Sustained Polymorphonuclear Leukocyte Transmigration Induces Apoptosis in T84 Intestinal Epithelial Cells

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Abstract. Acute colitis is characterized by a large number of polymorphonuclear leukocytes (PMNLs) migrating across the columnar epithelium in response to inflammatory stimuli. Several of these inflammatory factors have been characterized as proapoptotic inducers for intestinal epithelial cells. Our aim was to elucidate the role of PMNL transmigration in the onset of intestinal epithelial cell apoptosis. We found that PMNL migration, in response to *N*-formyl-methionyl-leucyl-phenylalanine across monolayers of intestinal epithelial cells (T84), was associated with activation of caspase-2, -3, and -9 and poly(ADP-ribose) polymerase cleavage within epithelial cells. Moreover, dihydrocytochalasin B treatment of T84 cells induced apoptosis

with similar characteristics. Although Fas and Fas ligand were expressed on T84 cells and PMNLs, treatment of epithelial cells with an antagonistic anti-Fas antibody failed to prevent apoptosis induced by migrating PMNLs. Owing to the F-actin reorganization accompanying PMNL transmigration, these findings indicate a direct relationship between PMNL migration and induction of apoptosis in epithelial cells. This apoptotic process appears to involve remodeling of the actin cytoskeleton of enterocytes independent of the Fas/Fas ligand pathway.

Key words: colitis • apoptosis • caspases • Fas • cytoskeleton

Introduction

Intestinal epithelial cells (IECs)[1] derive from stem cells located at the base of the crypt and differentiate during migration along the crypt–lumen axis, a course of 3–5 d (Moss et al., 1996). Enterocyte homeostasis in the colon depends on spontaneous apoptosis, mostly at the villous tip (Mancini et al., 1997; Metcalfe and Streuli, 1997; Efstathiou and Pignatelli, 1998; Mandal et al., 1998). Biopsies of normal colon provide evidence that apoptosis occurs in the region of shedding, but the number of apoptotic bodies markedly increases in case of inflammatory bowel diseases (IBDs) (Lee, 1993; Guy-Grand et al., 1998). Histological studies indicate a relationship between the degree of lesions observed in IBDs and the appearance of apoptotic and necrotic bodies (Fenoglio-Preiser et al., 1989; Iwamoto et al., 1996). Furthermore, the degree of polymorphonuclear leukocyte (PMNL) transepithelial migration

has been shown to correlate with the degree of intestinal epithelial barrier dysfunction (Teahon et al., 1991). Kinetic studies have shown a shortening in the life span of colonic epithelial cells in ulcerative colitis (Allan et al., 1985). The onset of apoptosis in epithelial cells seen in IBD occurs earlier than in normal cells and before reaching the luminal surface (Lee, 1993; Kim et al., 1998; Rudi et al., 1998). Because patients with ulcerative colitis have an increased risk of developing large bowel carcinoma (Serafini et al., 1981; Bedi et al., 1995), we thought it was of interest to characterize the apoptotic program of epithelial cells occurring during IBD.

The onset of physiological apoptosis depends on the intrinsic nature of the epithelium. Previous studies have shown that colonic stem cells, which barely undergo apoptosis, largely express *bcl-2*; however, the level of this suppressing gene decreases as cells migrate along the crypt axis, differentiate, and undergo apoptosis (Merritt et al., 1995; Metcalfe and Streuli, 1997). Conversely, it has been shown that expression of the apoptosis-inducing gene *bak* is increased in areas of epithelium undergoing apoptosis and in the human colon cancer cell line HT-29 treated with apoptogenic agents (Moss et al., 1996; Mandal et al., 1998). Apoptotic homeostasis can be disrupted by various extracellular factors generated by physiopathological condi-

tions. For instance, in graft versus host disease, intraepithelial lymphocytes or infiltrating lymphocytes expressing Fas ligand induce apoptosis of enterocytes which express Fas (Iwamoto et al., 1996; Strater et al., 1996; Guy-Grand et al., 1998; Kraus et al., 1998; Lin et al., 1998; Ueyama et al., 1998). It has been shown previously that bacterial invasion (Kim et al., 1998), toxin A and B produced by *Clostridium difficile* (Mahida et al., 1996; Fiorentini et al., 1998), peroxynitrite released by activated macrophages (Sandoval et al., 1997), or butyrate produced in the colon by symbiotic bacteria (Hague et al., 1997) can elicit apoptosis in colonic tumor cell lines. Ischemia and ischemia/reperfusion injury can also trigger the apoptotic process in colonic epithelial cells (Ikeda et al., 1998). Finally, disruption of the interaction between normal epithelial cells and extracellular matrix (Frisch and Francis, 1994; Wang et al., 1995; Frisch et al., 1996; Giancotti, 1997) or loss of cell–cell contact (Bates et al., 1994; Brancolini et al., 1997) has been shown to induce IEC anoikis.

Here, we present evidence that PMNL transmigration through an IEC monolayer (T84) is sufficient by itself to induce apoptosis of T84 cells. Transmigration has been shown to induce a profound remodeling of the epithelial actin cytoskeleton (Hofman et al., 1996). We present evidence that actin network disruption induced by massive PMNL transmigration is able to induce the early onset of apoptosis in the crypt epithelium.

Materials and Methods

Cell Culture

The human colonic carcinoma cell line T84 cells (passages 55–70) were obtained from the American Type Culture Collection. T84 cells were grown until they became confluent monolayers in a 1:1 mixture of DMEM and Ham's F-12 medium, supplemented with 15 mM N-2 Hepes buffer, pH 7.5, 14 mM $NaHCO_3$, 40 mg/ml of penicillin, 90 mg/ml of streptomycin, 8 mg/ml of ampicillin, and 5% FBS. Monolayers were used 6–14 d after plating. Steady-state resistance was reached after 4–6 d, with some variability largely related to the number of cell passages. Monolayers received one weekly feeding after initial plating. For transmigration assays performed in the physiological direction, inverted monolayers were grown on collagen-coated, 0.33-cm^2 ring–supported, permeable polycarbonate filters (Costar Corp.) were constructed to permit a basolateral to apical migration of PMNLs ("inverted inserts") as described previously by Madara et al. (1992). For transmigration realized in the nonphysiological direction, apical to basolateral, monolayers were grown and maintained confluent on collagen-coated 5-cm^2 polycarbonate filters adapted from a previously described method (Dharmsathaphorn and Madara, 1990).

Preparation of Human PMNLs

PMNLs were isolated from normal human volunteers using a gelatin sedimentation technique (Parkos et al., 1992). In brief, whole blood was collected in tubes containing heparin and centrifuged at 300 g for 20 min (20°C). The plasma and buffy coat were removed and the gelatin/cell mixture was incubated at 37°C for 30 min to remove contaminating red blood cells. Residual red blood cells were then lysed with isotonic ammonium chloride. After washing in HBSS (without Ca^{2+} and Mg^{2+}, with 10 mM Hepes, pH 7.4) (Sigma-Aldrich), PMNLs (95% pure) were counted and resuspended in HBSS at 5 × 10^7 PMNLs/ml. PMNLs with 98% viability by trypan blue exclusion were used for experiments within 1 h after isolation.

