



HAL
open science

Early Loss of E-cadherin from Cell-Cell Contacts Is Involved in the Onset of Anoikis in Enterocytes

Stéphane Fouquet, Verónica-Haydée Lugo-Martínez, Anne-Marie Faussat, Flore Renaud, Philippe Cardot, Jean Chambaz, Martine Pinçon-Raymond, Sophie Thenet

► **To cite this version:**

Stéphane Fouquet, Verónica-Haydée Lugo-Martínez, Anne-Marie Faussat, Flore Renaud, Philippe Cardot, et al.. Early Loss of E-cadherin from Cell-Cell Contacts Is Involved in the Onset of Anoikis in Enterocytes. *Journal of Biological Chemistry*, 2004, 279, pp.43061 - 43069. 10.1074/jbc.m405095200 . hal-03034648

HAL Id: hal-03034648

<https://hal.science/hal-03034648v1>

Submitted on 1 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Early Loss of E-cadherin from Cell-Cell Contacts Is Involved in the Onset of Anoikis in Enterocytes*

Received for publication, May 7, 2004, and in revised form, July 19, 2004
Published, JBC Papers in Press, August 2, 2004, DOI 10.1074/jbc.M405095200

Stéphane Fouquet‡§, Verónica-Haydée Lugo-Martínez‡, Anne-Marie Faussat¶, Flore Renaud||, Philippe Cardot‡, Jean Chambaz‡, Martine Pinçon-Raymond‡, and Sophie Thenet‡**

From the ‡UMR 505 INSERM-UPMC, Laboratoire de Pharmacologie Cellulaire et Moléculaire de l'EPHE, 75006 Paris, France, ¶INSERM EPI9912, IFR 58, 75006 Paris, France, and ||CNRS UPRES-A 8087, Laboratoire de Génétique Moléculaire et Physiologique de l'École Pratique des Hautes Etudes, Université de Versailles/Saint Quentin-en-Yvelines, 78000 Versailles, France

Anoikis, i.e. apoptosis induced by detachment from the extracellular matrix, is thought to be involved in the shedding of enterocytes at the tip of intestinal villi. Mechanisms controlling enterocyte survival are poorly understood. We investigated the role of E-cadherin, a key protein of cell-cell adhesion, in the control of anoikis of normal intestinal epithelial cells, by detaching murine villus epithelial cells from the underlying basement membrane while preserving cell-cell interactions. We show that upon the loss of anchorage, normal enterocytes execute a program of apoptosis within minutes, via a Bcl-2-regulated and caspase-9-dependent pathway. E-cadherin is lost early from cell-cell contacts. This process precedes the execution phase of detachment-induced apoptosis as it is only weakly modulated by Bcl-2 overexpression or caspase inhibition. E-cadherin loss, however, is efficiently prevented by lysosome and proteasome inhibitors. We also found that a blocking anti-E-cadherin antibody increases the rate of anoikis, whereas the activation of E-cadherin using E-cadherin-Fc chimera proteins reduces anoikis. In conclusion, our results stress the striking sensitivity of normal enterocytes to the loss of anchorage and the contribution of E-cadherin to the control of their survival/apoptosis balance. They open new perspectives on the key role of this protein, which is dysregulated in the intestinal epithelium in both inflammatory bowel disease and cancer.

The intestinal epithelium displays one of the most rapid turnover rates of any mammalian tissue. Enterocytes differentiate from proliferative cells, during their migration along the crypt-to-villus axis (1). After 2–6 days, these cells reach the villus tip and are shed into the intestinal lumen, with identical morphological features in human and mouse (2).

Apoptosis is thought to play a dual role in the maintenance of homeostasis in this epithelium: 1) at the base of the crypt (3) to control the number of stem cells and 2) in the shedding process of enterocytes at the villus tip by anoikis (1, 2, 4). Anoikis refers to apoptosis induced by a loss of cell-matrix

interactions and was initially described for epithelial and endothelial cells (5, 6). Integrins, which act as adhesion receptors, control cell survival in response to extracellular matrix binding and also affect the cellular response to other survival and death signals, thereby controlling the fate of cells as a function of their immediate environment (7, 8).

It has been suggested that cell-cell interactions also control cell survival, particularly via the classical cadherins, a family of transmembrane proteins promoting calcium-dependent cell-cell adhesion. Cadherin homophilic ligation initiates the assembly of large adhesion multimolecular complexes, which are connected to the actin cytoskeleton via proteins of the catenin family (9). This connection is essential for the stabilization and the strength of cell-cell junctions (10). Cadherin engagement also leads to the transduction of intracellular signals (11, 12) and has been shown to protect squamous carcinoma cells and normal proximal tubular cells from anoikis (13, 14).

The mechanisms controlling normal enterocyte survival are poorly understood. Electron microscopy studies suggest that cell-matrix and cell-cell interactions are disrupted sequentially in enterocytes reaching the villus tip (15). Furthermore, loss of E-cadherin function in intestinal epithelium via the targeted expression of a dominant negative N-cadherin mutant increases the frequency of apoptotic cells while perturbing migration and differentiation (16). We hypothesized that the remodeling of E-cadherin-cytoskeleton complexes upon the loss of anchorage might be involved in the apoptosis signaling cascade. Indeed, we recently showed that the targeting of E-cadherin to the upper lateral membrane and its co-localization with subcortical actin in Caco-2 enterocytic cells are controlled by cell-matrix interactions involving integrins (17). We tested our hypothesis with an original model of anoikis in normal villus epithelium, in which interactions between epithelial cells and the underlying basement membrane were disrupted while cell-cell interactions were maintained. We found that, upon the loss of anchorage, normal enterocytes executed a strikingly rapid apoptosis program. We provide evidence that the loss of E-cadherin from cell-cell contacts, which occurred via a lysosome/proteasome-dependent degradation pathway, preceded the execution phase of detachment-induced apoptosis. Moreover, modulation of E-cadherin engagement affected the rate of apoptosis, demonstrating that E-cadherin participates in the control of anoikis in villus enterocytes.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture—Intestinal villus epithelium was isolated as entire epithelial linings by a method modified from Perreault and Beaulieu (18). Adult B6CBA mice were killed by cervical dislocation. The small intestine (jejunum and ileum) was everted; washed in ice-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Association François Aupetit.

** To whom correspondence should be addressed: EPHE-INSERM U505, 15 rue de l'École de Médecine, 75006 Paris, France. Tel.: 33-1-42-34-69-34; Fax: 33-1-43-25-16-15; E-mail: Sophie.Thenet-u505@bhdc.jussieu.fr.

cold phosphate-buffered saline (PBS)¹ containing Ca²⁺, Mg²⁺, and 1 g/liter D-glucose (Invitrogen); and cut into 5-mm pieces. The fragments were incubated in MatrisperseTM (Becton Dickinson) at 4 °C for 2 h. Detachment of the epithelium from the mesenchyme was then induced by vigorous shaking and filtration through large-pored nylon mesh. After washing in ice-cold PBS, the detached epithelia were retained on a 70- μ m nylon strainer, resuspended in ice-cold Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/liter glucose and supplemented with 20 mM Hepes, 50 units/ml penicillin, 50 μ g/ml streptomycin, 5 ng/ml recombinant human epidermal growth factor (Invitrogen), 0.2 IU/ml insulin (Novo Nordisk), and 5% fetal bovine serum (AbCys), plated on 24-well culture plates (8 wells/intestine) and incubated at 37 °C in an atmosphere containing 5% CO₂. The purity of the epithelial fraction was confirmed by the staining of 100% of the cells with an anti-keratin antibody (not shown) and by the absence of α -smooth muscle actin detected in Western blot (see Fig. 4A). The villus origin of this fraction was assessed by sucrase isomaltase activity; sucrase isomaltase-negative crypt fractions were only obtained after further incubations in chelating solutions (not shown). Epithelial linings were isolated in the same way from *L-PK-bcl-2* transgenic mice expressing the human *bcl-2* gene under control of the *L*-pyruvate kinase promoter (19), and nontransgenic littermates were used as a control. When indicated, the following proteases inhibitors were added to MatrisperseTM and culture medium: the broad spectrum caspase inhibitor Z-VAD-fmk (50 μ M; Bachem), the proteasome inhibitor MG-132 (20 or 50 μ M; Calbiochem), or the lysosome inhibitor chloroquine (100 μ M; Sigma-Aldrich).

