

Fig. 3. Inhibition of epidermal growth factor receptor (EGFR) maintains E-cadherin at the cell-cell junctions and improves cell survival following loss of anchorage. A: double immunolabeling for E-cadherin (green on the merge image) and β-catenin (red) was performed on detached intestinal epithelial cells after a 30-min incubation in suspension in the presence of vehicle alone (control) or 100 nM AG-1478. Nuclei were stained by DAPI. Arrowheads indicate apoptotic nuclei, which are shown with a higher magnification and quantified in D. Bar: 10 μm. B: quantification of the percentage of cells with membrane E-cadherin in the experiment described in A. Cells exhibiting membrane E-cadherin labeling were counted on three independent sections. The results are the means ± SD of one representative experiment. ***P ≤ 0.001 as determined by Student’s t-test. C: cells treated as in A were immunolabeled for E-cadherin (green on the merge image) and α-catenin (red). Nuclei were stained by DAPI. Bar: 10 μm. D: quantification of apoptotic nuclei. The determination was performed by direct observation using the ×100 objective. A minimum of 1,000 nuclei/section was counted for each determination. The results are the means ± SD of three independent sections of one representative experiment. *P ≤ 0.05 as determined by Student’s t-test. Photographs show higher magnifications of the nuclei presented in A; a typical condensed nucleus is highlighted by an arrowhead. In A and C, dotted lines delineate zones of cells that have lost junctional E-cadherin staining.
A tyrosine kinase activity is involved in the disassembly of E-cadherin-mediated junctions in enterocytes undergoing anoikis. The destabilization of E-cadherin-mediated junctions has been shown to be under the control of several signaling molecules, among which the receptor and non-RTK play important roles (6, 9, 36). E-cadherin was maintained at cell-cell contacts in enterocytes detached in the presence of genistein, a broad spectrum tyrosine kinase inhibitor (Fig. 2). This suggested the involvement of a tyrosine kinase activity in the signaling pathway triggered by loss of anchorage and leading to E-cadherin loss from cell-cell junctions. Although Src family kinases (SFKs) have been shown to be involved in the destabilization of E-cadherin-mediated junctions in transformed cells during the epithelial-to-mesenchymal transition, as well as in normal cells (1, 37), E-cadherin loss from cell-cell junctions during enterocyte anoikis was not prevented by the SFK inhibitor PP2 (Fig. 2). By contrast, E-cadherin was maintained at cell-cell junctions, and intestinal epithelial cells remained more cohesive in the presence of AG-957, an inhibitor of Abl kinase, recently shown to be involved in the inhibition of N-cadherin-mediated cell adhesion (43). However, the involvement of Abl in E-cadherin loss from detached enterocytes was not confirmed, since no protective effect was observed in the presence of STI571, another specific inhibitor of Abl activity (Fig. 2). Considering that AG-957 has also been shown to inhibit EGFR tyrosine kinase activity (51), we next investigated the possible involvement of EGFR in E-cadherin loss upon enterocyte detachment.

Inhibition of EGFR maintains E-cadherin at the membrane and decreases anoikis. Figure 3A shows that the disappearance of E-cadherin from cell-cell contacts induced by detachment was prevented by the EGFR inhibitor tyrphostin AG-1478. The presence of AG-1478 resulted in a 2.5-fold increase in the percentage of cells exhibiting membrane E-cadherin labeling (Fig. 3B). As shown in Fig. 3, A and C, EGFR inhibition also maintained the cytoplasmic partners of E-cadherin, β-catenin, and α-catenin, at cell-cell contacts, suggesting that AJ were intact. The control peptide AG-9 (tyrphostin A1) had no statistically significant effect compared with nontreated cells (Supplemental Fig. S1; the online version of this article contains supplemental data).