Transmigration Assay

The PMNL transepithelial migration assay has been detailed previously (Nash et al., 1991). The physiologically (basolateral to apical) or the nonphysiologically (apical to basolateral) directed PMNL–transepithelial migration assays were performed as described (Dharmsathaphorn and Madara, 1990). In both cases, T84 cells were rinsed in HBSS to remove residual T84 medium. To allow a transepithelial chemotactic gradient to form, N-formyl-methionyl-leucyl-phenylalanine (fMLP) at 10^{-7} M was added in the inner chamber 15 min before the addition of PMNLs to the upper chambers. For basolateral to apical transmigration experiments, 2 × 10^6 PMNLs were added at time 0 on upper chambers of inverted monolayers grown on 0.33-cm^2 filters. To compensate for the less efficient apical to basolateral transmigration (Parkos et al., 1992), more PMNLs were added to upper chambers, 15 or 40 × 10^6 PMNLs/ml. Because apical to basolateral (nonphysiological) transmigration allowed more epithelial cells to be harvested, this path was systematically used for biochemical experiments.

Transmigration of neutrophils was assayed by quantification of azurophil granule marker myeloperoxidase as described previously (Madara et al., 1992). In brief, transmigrated PMNLs located in the inner chamber were solubilized in Triton X-100 containing HBSS. The pH was adjusted to 4.2 with a 1:10 dilution of 1.0 M sodium citrate, and the myeloperoxidase activity was assayed by the addition of an equal volume of 1 mM 2,2'-azino-di-(3-ethyl)dithiazoline sulfonic acid and 10 mM H_2O_2 in 100 mM citrate, pH 4.2. Color development was quantitated in a microtiter plate reader at 405 nm.

Morphological Studies

T84 monolayers were studied by EM at different times of PMNL basolateral to apical transmigration experiments. T84 cells grown on 0.33-cm^2 filters were prefixed for 2 h in 3.7% paraformaldehyde, then fixed in osmic acid for 1 h at 4°C. Cells were dehydrated in ethyl alcohol and embedded into Epoxy resin (Amersham Pharmacia Biotech). The number of epithelial cells showing typical features of apoptosis, i.e., a nuclear condensation and/or fragmentation and a cytoplasmic shrinkage associated with a loss of microvilli, were counted at 0, 4, and 12 h after the onset of PMNL physiologically directed transmigration. In each condition, apoptotic cells were counted in a random section of 6 monolayers among a total of 200 epithelial cells.

For immunoelectron microscopy, monolayers grown on 0.33-cm^2 filters were fixed for 30 min in 3.7% paraformaldehyde and embedded at low temperature into LR White™ Resin (London Resin System). Ultrathin sections were put on 300-mesh nickel grids, washed with PBS, then incubated for 60 min at room temperature with the anti–Fas ligand antibody (dilution 1:200; Santa Cruz Biotechnology, Inc.). After washing with PBS, the grids were incubated for 60 min with 15 nm colloidal gold–conjugated rabbit anti–mouse secondary antibody (dilution 1:20; British Biocell International). The grids were washed with PBS, then with distilled water and stained with uranyl acetate. Secondary antibody alone and Leu4 (anti-CD3) antibody (Immunotech) were used in each experiment as negative controls. Ultrathin sections were examined with a JEOL 1200 EXII electron microscope.

For immunofluorescence and confocal microscopy studies, cells grown on collagen-coated 0.33-cm^2 filters were fixed in 3.7% paraformaldehyde. Cells were stained with the anti-CD95 ligand antibody (10 μg/ml; Santa Cruz Biotechnology, Inc.), or with the anti-CD95 antibody (CH11, 10 μg/ml; Immunotech) for 1 h and then with a swine anti–rabbit antibody linked to FITC (dilution 1:20; Dako) for 1 h. Between each step, cells were washed in PBS and antibodies were diluted in buffer (2% gelatin, 1% BSA, and 2.5% goat serum diluted in PBS buffer). Anti-CD3 antibody was used for negative control and anti-β1 integrin antibody (K20, 10 μg/ml; gift from M. Ticchioni, INSERM U343, Nice, France) was used for positive control of basolateral staining. Slides were examined with a Leica TCS NT confocal microscope.

Flow Cytometric Analysis

T84 cells grown on 5-cm^2 filters were submitted to PMNL transmigration and dissociated using trypsin and 0.3% EDTA. In brief, 10^6 cells were resuspended in PBS with 0.1% BSA buffer, and stained using the anti-Fas antibody (ZB4, 10 μg/ml; Immunotech) and the rabbit anti–mouse linked to FITC (dilution 1:20; Dako). After washing, cells fixed in 0.4% formaldehyde were analyzed on a FACScan™ flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using CellQUEST™ software.

Biochemical Studies

DNA Fragmentation Assay. DNA was isolated from 5 × 10^6 control T84 monolayers grown on 5-cm^2 filters or from T84 cells exposed for various

times to PMNL apical to basolateral transmigration experiments (6, 12, and 16 h). Cells were lysed for 20 min at room temperature with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)/0.2% Triton X-100 and treated with RNase A (100 μg/ml; Boehringer) at 37°C for 30 min. Proteins were denatured by incubation with proteinase K (100 μg/ml) (Boehringer) at 30°C for 30 min. DNA was then precipitated in 0.5 M NaCl/isopropanol overnight at −20°C. DNA was then rinsed with 70% ethanol, mixed with TE buffer, heated for 30 min at 56°C, and then electrophoresed in a 1.5% agarose gel containing 10 mg/ml ethidium bromide. The gel was examined and photographed under UV light to evidence intranucleosomal DNA fragmentation (laddering) characteristic of apoptosis.