Antibodies—The following antibodies were used: anti-E-cadherin (ECCD-2; Zymed Laboratories Inc.), anti- β -catenin (clone 14; Transduction Laboratories), anti-mouse β_1 -integrin (clone MB1.2; Chemicon), anti-mouse β_4 -integrin (clone 346-11A; Pharmingen), anti-human Bcl2 (clone 124; DAKO), anti-actin (clone C4; Chemicon), anti- α -smooth muscle actin (clone 1A4; Sigma), anti-caspase-8 (AAP-118; StressGen), anti-caspase-6 (AAP-106; StressGen), anti-human caspase-3 (H-277; Santa Cruz), anti-human caspase-9 (clone 5B4; Immunotech), and an anti-active caspase-3 antibody (Pharmingen). We used the following secondary antibodies: horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences), horseradish peroxidase-conjugated anti-rat and anti-rabbit IgG (Production d'Anticorps, Réactifs Immunologiques et Services), Cy3-conjugated anti-mouse, Cy2-conjugated anti-rabbit, and Cy2-conjugated anti-rat IgG (Jackson ImmunoResearch).

Electron Microscopy—Immediately after MatrisperseTM treatment, the epithelial linings were fixed by incubation for 1 h at 4 °C in 2.5% glutaraldehyde and 0.5% tannic acid in 0.1 M cacodylate buffer, pH 7.4. The cells were incubated overnight at 4 °C in 0.6% glutaraldehyde and 0.5% tannic acid in 0.1 M cacodylate buffer, pH 7.4. The cells were postfixed by incubation for 2 h at 4 °C in 2% osmic acid in phosphate buffer, dehydrated, and embedded in Epon resin (Poly/Bed 812; Polysciences). Ultrathin sections (70 nm) were counterstained with uranyl acetate and lead citrate and examined with a Jeol 100CX II microscope.

Determination of Apoptosis—Internucleosomal cleavage was visualized by the extraction of small detergent-soluble fragments of DNA in 0.5% Triton X-100 (20), followed by electrophoresis in a 1.5% agarose gel, using a 100-bp ladder (Invitrogen) as the standard. Apoptosis was quantified by flow cytometry analysis of subdiploid DNA content. The cells were dissociated by vigorous pipetting in ice-cold 2 mM EDTA in PBS and fixed in ice-cold 70% ethanol. Each pellet was resuspended sequentially in 100 μ l of 1% Nonidet P-40, 50 μ l of ribonuclease A (1 mg/ml), and 500 μ l of propidium iodide (50 μ g/ml) and incubated for 15 min at 4 °C. Analysis was performed on an ALTRA cell sorter (Beckman Coulter) equipped with a UV/argon laser and Expo32 software. Cell doublets and clumps were gated out of the analysis, and a total of 10,000 single cells were analyzed for each sample. For some experiments, apoptosis was quantified by counting apoptotic nuclei on cryosections after staining with DAPI (100 ng/ml; Research Organics Inc). A minimum of 500 nuclei/section was counted for each determination.

Immunofluorescence—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 10- μ m cryosections were cut. The sections were sequentially incubated in blocking buffer (1% w/v bovine serum albumin, 0.2% w/v low fat milk powder, 0.3% v/v Triton X-100 in PBS) and then with primary and secondary antibodies diluted in blocking buffer (anti-E-cadherin 1/250, anti- β_1 -integrin 1/1000, anti- β_4 -integrin

1/50, anti-Bcl-2 1/10, and anti-active caspase-3 1/50). Polymerized actin was stained with 0.5 μ g/ml TRITC-labeled phalloidin (Sigma), and the nuclei were stained with 100 ng/ml DAPI in PBS or with 2 μ M TOTO-3 (Molecular Probes) in PBS-bovine serum albumin (1% w/v) after a 1 mg/ml RNase A treatment. The images were acquired on a DMR Leica epifluorescence microscope or on a Zeiss LSM510 confocal laser-scanning microscope, with contrast and brightness settings kept constant throughout image acquisition.

Western Blot Analysis—Total protein extracts were prepared from epithelial linings washed in PBS and solubilized in SDS buffer (1% SDS, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA). The protein concentrations were determined with the DC protein-assay (Bio-Rad). 80- μ g protein aliquots were subjected to SDS-PAGE in a 15% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Roche Applied Science). The following antibody dilutions were used: anti-caspase 3 (1/200), anti-caspase 6 (1/1000), anti-caspase 9 (1/1000), and anti-caspase 8 (1/1000).

For the separation of Triton X-100-soluble and insoluble fractions, the cells were incubated for 15 min at 4 °C in CSK buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂) containing a protease inhibitor mixture (20 μ l/ml; Sigma). Insoluble material was removed by centrifugation at 12,000 \times g for 30 min. The supernatant was defined as the Triton X-100-soluble fraction. The pellet was extracted with the same volume of SDS buffer boiled for 10 min; insoluble material was removed by centrifugation, and the supernatant was defined as the Triton X-100-insoluble fraction. For each sample, a 100- μ g protein aliquot of soluble fraction and an equal volume of insoluble fraction was separated by SDS-PAGE in 7.5% polyacrylamide gel and transferred. The blots were incubated sequentially with primary and secondary antibodies diluted in Tris-buffered saline containing 0.05% Tween 20 (v/v), 1% low fat milk powder (w/v), anti-E-cadherin (1/2500), anti- β -catenin (1/2000), anti-actin (1/2000), and anti- α -smooth muscle actin (1/1000) and analyzed with an ECL kit (Amersham Biosciences). Red Ponceau staining was used to ensure equal loading of proteins. The quantitative analyses were performed with a high performance calibrated imaging densitometer (Bio-Rad GS-800) using PD Quest and Image Quant 5.2 softwares.

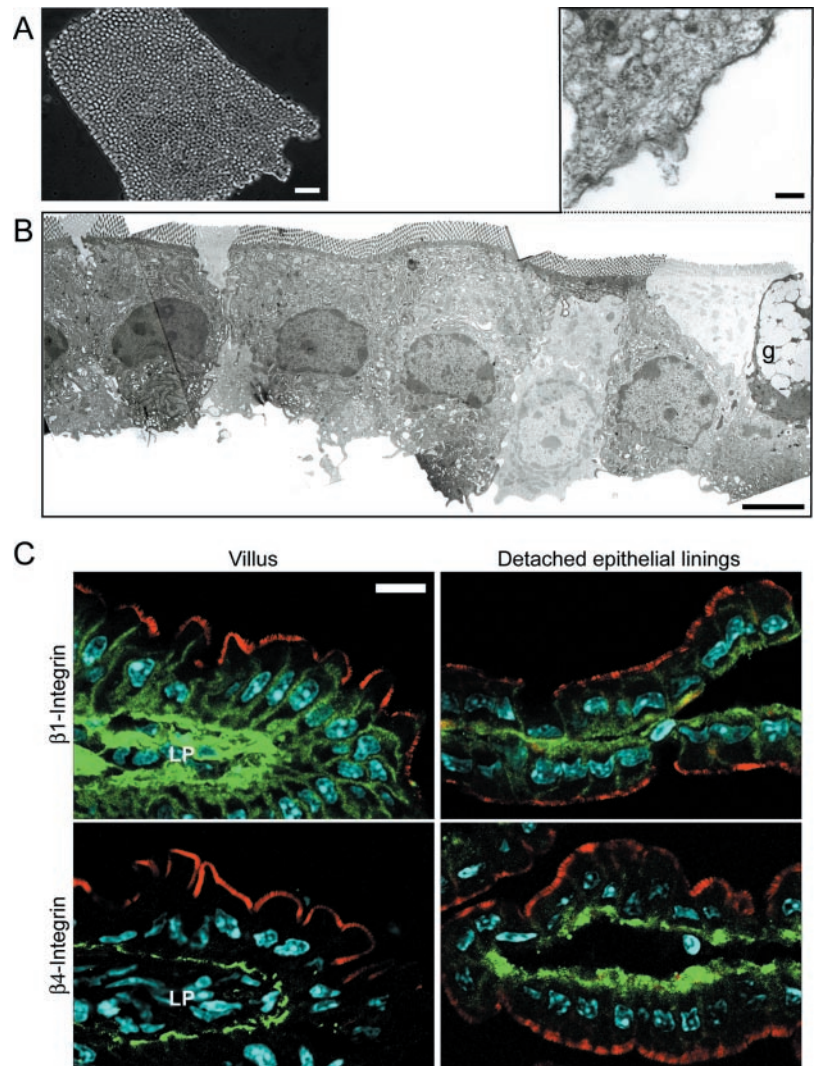
E-cadherin Perturbation Experiments—Detached epithelial linings were resuspended in culture medium containing either a function-blocking anti-E-cadherin antibody (DECCA-1 (11, 21), 200 μ g/ml (Sigma); or rat IgG as a control, 200 μ g/ml (Chemicon)), or a functional E-cadherin ligand (12) (dimeric E-cadherin-Fc chimera protein, *i.e.* mouse E-cadherin ectodomain fused to the Fc fragment of human IgG1, 10 μ g/ml; R & D Systems). In the latter case, E-cad-Fc was preincubated with epithelial linings for 15 min at 4 °C. Anti-human IgG specific for the Fc fragment (6 μ g/ml; Jackson ImmunoResearch) was added to trigger E-cad-Fc clustering at the cell membrane, as described for the clustering of activating antibodies (22), and the cells were immediately transferred to 37 °C. Controls were treated with anti-Fc IgG only. In both experiments, the cells were fixed with paraformaldehyde after 30 min, and apoptosis was assessed by counting apoptotic nuclei.