Fig. 4. EGFR is activated upon loss of anchorage from the basal lamina. Loss of E-cadherin and activation of EGFR occur in absence of EGF. A: double immunolabeling of E-cadherin (green on the merge image) and of EGFR phosphorylated (P-EGFR) on tyrosine-845 (pY-845) or on tyrosine-1173 (pY-1173) (red) was performed on intestinal epithelial cells immediately after detachment (time 0) or after a 10-min or 30-min incubation in suspension. Cells labeled for the activated EGFR were found in coherent sheets labeled for E-cadherin. B: E-cadherin immunolabeling and DAPI staining of nuclei were performed on intestinal epithelial cells after a 30-min incubation in suspension in standard culture medium or in medium devoid of EGF and serum (w/o EGF). Dotted lines delineate zones of cells that have lost junctional E-cadherin staining. Note that dismantling of epithelial sheets, loss of E-cadherin, and apoptosis, as shown by nuclei condensation (examples shown by arrowheads) occurred similarly under both conditions. Bar graphs show the quantification of E-cadherin loss using the TissueQuest software. The results are the means ± SD of one representative experiment and show no statistically significant difference according to the Student’s t-test. C: immunolabeling of EGFR phosphorylated on tyrosine-845 was performed on cells treated as in B. Activation of EGFR was detected under both conditions. Bars: 10 μm.
Since our laboratory previously showed that E-cadherin loss was involved in the onset of enterocyte anoikis (13), we investigated whether the maintenance of E-cadherin-mediated cell-cell adhesion through EGFR inhibition modulated cell death. EGFR inhibition by AG-1478 during incubation in suspension resulted in a 2.5-fold decrease in the percentage of cells exhibiting apoptotic nuclei (Fig. 3D), which our laboratory previously showed to be strictly correlated with the level of cells exhibiting activated caspase-3, sub-G1 DNA content, and internucleosomal degradation of DNA in our anoikis model (13).

These data suggest that activation of EGFR in response to loss of anchorage contributes to the dismantling of E-cadherin-mediated cell-cell junctions leading to anoikis.

**EGFR is activated in a ligand-independent manner upon loss of anchorage from the basal lamina.** To further implicate EGFR in the destabilization of E-cadherin junctions upon loss of anchorage, we analyzed EGFR activation in detached epithelial sheets. We performed immunofluorescence labeling using several antibodies directed against EGFR phosphorylated on various tyrosine residues involved in EGFR activation (31). Figure 4A presents the results obtained with the antibodies directed against EGFR phosphorylated on tyrosine 845 and on tyrosine 1173. Staining was very faint immediately after detachment (time 0). In contrast, after 10 min in suspension, a few individual cells exhibited an intense labeling, which extended to entire cell clusters after 30 min. Activated EGFR was detected in cells that were still included in coherent sheets exhibiting E-cadherin staining at cell-cell contacts. Although we found several examples of cell clusters exhibiting EGFR activation and a weaker membranous E-cadherin staining (Fig. 4A), EGFR activation was not associated with a significant decrease of E-cadherin intensity at cell-cell contacts (Supplemental Fig. S2). The very weak expression of EGFR in normal enterocytes compared with the enterocyte cell line Caco-2/TC7 (Supplemental Fig. S3), consistent with the well-described EGFR overexpression in carcinoma cells (46), did not allow us to clearly evidence EGFR activation by a biochemical method in our model.

Adhesion has been related to ligand-independent activation of several RTKs (8). We thus investigated whether EGFR activation and subsequent E-cadherin loss were dependent on binding of EGF to its receptor. The anoikis assay was performed in standard medium containing 5% fetal calf serum and 5 ng/ml EGF, or in medium devoid of EGF and serum. Absence of EGF did not modify E-cadherin disappearance or apoptosis induction (Fig. 4B). As shown in Fig. 4C, EGFR activation was detected in detached enterocytes in the absence of EGF. These data indicate that the activation of EGFR is triggered by loss of anchorage to the basal lamina, independently of the presence of its ligand.

**PI3K activity is involved in AJ disassembly leading to anoikis.** Activation of EGFR is known to activate a network of signaling pathways, resulting in changes in protein function. We focused on the PI3K pathway, which has been involved in the regulation of AJ stability (40, 45), and which can be activated following ligand-independent EGFR activation (8). The PI3K inhibitor LY-294002 strongly maintained E-cadherin and β-catenin at cell-cell contacts (Fig. 5A), as well as α-catenin (Supplemental Fig. S4). Quantification revealed a fourfold increase in the percentage of cells exhibiting E-cadherin labeling 30 min after detachment (Fig. 5B), which was accompanied by a 3.7-fold decrease in apoptosis (Fig. 5C). Therefore, the PI3K pathway is likely necessary for AJ disassembly and anoikis onset in enterocytes upon loss of anchorage.