Western Immunoblot Analysis. To analyze biochemical hallmarks of apoptosis, we used inserted T84 monolayers grown on 5-cm² filters and exposed for various times for transmigration experiments. Control monolayers and monolayers used for transmigration assays were washed in HBSS, then gently scraped in lysis buffer at 4°C (10 mM Hepes, 3.5 mM MgCl$_2$, 150 mM NaCl, 1% NP40, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 25 μM leupeptin, 5 mM benzamidin, 1 μM pepstatin, 25 μM aprotinin, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 0.5 mM DTT) at a 25×10^6 cells/ml density. Cell lysates were centrifuged for 15 min at 4°C and denatured by boiling in reducing SDS sample buffer. Protein lysates (50 μg) were analyzed by migration in SDS-PAGE polyacrylamide gels and electrophoretically transferred to nitrocellulose sheets. The sheets were incubated in blocking buffer then probed with the first antibody incubated overnight at 4°C. This labeling was visualized by peroxidase-conjugated secondary antibody (rabbit anti–mouse immunoglobulins, dilution 1:5,000, or goat anti–rabbit immunoglobulins, 1:10,000 dilution; both from Dako) and by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech). The different antibodies used were: anti–extracellular signal–regulated kinase (ERK)1 (dilution 1:2,000), anti–caspase-2 (1:1,000 dilution; Santa Cruz Biotechnology, Inc.); anti–caspase-9 (1:3,000 dilution) anti–poly(ADP-ribose) polymerase (PARP, 1 μg/ml), anti–caspase-6 (1 μg/ml; BD PharMingen); anti–caspase-3 (1:3,000 dilution), anti–caspase-7 (0.5 μg/ml; Transduction Laboratories); anti-Fas (250 ng/ml, CH11; Immunotech); and anti–p38 mitogen-activated protein kinase (1:1,000 dilution; Biolabs).

DEVD-pNA Cleavage Assay. Caspase activity was measured using a continuous colorimetric assay. In brief, control cells or cells undergoing 12 h of PMNL transmigration were gently scraped in PBS containing 2 mM DTT. After sonication, two times for 8 s each, lysates were centrifuged at 15,000 g and 50 μg of cell extract was incubated with 200 μM of Ac-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Alexis Corporation) preferentially cleaved by members of the CPP32 family of cysteine proteases. Liberation of pNA was monitored continuously at 37°C by using an excitation wavelength of 410 nm. Measurements were recorded over the linear range of assay, and caspase activity was controlled by adding in the cell extract an apopain/CPP32 inhibitor (DEVD-CHO; Alexis Corporation). Substrates without lysates served as negative control.

Results

Sustained Transepithelial Migration of PMNLs Induces Apoptosis of Human Colonic Epithelial T84 Cells

Monolayers of T84 cells grown on collagen-coated permeable supports were used for transmigration assays and apoptosis, using several methods. To reproduce massive PMNL transmigration, 40×10^6 PMNLs/ml were used for transmigration assays. As shown in Fig. 1 A, the majority of T84 cells observed before the onset of PMNL transmigration exhibited a regular brush border (Fig. 1 Aa), whereas cells subjected for 4 h to PMNL transmigration exhibited a profound disorganization of this structure (Fig. 1 Ab). Under these conditions, a low percentage of epithelial cells showed features of apoptosis (3 vs. 5% for cells observed at 0 and 4 h, respectively). Furthermore, after being exposed for 12 h to PMNL migration, an increased percentage of T84 cells adherent to the filters (25%) presented typical apoptosis features, e.g., nuclear condensation as well as cytoplasmic shrinkage associated with a loss of microvilli (Fig. 1 Ac). These morphologic apoptosis

hallmarks were accompanied by DNA fragmentation, suggesting activation of the caspase-activated deoxyribonuclease (CAD) (Fig. 1 B). DNA laddering was only detectable in T84 cells at 12 h after the initiation of PMNL transmigration. As shown in Fig. 1 Cb, this time corresponds to $\sim 5 \times 10^6$ transmigrated PMNLs per 5 cm² of epithelial monolayer. It is noteworthy that none of these molecular features of apoptosis were visible when the density of PMNLs was decreased to 15×10^6 PMNLs/ml, suggesting that the apoptotic program was activated by PMNL transmigration in a graded manner.

PMNL Transmigration Induces Procaspase-9 and PARP Cleavage

Biochemical analysis of activation of various caspases in apoptotic T84 cells was performed. The amount of procaspase-9 and PARP was analyzed by Western blotting after transmigration at a high density (40×10^6 PMNLs/ml) of PMNLs. As shown in Fig. 2 A, PMNL transmigration induced an $\sim$10-fold decrease in the level of procaspase-9 within 90 min, which was earlier than the appearance of morphological signs of apoptosis and DNA fragmentation. As shown in Fig. 2 B, intact PARP was cleaved into the expected 85-kD fragment within 4 h. When PMNLs were incubated with T84 cell monolayers for 4 h at the same density in the absence of fMLP, little or no fragmentation of PARP was detectable. Similarly, the procaspase-9 level was only slightly reduced under the same conditions. These results suggest that the mere contact between PMNLs and T84 cells was not sufficient to induce apoptosis in epithelial cells (Fig. 2 B). To confirm that procaspase-9 cleavage activity resulted from activation of upstream caspases, T84 cells were preincubated for 24 h with the fluoromethyl ketone inhibitor ZVAD-FMK (200 μM; Alexis Corporation), an irreversible inhibitor of caspases 1–10 (Garcia-Calvo et al., 1999) before being subjected to PMNL migration. Indeed, ZVAD-FMK inhibited procaspase-9 processing. However, this compound only partially inhibited the cleavage of the 116-kD form of PARP into the 85-kD fragment, indicating that other proteases distinct from caspases may participate in the migration-induced PARP cleavage (Fig. 2 B). We verified, by repeating the experiment in the presence of an excess of medium in the upper chamber, that PARP and procaspase-9 cleavage could not be accounted for by deprivation of nutrients or survival factors (Fig. 2 B).

Apoptosis of T84 Cells Induced by PMNL Transmigration Is Associated with a Late Activation of Caspase-2 and -3

Time course of procaspase-9 activation was compared with those of procaspase-2, -3, -6, and -7. Colonic epithelial T84 cells, grown on collagen-coated filters, were subjected to intensive fMLP-mediated PMNL transmigration for various times. After transmigration, T84 cells were lysed and lysates were probed with antibodies to procaspase-2, -3, -6, and -7. Fig. 3 A shows that procaspase-2 was significantly activated after 10 h of transmigration, as indicated by the cleavage of the 48-kD procaspase-2 band into its 18-kD active fragment. IECs contain abundant amounts of procaspase-3 (Grossman et al., 1998; Guy-
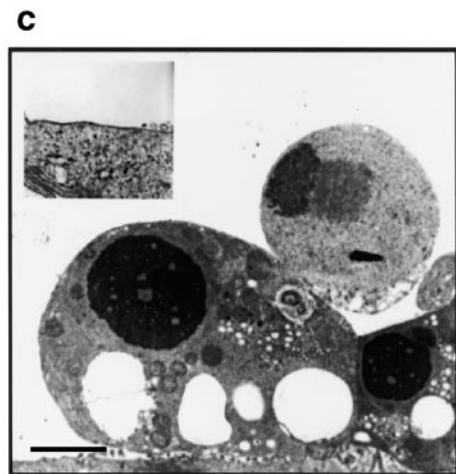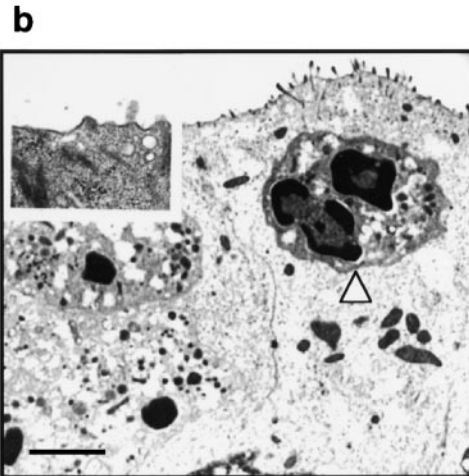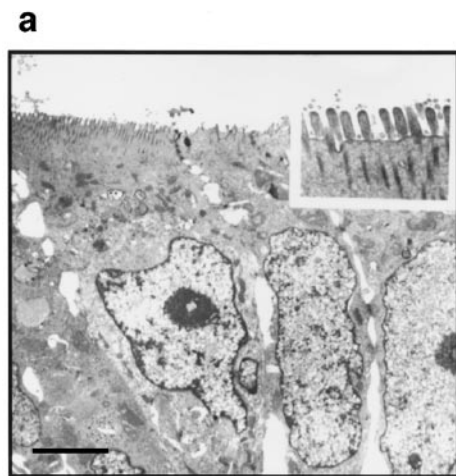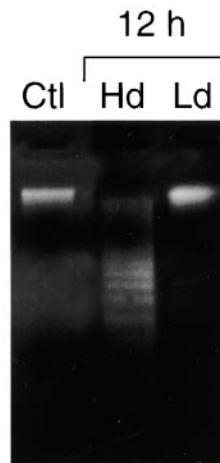
A