RESULTS

Characterization of Apoptosis Triggered by Loss of Cell-Matrix Anchorage in Villus Epithelial Linings—We studied the response of normal enterocytes to the loss of anchorage without disruption of cell-cell interactions by detaching mouse intestinal villus cells from mesenchyme as entire epithelial linings, using a nonenzymatic dissociating solution (MatrisperseTM). The resulting sheets of epithelial cells with conserved cell-cell contacts are shown in Fig. 1A. Ultrastructure analysis showed that the epithelial linings were still polarized, as shown by the basal position of the nuclei and the presence of a well organized brush border, and maintained cell-cell interactions (Fig. 1B). No basement membrane was present beneath the basal membrane (Fig. 1B, *inset*). We compared the distribution of the β_1 - and β_4 -integrins, the main integrin β chains present in the intestine (23), in the intact tissue and in the freshly detached epithelial linings. Detachment had no marked effect on β_1 -integrin distribution along the basolateral membrane of epithelial cells. In contrast, β_4 -integrin was strictly restricted to the basal membrane of enterocytes within the tissue, as previously observed in human intestine (23). Immediately after detachment, β_4 -integrin labeling was still restricted to the basal

¹ The abbreviations used are: PBS, phosphate-buffered saline; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; DAPI, 4',6-diamidino-2-phenylindole-dihydrochloride; TRITC, tetramethylrhodamine isothiocyanate.

FIG. 1. Characterization of intestinal villus epithelial linings detached from the mesenchyme with preservation of cell-cell interactions. *A*, phase contrast photograph of murine villus epithelial cells dissociated from mesenchyme as an entire epithelial lining by incubation in Matrisperse™. *Bar*, 40 μm . *B*, transmission electron micrograph of villus epithelial lining immediately after detachment. *Bar*, 5 μm . Enterocytes maintain close cell-cell interactions and remain polarized (note in particular the well preserved brush border microvilli). Note also the presence of a goblet cell (*g*). *Inset*, detail of the basal pole showing that no basement membrane is present beneath the plasmic membrane. *Bar*, 0.2 μm . *C*, confocal microscopy analysis of β_1 - and β_4 -integrins (green) in native intestinal villi (*left panel*) or in villus epithelial linings immediately after detachment (*right panel*). β_1 -Integrin is detected primarily along the basolateral membrane of all villus epithelial cells and is also present in large amounts in the lamina propria (*LP*), whereas β_4 labeling is restricted to the basal membrane of epithelial cells and is not detected in *LP*. The nuclei are stained with TOTO-3 (blue) and polymerized actin with TRITC-labeled phalloidin (red). *Bar*, 10 μm .



pole of the epithelial cells but was more diffusely distributed. Although expressing integrins at their cell surface, albeit with changes in the β_4 chain distribution, epithelial linings were unable to adhere when plated on collagen, laminin, or a native matrix deposited by mesenchymal cells (not shown).

We then studied the fate of epithelial linings maintained in suspension at 37 °C. Immediately after detachment, all of the nuclei were normal in morphology, but as early as 30 min later, 20–30% of the nuclei were condensed or fragmented, indicating that apoptosis was underway (Fig. 2A). We checked that apoptosis was triggered by detachment from the mesenchyme, and not simply by *ex vivo* incubation, by incubating fragments of whole intestinal mucosa (explants) at 37 °C; no apoptotic nuclei were detected in epithelial cells still attached to the villus mesenchyme within explants (Fig. 2A).

Study of internucleosomal DNA fragmentation (Fig. 2B) and flow cytometry quantification of subdiploid cells as a function of time (Fig. 2C) indicated that apoptosis was negligible immediately after detachment (0 min), becoming detectable within 15 min and rapidly increasing thereafter; 35% of sub-G₁ events were detected after 30 min and 75% after 3 h. These data demonstrate the apoptotic nature of the cell death program triggered by loss of anchorage and its striking rapidity in normal enterocytes.

Expression of the anti-apoptotic gene *bcl-2*, which protects against anoikis in epithelial cell lines (6), is weak in the small intestine. To ask whether sensitivity to anoikis could be alle-

viated by *bcl-2* overexpression in enterocytes, we used transgenic mice expressing the human *bcl-2* gene under control of the L-pyruvate kinase promoter (19). *Bcl-2* levels were higher in villus-associated enterocytes from *L-PK-bcl-2* transgenic mice than in those from wild-type littermates, as shown by immunostaining (Fig. 2D) and Western blotting (not shown). *Bcl-2* overproduction clearly delayed detachment-induced apoptosis (Fig. 2E). The rate of apoptosis was also markedly reduced in the presence of 50 μM Z-VAD (Fig. 2E), indicating the involvement of caspase activation in the execution of anoikis. We used Western blots to assess activation of the initiator caspases-8 and -9 and of the executioner caspases-3 and -6 (Fig. 2F). Caspase-9 was activated within 30 min, as shown by a 40% decrease in the amount of the 45-kDa proform, concomitant with an obvious appearance of the 35-kDa activated form. In contrast, the amount of caspase-8 proforms did not markedly vary, and we did not observe any processing of this caspase. The activation of executioner caspase-6 and caspase-3 was detected within 30 min by a 60% decrease in the amount of the proforms of these enzymes and the obvious appearance of the 11-kDa activated form of caspase-3. The activation of caspases-9, -3, and -6 was reduced by Z-VAD and *bcl-2* overexpression, with *bcl-2*-overexpressing enterocytes treated with Z-VAD presenting the most effective blockage. Quantitation of the cleaved forms indicated that caspase-3 activation was decreased by 90% upon Z-VAD treatment and was almost completely blocked by *bcl-2* overexpression. Z-VAD treatment and