**EGFR is activated in vivo, in individual cells of the intestinal villus epithelium.** Our ex vivo model of anoikis, in which detachment from the basal lamina triggers loss of E-cadherin-mediated cell-cell adhesion, is consistent with observations on native tissues. Previous electron microscopy studies described a “two-step” remodeling of cell adhesion in shedding enterocytes.
cytes at the villus tip, involving sequential loss of cell-matrix and cell-cell interactions (48). Furthermore, it has been recently reported that loss of E-cadherin precedes the extrusion of cells at the villus tip (29). We, therefore, investigated whether the destabilization of E-cadherin complexes through EGFR activation, shown in an ex vivo model, could also be involved in intestinal epithelial cell shedding in vivo. In accordance with this hypothesis, we detected, within the villus epithelial lining, individual cells labeled with antibodies directed against EGFR phosphorylated on either tyrosine 845 or tyrosine 1173 (Fig. 6). Such cells were observed at the villus tip, but also at lower positions in the villus. Their frequency was comprised between 0.1 and 1 cell per villus section, a range comparable to the frequency of apoptotic cells detected in the villi (12, 21). Cells exhibiting activated EGFR were positive for E-cadherin labeling. These cells were frequently displaced outside the epithelial lining, as shown by the position of their nucleus (Fig. 6, middle), suggesting that they could have already lost their interactions with the basal lamina.

To confirm this point, a correlative light and electron microscopy study was performed. Jejunum sections were labeled with the antibody directed against phosphorylated EGFR revealed by a fluoronanogold-conjugated secondary antibody. Labeled cells were precisely located by epifluorescence microscopy (Supplemental Fig. S5) before being examined by electron microscopy. Figure 7 shows that nonlabeled cells were systematically closely connected with each other, their basal pole being apposed to a continuous basal lamina (Fig. 7A). In contrast, cells exhibiting activated EGFR were clearly loosing their interactions with the basal lamina (Fig. 7B). Numerous cytoplasmic processes extending from their basal pole were visualized, as reported for shedding enterocytes (32, 48). It is worth noting that these cells were still closely interacting with adjacent cells through the upper part of their lateral membrane (Fig. 7B, inset b), in accordance with the presence of E-cadherin labeling (Fig. 6), but were loosing their interactions at the lower level of their lateral membrane. Occasionally, EGFR-activated cells undergoing quite complete extrusion were visualized (Fig. 7C). Altogether, these data show that, in accordance with our ex vivo anoikis model, EGFR activation in vivo coincides with a loss of interaction with the basal lamina and is related to the physiological shedding process of enterocytes.

**DISCUSSION**

Three lines of evidence in this study identify EGFR as a key actor in the process of disassembly of the E-cadherin-mediated junctions leading to enterocyte anoikis. First, the specific inhibition of EGFR tyrosine kinase activity by tyrphostin AG-1478 (31) during enterocyte detachment leads to the maintenance at cell-cell contacts of E-cadherin and its cytoplasmic partners, β- and α-catenin, and to a concomitant improvement of cell survival. Second, EGFR phosphorylated on tyrosine residues, which are important for its activity (10), was detected within the detached intestinal epithelial sheets, before E-cadherin disappearance. Finally, the same phosphorylated forms of EGFR were detected in vivo, within the intestinal epithelium of villi, in individual cells whose frequency, position, and ultrastructure were compatible with their imminent detachment from the epithelial lining.