a

b

c

B

12 h

Ctl Hd Ld

C

a

Number of PMNL transmigrated (millions)

Time of transmigration (hours)

b

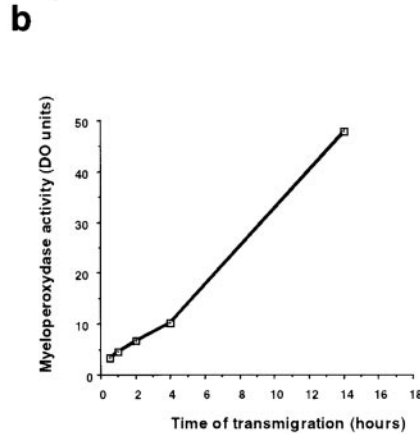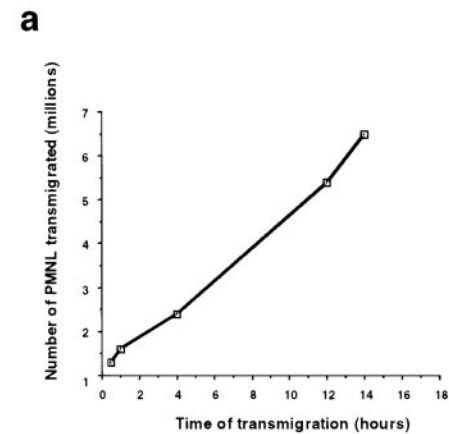Myeloperoxydase activity (DO units)

Time of transmigration (hours)

Figure 1. (A) Apoptosis of T84 cells after PMNL transmigration. Confluent T84 cells grown on 0.33-cm^2 filters were incubated with PMNLs for transmigration assays. Morphological hallmarks of apoptosis were analyzed by EM. (a) T84 cell controls. (b) T84 cells after 4 h of transmigration at a high density of PMNLs (40 × 10^6 PMNLs/ml). Arrowhead indicates a PMNL rolling across the epithelial barrier. Cells display a brush border disorganization. (c) After 12 h of transmigration at a high density of PMNLs (40 × 10^6 PMNLs/ml), T84 cells show cytoplasmic shrinkage and chromatin condensation and brush border disorganization. These experiments were performed three times in a basolateral to apical pathway. Bar: (a and b) 3.3 μm; (c) 2.3 μm; (inset) 0.8 μm. (B) DNA fragmentation of T84 cells after 12 h of transmigration at a high density of PMNL (Hd). At low density (Ld), DNA fragmentation remained undetectable. Ctl, T84 cell control. (C) Measurements of PMNLs transmigrated during a kinetic of transmigration at a high density of PMNLs (40 × 10^6 PMNLs/ml). (a) PMNLs located in the lower chamber were counted. (b) Myeloperoxidase activity of PMNLs transmigrated was analyzed during the same experiment as in Ca.

Grand et al., 1998), which decreased by ∼50% after 12 h of transmigration as evaluated by scanning densitometry of procaspase-3 immunoblots (Fig. 3 A). Thus, it appears that both caspase-2 and -3 were activated during the apoptotic program induced by PMNL transmigration. Under the same conditions, we failed to detect any trace of the p17 cleavage product of procaspase-6 and -7 during transmigration. Thus, it is likely that caspase-6 and -7 are not
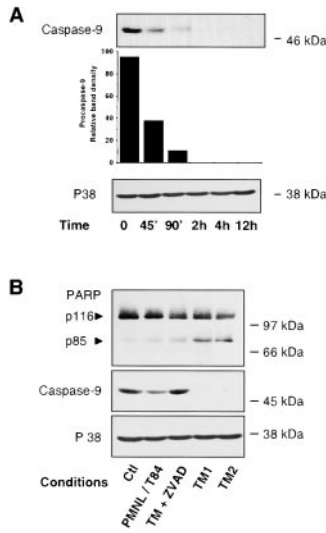
Figure 2. Early procaspase-9 and PARP cleavage in T84 cells after PMNL transmigration assays. (A) Immunoblot analysis of procaspase-9 disappearance during a time course of PMNL transmigration in T84 cell lysates (50 μg). Histogram of scanning densitometry of immunoblot of procaspase-9 indicates a decrease ∼90% of procaspase-9 expression after 90 min of PMNL transmigration. Immunoblot for p38 was performed to control for equal amounts of proteins. (B) Cleavage of PARP in T84 cells during PMNL transmigration was analyzed by the same technique using anti-PARP mAb. PARP cleavage is specifically induced by PMNL transmigration. PMNLs were added to T84 cells grown on filters for 4 h to induce contact with T84 cells without inducing transmigration with fMLP (PMNL/T84). Profile of PARP cleavage in T84 cells preincubated for 24 h with the caspases inhibitor ZVAD (200 μM; TM+ZVAD) was compared with the profile of PARP in T84 cells after 4 h of transmigration (TM1). To eliminate artifacts due to the loss of epithelial growth/survival factors, a 4-h PMNL transmigration assay was performed with a large excess of medium (2 ml instead of 0.5 ml) (TM2). The profile of procaspase-9 expression was analyzed in the same conditions. Procaspase-9 was cleaved after PMNL transmigration (TM1) and (TM2), whereas pretreatment of T84 cells with ZVAD inhibits this cleavage (TM+ZVAD), and the mere contact with PMNLs only induces a slight decrease in procaspase-9 expression. The same membrane was used for procaspase-9 and PARP expression; immunoblot for Erk was performed to control for equal amounts of proteins (transmigration assays performed in experimental conditions, *n* = 3).