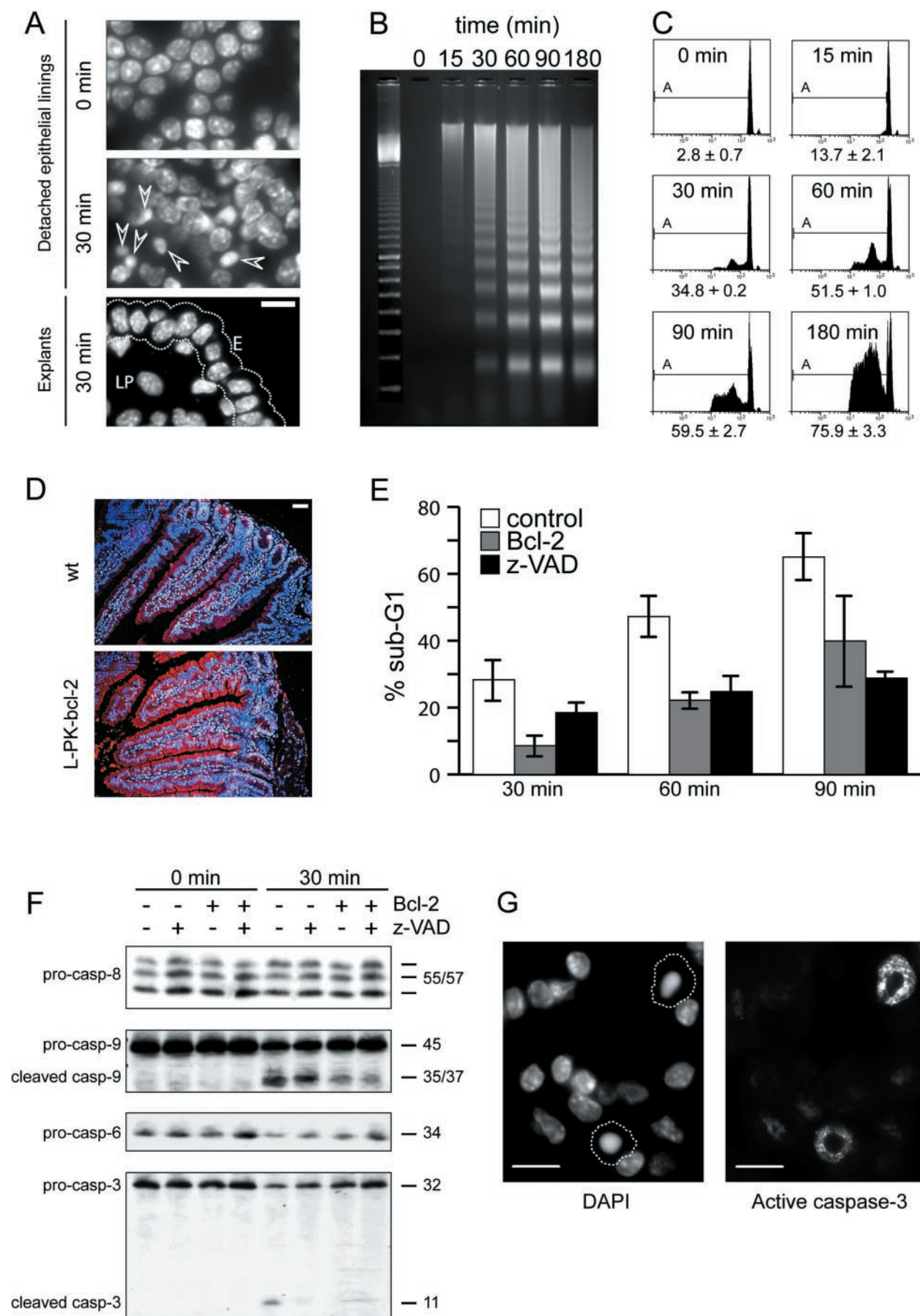
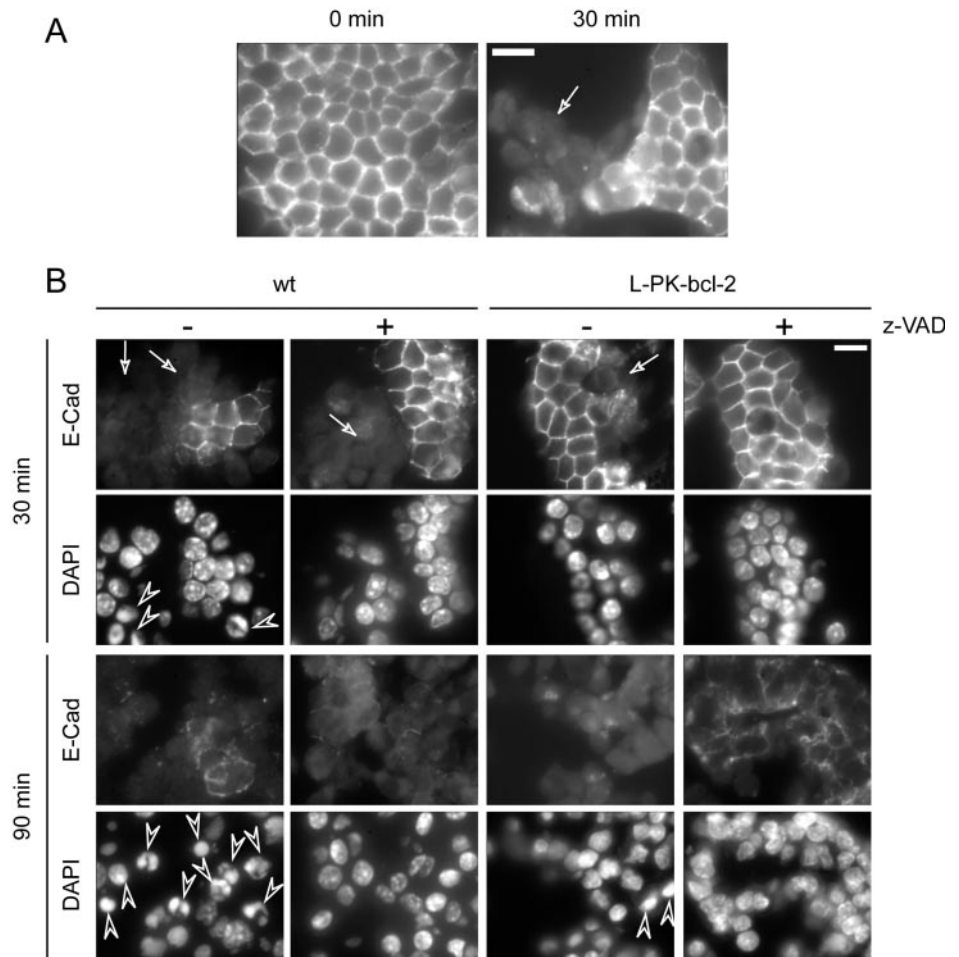


FIG. 2. **Characterization of apoptosis triggered by enterocyte detachment.** A, DAPI staining of nuclei from villus epithelial linings detached from the mesenchyme or maintaining interactions with the mesenchyme (“explants”) and incubated at 37 °C for the times indicated. The apoptotic nuclei were detected after 30 min in detached cells (examples indicated by *arrowheads*) but not in epithelial cells maintaining

FIG. 3. The kinetics of E-cadherin loss at cell-cell junctions is not affected by inhibitors of apoptosis. *A*, villus epithelial linings were fixed immediately after detachment (0 min) or after 30 min in suspension and immunostained for E-cadherin. The *arrow* indicates a group of cells that have lost E-cadherin staining. *Bar*, 10 μ m. *B*, villus epithelial linings from *L-PK-bcl-2* transgenic mice or from wild-type littermates (*wt*) were incubated in suspension for the times indicated in the absence (–) or presence of 50 μ M Z-VAD (+), fixed, and immunostained for E-cadherin. The nuclei stained with DAPI are shown for the same fields. *Bar*, 10 μ m. The *arrows* indicate examples of cell clusters at 30 min that have lost E-cadherin at the membrane but that still present a nonapoptotic nucleus. The *arrowheads* indicate nuclei with advanced apoptotic morphology (condensation or fragmentation). Note that cells with a normal nucleus presented either normal membranous or diffuse/weak E-cadherin staining but that cells with apoptotic nuclei systematically had diffuse or no E-cadherin staining.



bcl-2 overexpression decreased caspase-9 activation by 40 and 80%, respectively.

The activation of caspase-3 was also evidenced by immunofluorescence, using an antibody that binds to a conformational epitope exposed by activation-induced cleavage of the inactive pro-caspase-3. Consistently with Western blot analysis, this antibody did not label epithelial cells in intact intestine or immediately after detachment (not shown). By contrast, intense cytoplasmic staining was detected after 30 min in all cells showing a condensed, apoptotic nucleus (Fig. 2*G*). The proportion of active caspase-3-labeled cells increased with time together with the proportion of condensed or fragmented nuclei (not shown). Overall, villus enterocytes are equipped to activate programmed cell death within minutes after loss of anchorage, and apoptosis is executed in most cells within 3 h via a Bcl-2-regulated and caspase-9-dependent pathway.