We report, for the first time, the endocytosis of E-cadherin in cells whose fate is to enter apoptosis, while, in several other apoptosis models, E-cadherin has been shown to be cleaved by caspases or other proteases, as a consequence of activation of the death program (33, 44, 47, 50). We show that inhibiting clathrin-mediated endocytosis by chlorpromazin is sufficient to maintain intestinal cells as coherent epithelial sheets, suggesting that E-cadherin endocytosis could be a primary event in junction dismantling leading to apoptosis. This result is consistent with the recent finding that endocytosis could actively inhibit the cadherin adhesive machinery (54). Endocytic trafficking of E-cadherin is known to contribute to the regulation of AJ stability (6, 9, 60). At the lateral membrane, cadherin molecules undergo constitutive endocytosis and are recycled back to the plasma membrane. This endocytic/exocytic traffic is minimal in confluent and poorly motile epithelial monolayers and is markedly enhanced as cells disassemble their intercellular contacts and acquire a mesenchymal-like phenotype, upon calcium depletion (30), but also in response to activation of receptor or non-RTKs (9, 15, 26). Although the Src kinase has been shown to be largely involved in the dismantling of cadherin contacts (1, 14), we show here that its inhibition had...
no effect on the loss of junctional E-cadherin induced by the detachment of enterocytes from the basal lamina. This result could be explained by the observation in colon cancer cells that Src-induced deregulation of E-cadherin requires integrin signaling (2), with such signaling being interrupted upon loss of Src-induced deregulation of E-cadherin requires integrin signaling. The operating event in the cascade leading to enterocyte anoikis (13), a result that has been recently extended to colonic epithelial cells (24). However, both EGFR and PI3K pathways are well-described positive regulators of cell survival. These conflicting data illustrate the complexity of the interactions occurring between signaling pathways and adhesion molecules. In carcinoma cell lines, the disruption of E-cadherin junctions mediated by tyrosine kinases invariably leads to the acquisition of a migratory

to engagement of either integrin or cadherin (8). Interestingly, tyrosine phosphorylation of β-catenin through its association with EGFR in a ligand-independent manner has also been reported to be induced by detachment of normal breast epithelial cells (52). Altogether, these observations suggest that both adhesion or detachment, as we report here, would be able to activate EGFR. The maintenance of E-cadherin-mediated cell-cell adhesion in detached enterocytes in the presence of the PI3K inhibitor suggests that this pathway, which is a known target of activated EGFR, could also play a role in E-cadherin endocytosis, as shown by others during HGF- or TGF-β-induced disassembly of AJ (26, 40, 45).

It should be noted that blocking either EGFR or PI3K pathways is sufficient to decrease anoikis. The fold increase of E-cadherin-positive cells is identical to the fold decrease of apoptotic cells when either of these two pathways is inhibited. This result supports our previous finding that the disassembly of E-cadherin-mediated intercellular junctions is an upstream event in the cascade leading to enterocyte anoikis (13), a result that has been recently extended to colonic epithelial cells (24). However, both EGFR and PI3K pathways are well-described positive regulators of cell survival. These conflicting data illustrate the complexity of the interactions occurring between signaling pathways and adhesion molecules. In carcinoma cell lines, the disruption of E-cadherin junctions mediated by tyrosine kinases invariably leads to the acquisition of a migratory

EGFR-activated cells in the intestinal epithelium are engaged in the shedding process. Correlative light electron microscopy was performed to examine the interactions of EGFR-activated cells with the basal lamina and with their neighboring cells in the intestinal epithelium. Immunolabeling was performed on jejunal sections using the antibody against EGFR phosphorylated on tyrosine-845 revealed by a fluoronanogold-conjugated secondary antibody. Sections were examined by electron microscopy after location of the EGFR-labeled cells by epifluorescence microscopy. In all three panels, the photographs labeled with a lowercase letter show higher magnifications of the regions delineated by rectangles in the left-side photographs, and schematic drawings of the cells are shown, in which the EGFR-labeled cell is shaded dark gray. A: a field of nonlabeled cells is shown. Cells are well aligned, their upper lateral membrane are in close contact with adjacent cells (arrowheads in a), and their basal membrane is in contact with the basal lamina (bl; arrowheads in b). C: an extruded cell labeled for activated EGFR is shown. The labeling detected by silver-enhanced gold particles is better visualized in b, which was obtained from an adjacent ultrathin section, explaining the absence of the debris observed in the left-side image. As for the nonlabeled cells, upper lateral membranes are closely apposed to those of adjacent cells (arrowheads in b). However, the lower lateral membranes are disconnected from the neighboring cells (arrows in B), and the contact with the basa lamina is almost completely disrupted (b'). C: an extruded cell labeled for activated EGFR is shown. The cells are retained to the adjacent enterocytes only by a thin cytoplasmic strand (c). Note that the protocol of immunolabeling on cryosections before retixation and procession for electron microscopy does not allow an optimal preservation of the cell structures, explaining the nuclei alterations in A and C or the presence of cell debris in B. Bars: 5 μm in left-side photographs and 1 μm in insets. gc, Goblet cell.