Figure 3. Experimental conditions mimicking pathological conditions of PMNL transmigration induce caspase-3 and -2 activation in T84 cells. (A) Western blot analysis of caspase-3 and -2 activation in IECs in response to PMNL transmigration. Lysates from control or after various times of transmigration were prepared as described previously. 50 μg of supernatant was analyzed by immunoblot for expression of different caspases using anti–caspase-3, -2, -6, or -7 antibodies. Histogram of scanning densitometry of procaspase-3 immunoblot depicts a regular decrease in procaspase-3 expression until 50% of disappearance at 12 h. Caspase-2: procaspase-2 (48 kD) was cleaved after 10 h of PMNL transmigration leading to the appearance of the p18 active fragment. Procaspase-7 (35-kD band) and procaspase-6 (34-kD band) remained uncleaved during transmigration assays. Activation of caspase-7 in T84 cells was checked with St, yielding the p17 active fragments. ERK: immunoblot for ERK was performed on each membrane to control for equal amounts of proteins (*n* = 3). (B) Caspases and PARP cleaved in T84 cells during PMNL transmigration were specifically expressed by T84 cells. PMNLs were kept in HBSS($-$) buffer at 4°C (Ctl) or in 10^{-7} M fMLP diluted in HBSS($+$) buffer at 37°C for 4 h (fMLP), and lysates were analyzed by immunoblot for expression of procaspase-9, PARP, and procaspase-3, as described above. Lysates from T84 cell controls (Ctl), or after 4 h of PMNL transmigration (TM), were analyzed in the same conditions. (C) Stimulation of caspase-3–like activity in T84 cells during transmigration assays. Extracts, prepared as described in Materials and Methods, were assessed for Ac-DEVD-pNA hydrolyzing activity, and measurements of activity were recorded over 24 h. *N*-acetyl-Asp-Glu-Val-Asp-pNa hydrolyzing activity was 98% blocked by the addition of Ac-DEVD-aldehyde (CHO, 100 μM) to extracts before addition of chromogenic substrate (not shown). Two transmigration assays (TM1 and TM2) were performed, and when indicated T84 cells were preincubated with DEVD-CHO (TM + DEVD-CHO) for 24 h before 12 h of transmigration assay. One of three transmigration assays is shown with error bars corresponding to triplicates (transmigration assays performed in experimental conditions).

involved in the onset of T84 cell apoptosis as induced by PMNL migration, whereas caspase-7 was shown to be activated when T84 cells apoptosis was induced by incubation with staurosporine (St) (10 μM) for 6 h (lane 8). To exclude possible contamination by caspases expressed by PMNLs bound to T84 monolayers, we compared procaspase-9 and -3 and PARP cleavage in both cell types. First, our experiments confirm a previous study indicating absence of PARP expression in PMNLs (Fig. 3 B) (Shangavi et al., 1998). Second, they also show that procaspase-9 and -3 are not, or only weakly, expressed by these cells compared with T84 cells (Fig. 3 B).

Because the p17 caspase-3 active form remained undetectable by Western blot measurement, we investigated global caspase activity using a colorimetric substrate assay. T84 cells were subjected for 14 h to PMNL transmigration before being lysed. Thereafter, cell extracts were incubated for 24 h with DEVD-pNA, a colorimetric substrate of caspase-3 (see Materials and Methods). Specificity of the cleavage was evaluated by adding the caspase-3 inhibitor (DEVD-CHO, 100 μM) to each extract. As shown in Fig. 3 C, though we failed to detect the p17 caspase-3 ac-
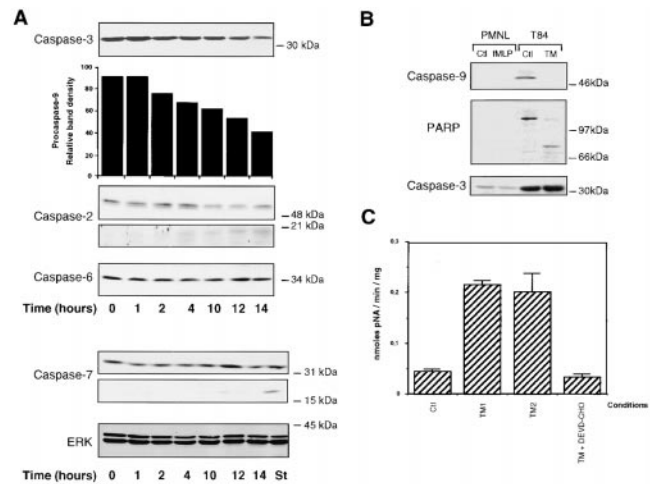
tive form by immunoblot (Fig. 3 A*)*, caspase-3 activity was weakly active in T84 cells after 14 h of exposure to PMNL transmigration.

T84 Cells Constitutively Express Fas and Fas Ligand

Since Fas receptor expression has been reported to be up-regulated by various proapoptotic stimuli, it was of inter-
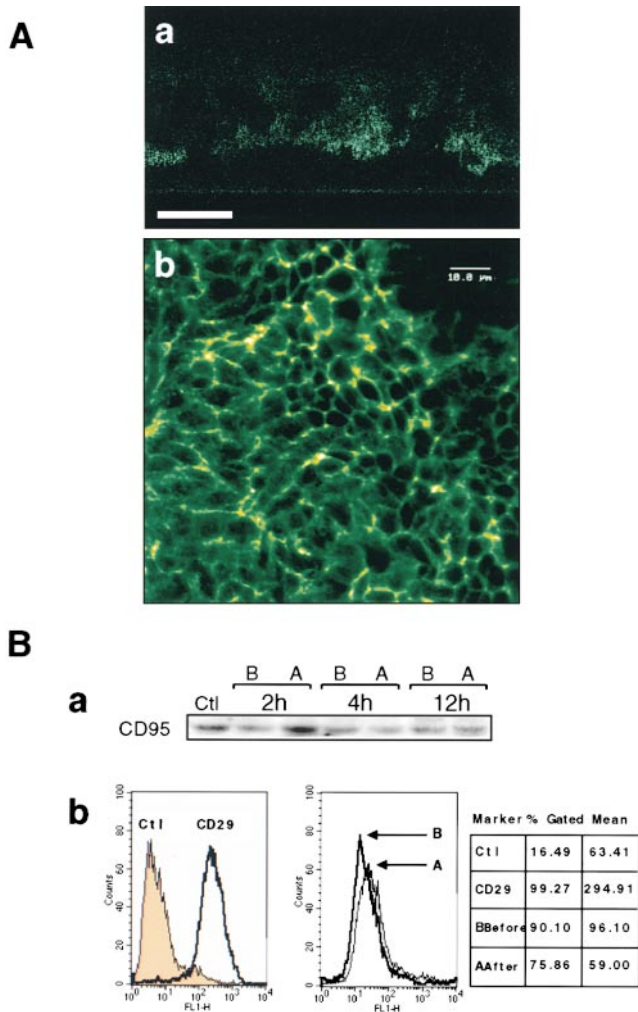
Figure 5. T84 cells constitutively express Fas ligand. (A) Confocal microscopy of confluent T84 cells stained with anti–human CD95 ligand–specific IgG1 and secondary antibodies linked to FITC showed basolateral and apical Fas ligand expression. Bar: (A) 15 μm. (B) EM studies. Confluent T84 cells staining with anti–human CD95 ligand followed by an immunogold staining confirm basolateral (b) and apical (a) expression. Bar: (a and b) 1.4 μm.