Early Loss of E-cadherin from Cell-Cell Junctions Occurs during Anoikis and Is Insensitive to Apoptosis Inhibitors—In the course of our studies, we also observed progressive dissociation of the epithelial linings (not shown). We therefore analyzed E-cadherin by immunofluorescence staining during anoikis. E-cadherin staining was intense and restricted to cell-cell contacts immediately after detachment (0 min) and decreased and became diffuse in ~50% of cells after 30 min (Fig. 3*A*). Furthermore, the combined analysis of E-cadherin localization and of nuclear morphology showed that after 30 min, E-cadherin was either lost or preserved at cell-cell contacts in cell clusters that still presented intact nuclei, whereas E-cadherin labeling was very faint and diffuse in all cells showing apoptotic nuclear morphology (Fig. 3*B*, upper left panel). After 90 min, E-cadherin staining was almost undetectable in ~90% of cells, whereas only 60% of cells were apoptotic (Fig. 3*B*, lower left panel). Among the thousands cells observed, no cell dis-

interactions with the mesenchyme. The *dotted line* delineates the epithelium (*E*). *LP*, lamina propria. *Bar*, 10 μ m. *B*, analysis of internucleosomal DNA fragmentation. Villus epithelial linings were incubated in suspension for the indicated times, and Triton X-100-soluble DNA was extracted and processed for electrophoresis. Note that no small detergent-soluble fragment of DNA was detected immediately after detachment (0 min). *C*, quantification of apoptosis by flow cytometry analysis of DNA content. Percentages of cells in the sub- G_1 region (labeled *A*) are indicated (means \pm S.D. of one representative experiment performed in triplicate). *D*, immunostaining of the Bcl-2 protein (*red*) in jejunum sections of wild-type mice (*wt*) or *L-PK-bcl2* transgenic mice. The nuclei are stained with DAPI (*blue*). *Bar*, 40 μ m. *E*, quantification of apoptosis by flow cytometry in villus epithelial linings taken from *L-PK-bcl-2* transgenic mice (*gray bars*) or from wild-type littermates incubated for the indicated times in the absence (*white bars*) or presence of 50 μ M Z-VAD (*black bars*). The results are means \pm S.D. from at least four independent experiments. At each time point, the percentage of apoptosis in *bcl-2*-overexpressing or Z-VAD-treated enterocytes differed significantly from that in control enterocytes ($p < 0.001$ as determined by Student's *t* test.). *F*, Western blot analysis of the activation of caspases-8, -9, -6, and -3. Villus epithelial linings from *L-PK-bcl-2* transgenic mice (+) or from wild-type littermates (–) were incubated in suspension for the times indicated in the absence (–) or presence (+) of 50 μ M Z-VAD. The estimated molecular masses (kDa) of the various pro-caspases and cleaved caspases are indicated on the *right*. *G*, immunofluorescent staining of activated caspase-3. The nuclei stained with DAPI are shown for the same field. Note that only cells exhibiting a condensed nucleus (surrounded by a *dotted line*, *left panel*) are labeled with the anti-activated caspase-3 antibody (*right panel*). *Bar*, 10 μ m.

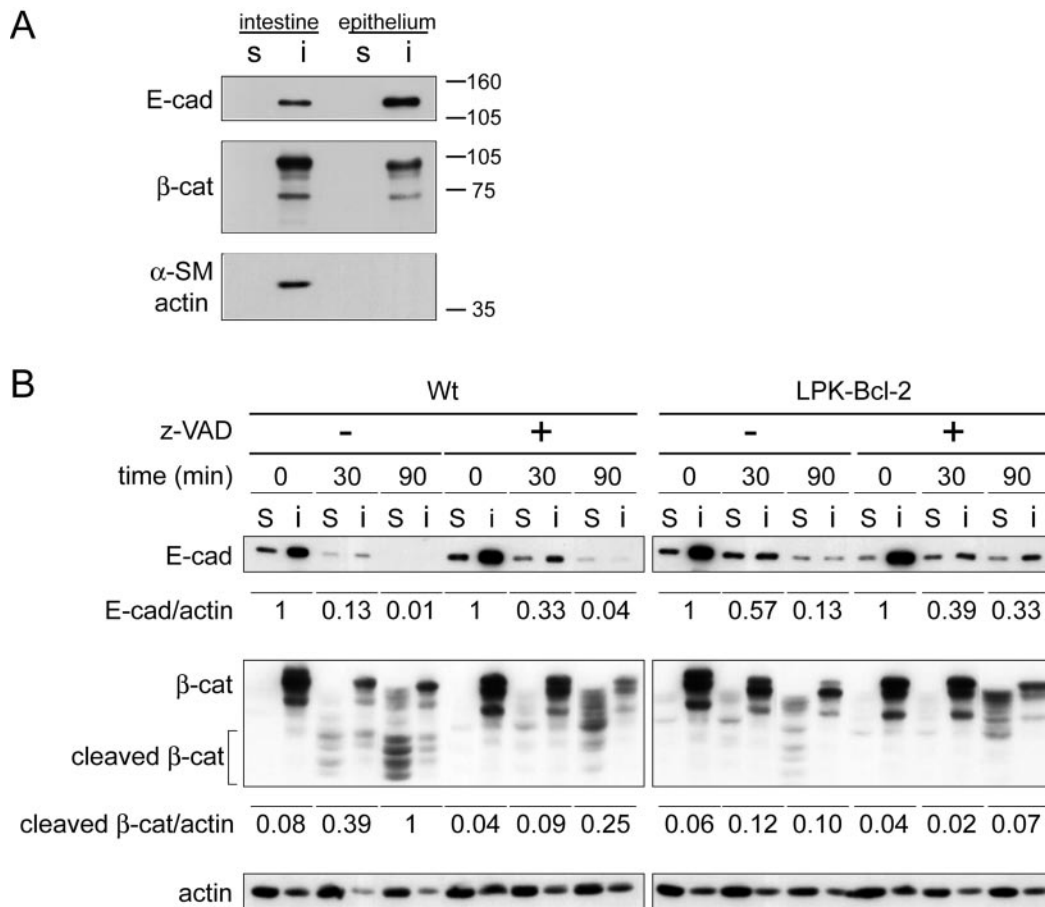


FIG. 4. Western blot analysis of E-cadherin and β -catenin levels during anoikis. *A*, extracts from whole intestinal mucosa (intestine) and from villus epithelial linings immediately after detachment by MatrisperseTM (epithelium) were compared. Aliquots of proteins of the Triton X-100-soluble fractions (s) and equivalent volumes of the insoluble fractions (i) were separated by SDS-PAGE, and the immunoblots were probed with anti-E-cadherin, anti- β -catenin, and anti- α -smooth muscle actin antibodies (α -SM actin). α -Smooth muscle actin, a marker of mesenchyme, was detected in native intestine but not in the detached epithelium, confirming the purity of this preparation. Positions of the molecular weight markers are indicated on the right. *B*, effect of Z-VAD treatment and of *bcl-2* overexpression on decreases in E-cadherin levels and on β -catenin cleavage. Villus epithelial linings from *L-PK-bcl-2* transgenic mice or from wild-type littermates (*wt*) were incubated in suspension for the times indicated in the absence (–) or presence (+) of 50 μ M Z-VAD and processed as in *A*. The immunoblots were probed with anti-E-cadherin, anti- β -catenin, and anti-actin. Note that actin levels in the Triton-soluble fractions (s) are constant throughout the kinetics, because of equivalent protein loading of these fractions, but that the proportion found in the insoluble fractions varies. Densitometric analysis of total E-cadherin levels related to total actin (*E-cad/actin*) is reported below the blot, and the figure represents a normalized value where the time 0 min for each series represent the value 1.0. Similarly, densitometric analysis of cleaved β -catenin levels related to total actin (*cleaved β -cat/actin*) is reported below the blot and is normalized to the maximal amount observed in the wild-type untreated condition at 90 min.

played both an apoptotic nucleus and membranes stained for E-cadherin. These results suggest that E-cadherin is lost from cell-cell contacts before the execution of apoptosis in our system.

We investigated the effect of apoptosis inhibition on the fate of E-cadherin. Bcl-2 overproduction or Z-VAD treatment decreased the proportion of apoptotic nuclei at both 30 and 90 min (Fig. 3*B*, three right panels, consistent with Fig. 2). However, these treatments had no major effect on the loss of E-cadherin throughout anoikis. A delay in the disappearance of E-cadherin was nevertheless observed in enterocytes overexpressing *bcl-2* and treated by Z-VAD, suggesting that the loss of E-cadherin is, at most, secondarily amplified upon apoptosis. These data support the hypothesis that E-cadherin loss at the cell membrane precedes both caspase activation and appearance of the morphological hallmarks of apoptosis.