beside SFKs, EGFR is one of the RTK that have been found to modulate cadherin adhesive function (8, 36). EGF induces morphological changes in carcinoma cell lines through functional alteration of the E-cadherin complex (22, 25, 34). While the molecular mechanisms orchestrating E-cadherin endocytosis in Src-transformed cells have been defined through the isolation of the E3 ubiquitin-ligase Hakai (15), the mechanisms by which EGFR activation induces the dismantling of the E-cadherin complex and its internalization remain to be elucidated. EGF has been recently shown to induce E-cadherin macropinocytosis and recycling without overt loss of cell-cell adhesion in breast adenocarcinoma cells (5). The operating mechanisms in our model are certainly different, since we show that EGFR activation following detachment and the consequent loss of E-cadherin occur independently of the presence of EGF. Ligand-independent activation of several RTK can occur in an adhesion-dependent manner, in response to engagement of either integrin or cadherin (8). Interestingly, tyrosine phosphorylation of β-catenin through its association with EGFR in a ligand-independent manner has also been reported to be induced by detachment of normal breast epithelial cells (52). Altogether, these observations suggest that both adhesion or detachment, as we report here, would be able to activate EGFR. The maintenance of E-cadherin-mediated cell-cell adhesion in detached enterocytes in the presence of the PI3K inhibitor suggests that this pathway, which is a known target of activated EGFR, could also play a role in E-cadherin endocytosis, as shown by others during HGF- or TGF-β-induced disassembly of AJ (26, 40, 45).

AJP-Gastrointest Liver Physiol • VOL 296 • FEBRUARY 2009 • www.ajpgi.org
or mesenchymal-like phenotype, a process referred to as epithelial-to-mesenchymal transition (9), which is generally associated with resistance to anoikis (17). However, the acquisition of this invasive phenotype is not the sole biological outcome triggered by disruption of cadherin complexes via activation of tyrosine kinases. For example, in normal endothelial cells, the VEGFR-2-induced disruption of the VE-cadherin-mediated endothelial barrier has been recently shown to be responsible for the impact of VEGF on vascular leakage (16). Therefore, the consequences of AJ dismantling probably depend on cell type and transformation status.

One important point raised by our results is the in vivo activation of EGFR shown within the epithelial lining. These activated cells still interact with their neighbors and present E-cadherin at their lateral membranes, but are loosing their interactions with the basal lamina, a morphology that has been reported in previous ultrastructural studies of the shedding process (32, 48). This observation, together with the frequency of apoptotic cells (12, 21), supports the hypothesis that EGFR may contribute to the control of physiological enterocyte exfoliation. Our observations are consistent with a scenario in vivo in which EGFR activation would occur upstream from loss of E-cadherin, which, in turn, would lead to anoikis and complete extrusion of the cell. The signal responsible for EGFR activation under physiological conditions remains to be identified and could possibly be a very localized modification of signaling from the basal basement membrane.

In conclusion, we have demonstrated an unexpected contribution of EGFR to enterocyte anoikis. Based on the data obtained from the ex vivo anoikis model we developed, together with in vivo observations, our results suggest that one possible mechanism for enterocyte elimination in vivo would be activation of EGFR upon loss of anchorage from the basal lamina, leading to the disruption of E-cadherin-dependent cell-cell junctions, which would, in turn, trigger induction of the apoptosis program. Since both E-cadherin and EGFR are dysregulated in inflammatory bowel disease and in cancer, it would be of interest to further characterize the functional relationships between these two proteins in the intestinal epithelium.

ACKNOWLEDGMENTS

Confocal and electron microscopy analyses were performed using the image facilities of the Centre de Recherche des Cordeliers. We thank Julika Biscan for excellent technical assistance and Susan Saint-Just for editing the manuscript.

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GRANTS

V.-H. Lugo-Martinez was the recipient of a grant from Consejo Nacional de Ciencia y Tecnología (175581/188879). C. Petit was the recipient of a fellowship from the French Ministère de l’Enseignement Supérieur et de la Recherche. S. Fouquet was the recipient of a grant from the association François Aupertit. This work was supported by institutional funding from Institut National de la Santé et de la Recherche Médicale and Université Pierre et Marie Curie.

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