Figure 4. Transmigration failed to affect Fas expression on T84 cells. (A) T84 cells constitutively express the death receptor Fas on their basolateral side. Confocal analysis of T84 cells stained with anti-Fas IgM (CH11) followed by secondary antibodies linked to FITC. Analysis of T84 cells staining in a vertical axis (a), or in a horizontal axis (b). Bar: (a and b) 10 μm. (B) (a) Immunoblot analysis of Fas expression by T84 cells before and after a time course of PMNL transmigration. Lysates prepared from T84 cells (50 μg) before (B) and after (A) 2, 4, and 12 h of PMNL transmigration were immunoblotted with anti-Fas (CH11) mAbs. (b) Analysis of cell surface Fas expression by flow cytometry using the specific mAb to Fas (ZB4; Immunotech). T84 cells were stained before (B) and after (A) 4 h of PMNL transmigration experiments. Staining with antibodies to CD29 was used as a positive control (CD29) and irrelevant IgG1 as a negative control (Ctl) (transmigration assays performed in experimental conditions).

est to determine whether PMNL transmigration can induce Fas upregulation in T84 cells (Micheau et al., 1997). As shown in Fig. 4 A, Fas expression was mainly located at the basolateral side of T84 cells and remained unchanged after 12 h of transmigration, as assessed by probing lysates with anti-Fas antibodies (Fig. 4 Ba). Nonetheless, a slight decrease in cell surface expression was observed when we analyzed Fas staining on T84 cells before and after 4 h of PMNL transmigration by flow cytometry (Fig. 4 Bb).

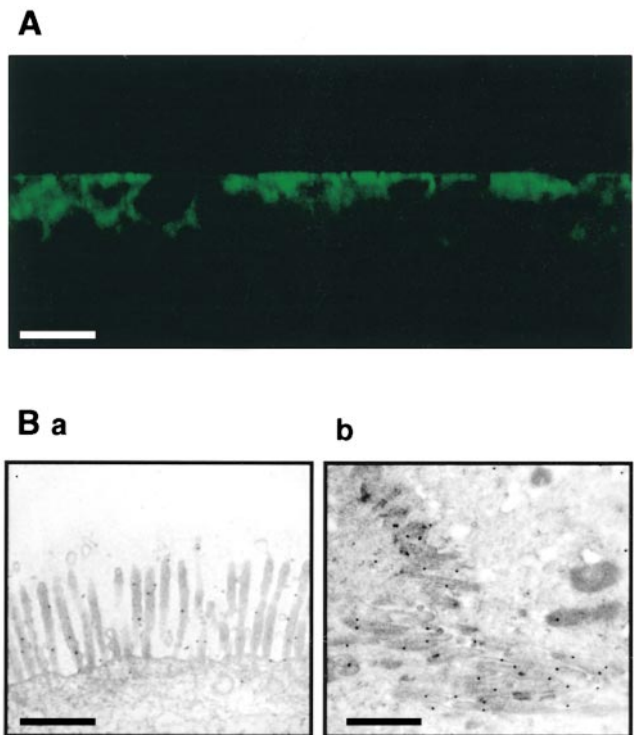When confluent monolayers grown on collagen-coated filters were stained by anti–human Fas ligand antibodies and analyzed by confocal microscopy, both basolateral and apical sides were clearly labeled (Fig. 5 A). EM analysis confirmed the location of Fas receptor at the basolateral side of T84 cells (not shown), while Fas ligand was expressed both at the apical (Fig. 5 Ba) and the basolateral sides of the epithelial cells (Fig. 5 Bb).

Fas Pathway Is Not Involved in T84 Cell Apoptosis Induced by PMNL Transmigration

To determine whether the Fas pathway was functional in T84 cells, two series of experiments were conducted. First, DNA fragmentation was assayed after 4 h of incubation with the agonistic anti-Fas mAb CH11 (1 μg/ml) and TNF-α (10 ng/ml) in the presence of cycloheximide (0.5 μM). St (10^{-7} M) incubated for 6 h was used as a positive control for DNA fragmentation. Treatment of T84 cells with CH11, similar to TNF-α or St, resulted in a typical profile of DNA fragmentation characterized by the appearance of DNA ladders (Fig. 6 A), confirming that Fas and TNF-α death receptors were functional in T84 cells, as in other human colon carcinoma cell lines (Micheau et al., 1997). Second, to test a possible involvement of the Fas/Fas ligand pathway, T84 cells were incubated with an antagonistic mAb to Fas (ZB4) for 1 h before the beginning of PMNL transmigration. Inhibition of the proapoptotic
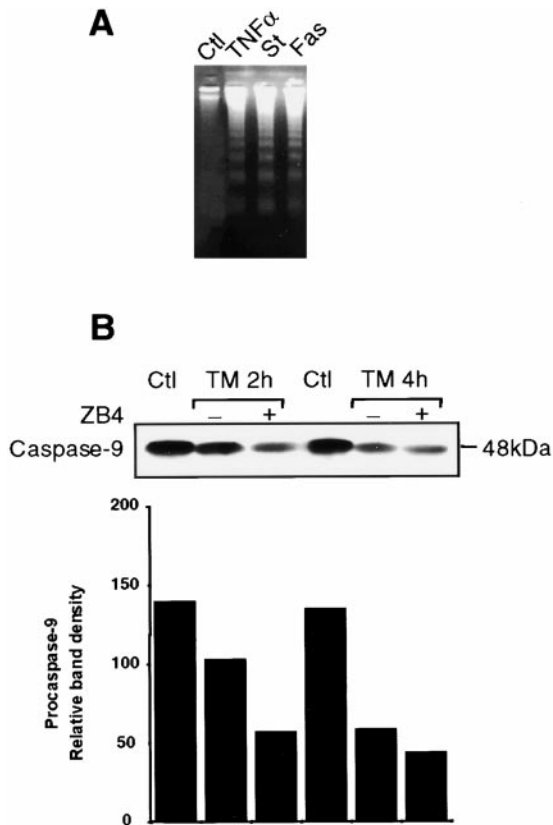
A