Apoptosis Inhibitors Efficiently Inhibit β -Catenin Cleavage but Have Little Effect on the Decrease in E-cadherin Levels—To clarify the fate of E-cadherin in epithelial linings upon loss of anchorage, immunofluorescence studies were supplemented by Western blot analyses of Triton-soluble and insoluble fractions at various time points. Just after detachment of epithelial linings,

E-cadherin was primarily located in the detergent-insoluble fraction, which comprises polymerized actin (10), as in native whole intestine mucosa (Fig. 4*A*). This result and our morphological observations indicate that most of the E-cadherin was retained in the junctional complexes after MatrisperseTM-induced detachment. The majority of β -catenin was also detected in the detergent-insoluble fraction, and its pattern of migration was identical in native intestine and in detached epithelium.

Upon incubation in suspension, we observed a dramatic time-dependent decrease in the amount of E-cadherin associated with both the insoluble and soluble fractions (Fig. 4*B*). When related to total actin, only 1% of the initial amount of total E-cadherin was detected after 90 min. Consistent with immunofluorescence studies (Fig. 3*B*), the kinetics of this decrease in E-cadherin levels was not markedly affected by Z-VAD treatment or by Bcl-2 overproduction; only 4 and 13% of the initial amounts, respectively, were still detected after 90 min (Fig. 4*B*). The protective effects by apoptosis modulators visualized at 30 min were no more observed at 90 min, except when the two modulators were combined. It has been shown that during apoptosis, E-cadherin is cleaved on its intracytoplasmic domain in a caspase-dependent manner and on its extracellular domain by a metalloproteinase

(24, 25). In the present model of enterocyte anoikis, no accumulation of cleaved fragments was detected, when using antibodies directed against the ectodomain or the cytodomain of E-cadherin (not shown). Because β -catenin, one of the cytoplasmic partner of E-cadherin, has also been shown to be cleaved by caspases in several models of apoptosis (26), we analyzed β -catenin degradation upon enterocyte anoikis. In contrast to E-cadherin, β -catenin was clearly progressively cleaved into several fragments that were detected mainly in the Triton-soluble fraction (Fig. 4B). This cleavage process was inhibited by 75% upon Z-VAD treatment and by 90% upon Bcl-2 overproduction (Fig. 4B). Interestingly, despite protecting against β -catenin cleavage, Z-VAD treatment or Bcl-2 overproduction were able to delay, but not to fully prevent, the redistribution of β -catenin from the insoluble to the soluble fraction. These observations are consistent with a remodeling of the multimolecular E-cadherin- β -catenin-actin complexes that still occurs when apoptosis is inhibited and when β -catenin is not cleaved.

E-cadherin Degradation during Anoikis Occurs via a Proteasome- and Lysosome-dependent Pathways—Our results indicate that E-cadherin loss precedes apoptosis induction and does not involve degradation by caspases. The proteasome and the lysosome have been involved in the degradation of E-cadherin and of other classical cadherin when junctional complexes are destabilized (27, 28). We asked whether E-cadherin degradation upon enterocyte anoikis could occur via these pathways. Anoikis was induced in the presence of the lysosomal inhibitor chloroquine or in the presence of the proteasome inhibitor MG-132, and E-cadherin levels were studied 90 min after detachment, a time at which the caspase inhibitor Z-VAD displayed no protective effect (Fig. 4B). Both chloroquine and MG-132 markedly inhibited the reduction of the E-cadherin level, with MG-132 presenting the most potent effect (Fig. 5A). Immunofluorescent analysis showed that more cells maintained E-cadherin at cell-cell contacts and remained cohesive in presence of MG-132 than in untreated epithelial linings (Fig. 5B). This observation is consistent with previous studies showing that the proteasome activity is required for internalization of some transmembrane receptors, including VE-cadherin (28, 29). Interestingly, the amount of apoptotic nuclei fell to 55% of untreated controls upon MG-132 treatment (not shown). The simultaneous inhibition of apoptosis and E-cadherin degradation we observed points out the involvement of E-cadherin degradation in the onset of apoptosis.

Effects of the Modulation of E-cadherin Function on Anoikis—We thus investigated whether anoikis could be modulated by affecting E-cadherin adhesion and/or signaling function. We performed the anoikis assay in the presence of an anti-E-cadherin antibody (DECMA-1), which recognizes the ectodomain and has been reported to block cell-cell adhesion (11). At 30 min, 23% of the nuclei were apoptotic and a 2-fold increase of this proportion was observed in the presence of the DECMA-1 antibody (Fig. 6A). To mimic E-cadherin engagement and intracellular signaling, we then used a dimeric E-cadherin-Fc chimera protein containing the E-cadherin ectodomain fused to the IgG Fc fragment (Ecad-Fc). This molecule has been shown to act as functional cadherin ligand, inducing the recruitment of cadherin as a functional complex anchored to the actin cytoskeleton (12). In presence of E-cad-Fc, the rate of apoptosis was decreased by 2-fold (Fig. 6B). Thus, ligands known to modulate functional E-cadherin complexes also modulate anoikis, indicating that E-cadherin controls anoikis, at least partly, following the disruption of cell-matrix interactions.

DISCUSSION

In this study, we characterized an original model of anoikis in normal villus epithelium in which cell-matrix and cell-cell

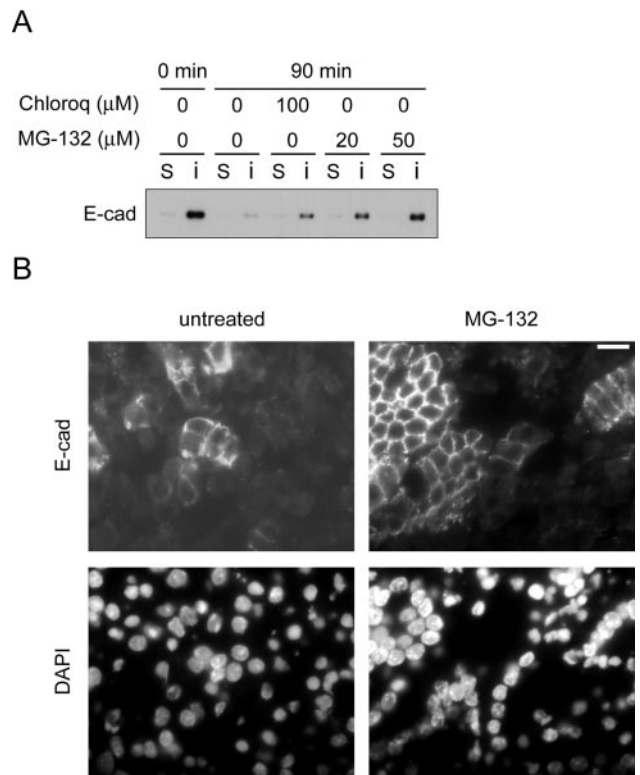


FIG. 5. E-cadherin loss during anoikis occurs through a lysosome and proteasome dependent degradation pathway. *A*, villus epithelial linings were incubated in suspension for the times indicated in presence of the lysosomal inhibitor chloroquine (100 μ M) or the proteasome inhibitor MG-132 (20 or 50 μ M). Aliquots of proteins of the Triton X-100-soluble fractions (s) and equivalent volumes of the insoluble fractions (i) were separated by SDS-PAGE, and the immunoblots were probed with an anti-E-cadherin antibody. *B*, villus epithelial linings were incubated in suspension for 90 min in presence of Me₂SO (untreated) or 50 μ M MG-132, then fixed, and immunostained for E-cadherin. The nuclei stained with DAPI are shown for the same fields. Bar, 10 μ m.

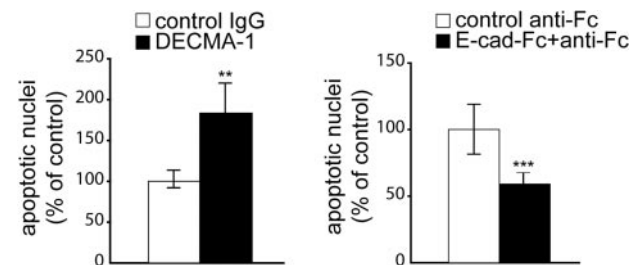


FIG. 6. Modulation of anoikis by a blocking anti-E-cadherin antibody or by an agonist E-cad-Fc chimera protein. *A*, villus epithelial linings were incubated in suspension for 30 min with anti-E-cadherin antibody (DECMA-1) or with IgG (control), fixed, and stained with DAPI. The apoptotic nuclei were counted in three independent sections (at least 500 nuclei/section). The results are expressed as percentages of control and are the means \pm S.D. from two independent experiments. **, $p < 0.01$ as determined by Student's *t* test. *B*, the same experiment was performed as in *A*, except that cells were incubated with E-cad-Fc chimera protein together with anti-Fc IgG to induce aggregation or with anti-Fc IgG alone (control). The results are expressed as percentages of control and are the means \pm S.D. from three independent experiments. ***, $p < 0.001$ as determined by Student's *t* test.

interactions are sequentially disrupted. We report, for the first time, the early loss of E-cadherin from cell-cell contacts after detachment, in a process that does not depend on the caspase-dependent execution phase of apoptosis but rather contributes to its onset.