Ctl TNFα St Fas

B

Ctl TM 2h Ctl TM 4h
ZB4
    − +   − +
Caspase-9 ——— ◄ 48kDa

Procaspase-9 Relative band density

Figure 6. Different requirements for Fas- and transmigration-induced apoptosis in T84 cells. (A) DNA fragmentation assay. Control T84 cells grown on 6-well plates were treated for 4 h with either CH11 antibody (1 μg/ml, Fas), TNF-α (10 ng/ml) plus cycloheximide (0.5 μM, TNFα), or St (1 μM), which is used as positive control for DNA fragmentation. (B) Immunoblot analysis of Fas-dependent apoptosis during PMNL transmigration. To impair Fas–Fas ligand interaction, T84 cells were preincubated for 1 h with the antagonistic antibody to Fas receptor (ZB4, 1 μg/ml), and PMNL transmigration was continued in the presence of the same antibody. After 2 h (TM 2h) and 4 h (TM 4h) of transmigration, T84 cells lysates (50 μg) were analyzed by immunoblot with the mAb to caspase-9. Histogram of scanning densitometry of immunoblot of procaspase-9 indicates a twofold decrease in procaspase-9 expression after 4 h of PMNL transmigration. This histogram also shows the absence of a clear ZB4 effect in procaspase-9 expression (transmigration assays performed in experimental conditions).

effect of CH11 antibodies by preincubation with ZB4 mAbs was verified (data not shown). Lysates corresponding to T84 cells exposed for 2 and 4 h of PMNL transmigration were probed with anti–procaspase-9 antibody. As shown in Fig. 6 B, scanning of procaspase-9 immunoblots indicated that PMNL transmigration induced activation of procaspase-9, regardless of the presence of the antagonistic antibody. This eliminates the possibility that Fas–Fas ligand interaction was involved in the apoptotic process induced by PMNL transmigration.

Prolonged Actin Depolymerization Induces Apoptosis in T84 Cells

During PMNL transmigration, epithelial cells undergo

major rearrangement of cortical F-actin in association with the passage of PMNLs along the paracellular pathway and across tight junctions (Shapiro et al., 1991; Matthews et al., 1994; Hofman et al., 1996). To address if epithelial actin cytoskeleton reorganization was a potential inducer of T84 cell apoptosis, T84 cells were incubated for 6 h with dihydrocytochalasin B (DHCB, 3×10^{-4} M), a potent F-actin depolymerization agent. As shown in Fig. 7 A, DHCB induced the concomitant cleavage of procaspase-9 and PARP. Further, the histogram for caspase-2 immunoblot shows that DHCB induced the same profile of caspase-9 and -2 activation as that observed after a 12-h exposure of T84 cells to PMNL transmigration. Activation of caspases, as well as DNA fragmentation, appeared after a 10-h treatment of T84 cells with 3×10^{-4} M DHCB (Fig. 7 B), reminiscent of the apoptotic program induced by PMNL migration. Finally, to verify the specific involvement of actin cytoskeleton depolymerization in the commitment of the apoptotic program, T84 cells were pretreated with phallacidin (30 μM), an actin polymerizing agent, to antagonize the disrupting action of DHCB. As shown in Fig. 7 B, phallacidin totally inhibited the apoptogenic effect of DHCB, indicating that a long-lasting cytoskeleton actin depolymerization is responsible for the epithelial cells apoptosis. To ascertain if the prolonged cytoskeletal depoly-
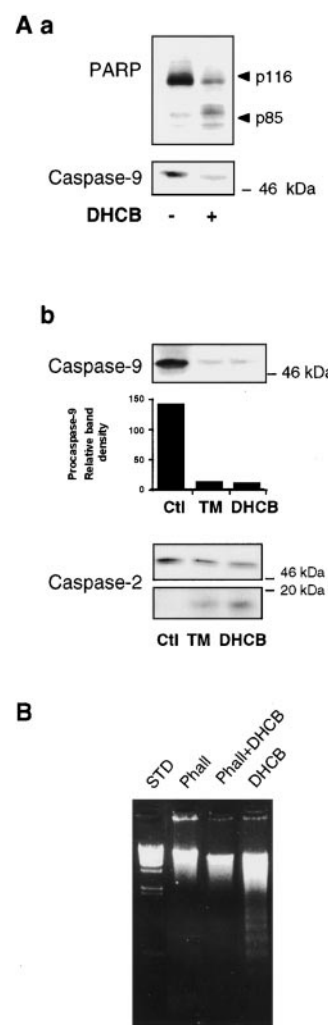

A a

PARP ◄ p116 ◄ p85
Caspase-9 — 46 kDa
DHCB − +

b

Caspase-9 — 46 kDa
Procaspase-9 Relative band density
Ctl TM DHCB

Caspase-2 — 46 kDa
— 20 kDa
Ctl TM DHCB

B

STD Phall Phall+DHCB DHCB

Figure 7. Actin depolymerization mediated apoptosis in T84 cells. (Aa) Immunoblot analysis of caspase activation induced by actin depolymerization. T84 cells were treated with DHCB (3×10^{-4} M) for 10 h and protein lysates (50 μg) were analyzed by Western blot using anti-PARP or anti-caspase-9 antibodies. (b) After 14 h of transmigration assay (TM), protein lysates from T84 cells were immunoblotted with anti-caspase-9 or -2, and DHCB and transmigration effects was compared. Scanning densitometry of immunoblot of procaspase-9 shows an ∼14-fold decrease in procaspase-9 expression in T84 cells after 14 h of PMNL transmigration or after DHCB treatment. (B) T84 cells were analyzed for DNA fragmentation. T84 cells grown on 6-well plates were preincubated with DHCB (3×10^{-4} M, DHCB) for 10 h, with phallacidin (30 μM, Phall) for 16 h, or with phallacidin (30 μM) during 16 h and DHCB for the last 6 h (Phall + DHCB) (transmigration assays performed in experimental conditions).
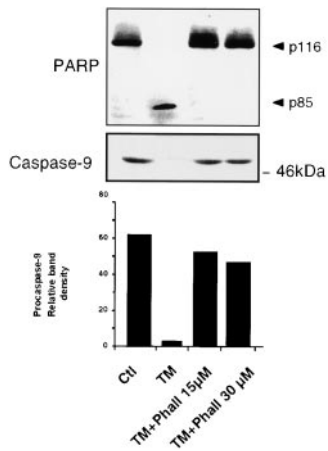
Figure 8. T84 cells apoptosis induced by PMNL transmigration is inhibited by actin rigidification. Immunoblot analysis of PARP and procaspase-9 cleavage inhibition by phallacidin treatment. T84 cells were treated with 15 or 30 μM phallacidin for 12 h and then subjected to PMNL transmigration for 4 h. Levels of PARP and procaspase-9 expressed by T84 cells control (Ctl) or after 4 h of PMNL transmigration (TM) were analyzed by immunoblot using antibodies to PARP or to procaspase-9. These effects were compared with those obtained after T84 cells pretreatment with 15 μM phallacidin (TM+Phall 15 μM) or with 30 μM phallacidin (TM+Phall 30 μM). Histogram corresponds to the densitometric scanning of caspase-9 immunoblot (transmigration assays performed in experimental conditions, $n = 3$).

merization induced by transmigration was responsible for the commitment of the apoptotic program, actin depolymerization was prevented by pretreating T84 cells with 15 or 30 μM of phallacidin before exposing cells to PMNL transmigration. PARP immunoblot and scanning densitometry of anti–caspase-9 blot show that phallacidin inhibited both PARP and procaspase-9 cleavage induced by PMNL transmigration (Fig. 8).