We showed that the activation and execution of enterocyte

anoikis was extremely rapid in our model. This seems consistent with the rapid renewal of the intestinal epithelium *in vivo*. The activation of caspases and other hallmarks of apoptosis were detected within 30 min of detachment, and most cells died within 3 h. This process is very different from that observed in other cell systems. Indeed, sensitivity to anoikis depends on cell type, oncogene status, and extracellular cues. Sensitivity to anoikis is lost or decreased upon epithelial-mesenchymal transition (7) and upon transformation by oncogenes, *e.g.* *ras*, *src*, and *bcl-2* (6, 30), and resistance to anoikis contributes to cell malignancy (31, 32). In current intestinal cell models, apoptosis is not detected until several hours after detachment from the culture support in nondifferentiated/non transformed cell lines such as IEC18 (30), whereas carcinoma cell lines, *e.g.* Caco-2 or HT29, display no triggering of apoptosis upon loss of anchorage,² unless this loss is associated with serum deprivation (33). Only normal colonic crypt cells have been reported to execute anoikis with a time course similar to that reported here for normal enterocytes (4, 34). These kinetics, together with the demonstration that the execution of anoikis in normal colonocytes is not dependent on protein synthesis (35), suggest that the apoptotic machinery is readily recruitable in both enterocytes and colonocytes.

Cell survival depends on the efficient inactivation of proapoptotic molecules and/or on strong survival signals that counteract apoptotic signals. Anoikis may therefore result from the abolition of survival pathways and/or the induction of specific death signals (7). The initiator caspases activated depend on the death signal. They, in turn, activate executioner caspases, which are responsible for the cleavage of intracellular components. Caspase-9 is activated by the mitochondrial pathway, on which most apoptotic signals converge, whereas caspase-8 is essentially activated by the oligomerization of death receptors upon ligand binding. We found that caspase-8 was not activated during the anoikis of normal enterocytes, or at most late and weakly, contrary to what was reported in Madin-Darby canine kidney cells (36, 37). Consistently, caspase-8 was shown to be cleaved only after the activation of caspase-2 and caspase-9 during colonocyte anoikis (4). Thus, in normal intestinal epithelial cells, caspase-8 is clearly not involved in the initiation of anoikis, as shown recently in mammary epithelial cells (38).

We show here that the disruption of cell-matrix interactions triggers a rapid loss of E-cadherin from cell-cell contacts in normal enterocytes. Consistent with this, we previously showed that the extracellular matrix controls cell-cell adhesion by strengthening functional E-cadherin-actin adhesion complexes in Caco-2 enterocytic cells (17). E-cadherin cleavage upon apoptosis has been described in other models and is thought to result from the activation of caspases (24, 25) and/or other proteases (25, 39, 40). We found that the disappearance of E-cadherin from the membrane in enterocyte anoikis was only modestly prevented by Z-VAD or by *bcl-2* overexpression. We also detected no cleavage fragments of E-cadherin, whereas we did observe progressive Z-VAD-sensitive and Bcl-2-sensitive cleavage of β -catenin, as reported in other studies (26, 41). In contrast, our data strongly suggest that E-cadherin degradation upon anoikis occurs via a mechanism that involves the lysosome and the proteasome. Such mechanisms have been involved in junction disorganization and cadherin degradation in endothelial cells and in epithelial cell lines, upon hepatocyte growth factor or Src-induced cell scattering (42), p120^{ctn} inactivation (27), or expression of a dominant negative cadherin mutant (28), but, to our knowledge, never in a context of apo-

ptosis. Although data reported until now in the literature suggest that E-cadherin and β -catenin are processed by the same degradation pathway, which involves the lysosome and the proteasome upon junction disorganization (43) and caspases upon apoptosis (25, 26), our data suggest that during enterocyte anoikis, E-cadherin is degraded through the proteasome and the lysosome, and β -catenin is processed by caspases. Our model would assume a remodeling of the junctional complex in the course of which E-cadherin/ β -catenin dissociation would be an early step. Such an early dissociation between E-cadherin and β -catenin has already been described upon disruption of cell-cell adhesion induced by epidermal growth factor in tumor cells (44) and could explain the transient retention of β -catenin in the Triton-insoluble fraction observed upon Z-VAD treatment (Fig. 4B) at a time (30 min) at which E-cadherin level has already dramatically decreased. The individual fate of each protein of the junctional complex may also depend on the internalization pathway of E-cadherin, which has been suggested to vary according to the junction disrupting signal and the cellular model. They include clathrin-mediated endocytosis (43, 45, 46), caveolar-mediated internalization (44, 47), and macropinocytosis (48).

Although the precise mechanisms of E-cadherin complexes remodeling and degradation remain to be determined, we present strong evidence that the loss of E-cadherin-mediated adhesion is involved in the onset of apoptosis after detachment and that, conversely, the engagement of E-cadherin helps to maintain enterocyte survival. The DECMA-1 antibody, which inhibits E-cadherin mediated cell-cell adhesion (21) and blocks activation of the PI3K/Akt pathway (11) and Rac1 (49), induced a doubling of the rate of apoptosis. Conversely, we observed a halving of the rate of apoptosis upon E-cad-Fc ligation and clustering, which is known to recruit and activate PI3K and Rac (12). Rac recruitment by E-cadherin has been involved in the stabilization of anchoring of the E-cadherin-catenin complex to actin (50). Activation of PI3K/Akt signaling has been shown to mediate survival signals triggered by the engagement of E-cadherin (14) and other classical cadherins (51, 52). Rac1 has also been involved in the control of anoikis (53, 54). We propose that loss of anchorage triggers immediate modification of the E-cadherin complex resulting in 1) modification of cell survival signaling and 2) destabilization and degradation of E-cadherin. The resulting loss of ligand for E-cadherin in adjacent cells would amplify the phenomenon in our model in which all enterocytes are simultaneously detached from the basement membrane, but not in the intestine, in which only one cell at a time is assumed to lose its attachment.

Our results demonstrate the contribution of E-cadherin in the control of the death/survival balance in cells highly susceptible to apoptosis. The disruption of E-cadherin-mediated adhesion is likely a critical checkpoint in the modification of adhesion at the villus tip, leading to enterocyte anoikis. E-cadherin has been shown to be involved in the control of cell survival in other physiological models of apoptosis, prostate and mammary involution upon hormone withdrawal, but via a different mechanism, involving truncation of the β -catenin-binding domain (55). Strikingly, the original paper describing anoikis reported that cells have to be engaged in cell-cell interactions to be sensitive to this process (6). Moreover, cells that lose the ability to produce epithelial proteins, following epithelial-mesenchymal transition for example, become less sensitive to apoptosis (7). E-cadherin production is down-regulated during the epithelial-mesenchymal transition, during the progression of carcinoma toward more malignant states (56) but also in inflammatory bowel disease (57). Our data, combined with these observations, suggest that E-cadherin plays a complex

² S. Fouquet and S. Thenet, unpublished observations.

role both in sensitizing cells to apoptotic signals, in particular to loss of anchorage, and in maintaining survival signals. These findings open perspectives on the key role of this protein, which is dysregulated in the intestinal epithelium in both inflammatory bowel disease and in cancer.

Acknowledgments—We thank Caspar Ohnmacht, Anna Nölle, and Danièle Citadelle for their contributions; Axel Kahn for generously providing us with *L-PK-bcl-2* transgenic mice; Mireille Lambert, Johanne Le Beyec, Thélia Lesuffleur, René-Marc Mège, Monique Rousset, Alicia Torriglia, and Jean-Luc Vayssièrre for fruitful discussions; and Elena Bauer for assistance with artwork. We also appreciate the use of the facilities of IFR 58 for the electron microscopy, confocal microscopy, and flow cytometry analyses.

REFERENCES

- Gordon, J. I., and Hermiston, M. L. (1994) *Curr. Opin. Cell Biol.* **6**, 795–803
- Mayhew, T. M., Myklebust, R., Whybrow, A., and Jenkins, R. (1999) *Histol. Histopathol.* **14**, 257–267
- Potten, C. S. (1997) *Am. J. Physiol.* **273**, G253–G257
- Grossmann, J., Walther, K., Artinger, M., Kiessling, S., and Scholmerich, J. (2001) *Cell Growth & Differ.* **12**, 147–155
- Merredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993) *Mol. Biol. Cell* **4**, 953–961
- Frisch, S. M., and Francis, H. (1994) *J. Cell Biol.* **124**, 619–626
- Frisch, S. M., and Screaton, R. A. (2001) *Curr. Opin. Cell Biol.* **13**, 555–562
- Stupack, D. G., and Cheresch, D. A. (2002) *J. Cell Sci.* **115**, 3729–3738
- Steinberg, M. S., and McNutt, P. M. (1999) *Curr. Opin. Cell Biol.* **11**, 554–560
- Nagafuchi, A., and Takeichi, M. (1988) *EMBO J.* **7**, 3679–3684
- Pece, S., Chiariello, M., Murga, C., and Gutkind, J. S. (1999) *J. Biol. Chem.* **274**, 19347–19351
- Kovacs, E. M., Ali, R. G., McCormack, A. J., and Yap, A. S. (2002) *J. Biol. Chem.* **277**, 6708–6718
- Kantak, S. S., and Kramer, R. H. (1998) *J. Biol. Chem.* **273**, 16953–16961
- Bergin, E., Levine, J. S., Koh, J. S., and Lieberthal, W. (2000) *Am. J. Physiol.* **278**, F758–F768
- Shibahara, T., Sato, N., Waguri, S., Iwanaga, T., Nakahara, A., Fukutomi, H., and Uchiyama, Y. (1995) *Arch. Histol. Cytol.* **58**, 205–219
- Hermiston, M. L., and Gordon, J. I. (1995) *J. Cell Biol.* **129**, 489–506
- Schreider, C., Peignon, G., Thenet, S., Chambaz, J., and Pincon-Raymond, M. (2002) *J. Cell Sci.* **115**, 543–552
- Perreault, N., and Beaulieu, J. F. (1998) *Exp. Cell Res.* **245**, 34–42
- Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., Joulin, V., and Kahn, A. (1996) *Nature Med.* **2**, 80–86
- Walker, P. R., Leblanc, J., Smith, B., Pandey, S., and Sikorska, M. (1999) *Methods* **17**, 329–338
- Vestweber, D., and Kemler, R. (1985) *EMBO J.* **4**, 3393–3398
- Betson, M., Lozano, E., Zhang, J., and Braga, V. M. (2002) *J. Biol. Chem.* **277**, 36962–36969
- Beaulieu, J. F. (1997) *Prog. Histochem. Cytochem.* **31**, 1–78
- Schmeiser, K., and Grand, R. J. (1999) *Cell Death Differ.* **6**, 377–386
- Steinhusen, U., Weiske, J., Badock, V., Tauber, R., Bommert, K., and Huber, O. (2001) *J. Biol. Chem.* **276**, 4972–4980
- Steinhusen, U., Badock, V., Bauer, A., Behrens, J., Wittman-Liebold, B., Dorken, B., and Bommert, K. (2000) *J. Biol. Chem.* **275**, 16345–16353
- Davis, M. A., Ireton, R. C., and Reynolds, A. B. (2003) *J. Cell Biol.* **163**, 525–534
- Xiao, K., Allison, D. F., Kottke, M. D., Summers, S., Sorescu, G. P., Faundez, V., and Kowalczyk, A. P. (2003) *J. Biol. Chem.* **278**, 19199–19208
- van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (2000) *J. Biol. Chem.* **275**, 1575–1580
- Rosen, K., Rak, J., Leung, T., Dean, N. M., Kerbel, R. S., and Filmus, J. (2000) *J. Cell Biol.* **149**, 447–456
- Weaver, V. M., and Bissell, M. J. (1999) *J. Mammary Gland Biol. Neoplasia* **4**, 193–201
- Rak, J., Mitsuhashi, Y., Sheehan, C., Krestow, J. K., Florenes, V. A., Filmus, J., and Kerbel, R. S. (1999) *Neoplasia* **1**, 23–30
- Vachon, P. H., Harnois, C., Grenier, A., Dufour, G., Bouchard, V., Han, J., Landry, J., Beaulieu, J. F., Vezina, A., Dydensborg, A. B., Gauthier, R., Cote, A., Drolet, J. F., and Lareau, F. (2002) *Gastroenterology* **123**, 1980–1991
- Strater, J., Wedding, U., Barth, T. F., Koretz, K., Elsing, C., and Moller, P. (1996) *Gastroenterology* **110**, 1776–1784
- Grossmann, J., Mohr, S., Lapentina, E. G., Fiocchi, C., and Levine, A. D. (1998) *Am. J. Physiol.* **274**, G1117–G1124
- Frisch, S. M. (1999) *Curr. Biol.* **9**, 1047–1049
- Rytomaa, M., Martins, L. M., and Downward, J. (1999) *Curr. Biol.* **9**, 1043–1046
- Wang, P., Valentijn, A. J., Gilmore, A. P., and Streuli, C. H. (2003) *J. Biol. Chem.* **278**, 19917–19925
- Rios-Doria, J., Day, K. C., Kuefer, R., Rashid, M. G., Chinnaiyan, A. M., Rubin, M. A., and Day, M. L. (2003) *J. Biol. Chem.* **278**, 1372–1379
- Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarnier, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T., and Robakis, N. K. (2002) *EMBO J.* **21**, 1948–1956
- Brancolini, C., Lazarevic, D., Rodriguez, J., and Schneider, C. (1997) *J. Cell Biol.* **139**, 759–771
- Tsukamoto, T., and Nigam, S. K. (1999) *J. Biol. Chem.* **274**, 24579–24584
- Pece, S., and Gutkind, J. S. (2002) *Nat. Cell Biol.* **4**, E72–E74
- Lu, Z., Ghosh, S., Wang, Z., and Hunter, T. (2003) *Cancer Cell* **4**, 499–515
- Le, T. L., Yap, A. S., and Stow, J. L. (1999) *J. Cell Biol.* **146**, 219–232
- Ivanov, A. I., Nusrat, A., and Parkos, C. A. (2004) *Mol. Biol. Cell* **15**, 176–188
- Akhtar, N., and Hotchin, N. A. (2001) *Mol. Biol. Cell* **12**, 847–862
- Paterson, A. D., Parton, R. G., Ferguson, C., Stow, J. L., and Yap, A. S. (2003) *J. Biol. Chem.* **278**, 21050–21057
- Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N., and Kaibuchi, K. (2001) *J. Cell Sci.* **114**, 1829–1838
- Braga, V. M. (2002) *Curr. Opin. Cell Biol.* **14**, 546–556
- Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compernelle, V., Bono, F., Balconi, G., Spagnuolo, R., Oostuyse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M. C., Gittenberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J. M., Collen, D., and Dejana, E. (1999) *Cell* **98**, 147–157
- Tran, N. L., Adams, D. G., Vaillancourt, R. R., and Heimark, R. L. (2002) *J. Biol. Chem.* **277**, 32905–32914
- Coniglio, S. J., Jou, T. S., and Symons, M. (2001) *J. Biol. Chem.* **276**, 28113–28120
- Zugasti, O., Rul, W., Roux, P., Peyssonnaud, C., Eychene, A., Franke, T. F., Fort, P., and Hibner, U. (2001) *Mol. Cell Biol.* **21**, 6706–6717
- Vallorosi, C. J., Day, K. C., Zhao, X., Rashid, M. G., Rubin, M. A., Johnson, K. R., Wheelock, M. J., and Day, M. L. (2000) *J. Biol. Chem.* **275**, 3328–3334
- Thiery, J. P. (2002) *Nat. Rev. Cancer* **2**, 442–454
- Gassler, N., Rohr, C., Schneider, A., Kartenbeck, J., Bach, A., Obermuller, N., Otto, H. F., and Autschbach, F. (2001) *Am. J. Physiol.* **281**, G216–G228