Discussion

PMNL infiltrate is a common hallmark of several large bowel diseases, such as ulcerative colitis, Crohn's disease, bacterial colitis, drug-induced colitis, or ischemia injury (Nash et al., 1987; Lee, 1993). However, the role of PMNL–epithelial cell interaction as a proapoptotic factor in IECs was not explored. To determine whether the transmigration of PMNLs could interfere with IEC apoptosis, we used an in vitro model described by Dharmsathaphorn and Madara (1990) to study PMNL–IEC interaction. IEC apoptosis mechanisms have been assessed by both morphological and biochemical methods. Here, we provide the first evidence that neutrophil transmigration across cultured intestinal cell monolayers triggers apoptosis of epithelial cells. After PMNL transmigration for 12 h, T84 cells exhibited the morphological features of apoptotic cell death, such as brush border alteration, chromatin condensation, and cytoplasmic shrinkage. These cellular modifications have already been described in normal mice by Guy-Grand et al. (1998) for apoptotic epithelial cells lining the small bowel villi in histological sections. The morphological characteristics of apoptosis induced in T84 cells by a prolonged PMNL transmigration was confirmed by a DNA fragmentation assay showing a typical DNA laddering. Here, we verified that PMNL transmigration alone could induce T84 cell apoptosis, since the mere contact between epithelial cells and PMNL was not sufficient to induce PARP and procaspase-9 cleavage (Fig. 2) or DNA fragmentation (data not shown).

It is well documented that DNA fragmentation is preceded by the sequential conversion of inactive procaspases into active heterotetramers (Cohen, 1997). Caspases constitute a family of cysteine proteases that cleave target proteins after Asp residues. Substrates for caspases include structural and functional proteins, such as cytoskeletal proteins, oncoproteins, or proteins involved in the DNA repair system, like PARP (Kayalar et al., 1996; Takahashi et al., 1996). Cleavage of these proteins is believed to be responsible for the dramatic morphological changes observed during apoptosis (Brown et al., 1997; Kirschner et al., 1997).

There is growing evidence that for the initiation of programmed cell death, two distinct caspase activation routes are used, depending on the nature of the stimulus. The first pathway implicates the engagement by trimeric ligands of death receptors that utilize adaptor proteins such as Fas-associated death domain protein (FADD [MORT-1]) to induce the recruitment of procaspase-8 and -2. Association of these caspases to the death-inducing signaling complexes results in their processing and activation. The second pathway requires the release of cytochrome c from the mitochondria into the cytosol following opening of the permeability transition pore (Susin et al., 1999). In that case, caspase-9 binds to apoptosis protease-activating factor 1 in a cytochrome c– and dATP-dependent fashion and initiates its autocatalytic processing (Srinivasula et al., 1998). These two pathways converge in the activation of caspase-3, which plays a crucial role in the execution phase of programmed cell death, especially because of its proteolytic activity toward ICAD (inhibitor of CAD), a necessary step for releasing CAD, hence leading to internucleosomal DNA fragmentation (Orth et al., 1996; Enari et al., 1998; Sakahira et al., 1998).

After demonstrating that intensive PMNL transmigration could induce T84 cell apoptosis, we investigated how the apoptotic program is triggered in epithelial cells by the intensive migration of PMNLs. As the Fas receptor is constitutively expressed on the basolateral membrane of normal colon epithelium, and PMNLs constitutively expressed Fas ligand (Moller et al., 1994; Abreu-Martin et al., 1995; Hsieh et al., 1997; Rudi et al., 1998; Seino et al., 1998), we first explored the possible involvement of the Fas/Fas ligand pathway. In contrast with this hypothesis, we have shown that although Fas receptors expressed by T84 cells were functional, antagonistic antibodies to Fas did not alter the PMNL migration–mediated apoptotic pathway. This result is in agreement with data from Kim et al. (1998), which showed that the Fas-dependent pathway is not implicated in apoptosis of the human colon epithelial cell line (HT-29) induced by bacterial invasion. Thus, we considered the possibility that the PMNL transmigration–mediated T84 apoptosis instead was due to a loss of the mitochondrial function. In favor of this hypothesis, we found that procaspase-9 was indeed activated within 45 min after the initiation of PMNL transmigration, earlier than PARP cleavage (as no reproducible PARP cleavage was seen before 4 h; data not shown). In contrast, activation of procaspase-2 and -3 was observed only after 10 and 12 h of transmigration, respectively. Although PARP has been shown to be preferentially cleaved by caspase-3 or -7 (Orth et al., 1996), our data suggest that caspase-9 could

be responsible for PARP cleavage. This is in accordance with previous studies by Duan et al. (1996). Li et al. (1997) and Srinivasula et al. (1998) showed that caspase-9 and apoptosis protease-activating factor 1 bind to each other in the presence of dATP and cytochrome c and that this interaction leads to a rapid activation (30 min) of caspase-3 in vivo or in an acellular system. This is in contradiction to our results, which show that an unusually long delay of 11 h was necessary between the activation of caspase-9 and that of caspase-3, suggesting the existence of indirect mechanisms. The possibility that caspase-6 and -7 could intervene in activation of caspase-3 can be ruled out because of their inability to be activated, even after 14 h of transmigration. Instead, caspase-2 was considered a possible intermediate in the activation cascade leading to caspase-3, since it was activated after only 10 h. This is in accord with recent studies by Harvey et al. (1997) showing that Nedd2/caspase-2 is activated earlier than CPP32 cleavage, leading to the activation of postmitochondrial executioner caspases such as caspase-3, -6, and -7 (Susin et al., 1999). Nevertheless, other intermediates could be involved in the delay of >9 h that lasted between the activation of caspase-9 and -2.

Previous studies by Madara et al. (1988) and Shapiro et al. (1991) have shown that PMNL transmigration could induce reorganization of the epithelial cortical F-actin network. Moreover, interaction of integrins with the extracellular matrix has been shown to be necessary for maintaining the actin cytoskeleton organization and the protection of adherent cells against programmed cell death or anoikis (Frisch and Francis, 1994). Thus, the emerging question is how the permanent remodeling of the actin filaments in T84 cells, produced by a sustained PMNL migration, was able by itself to induce the onset of apoptosis. This was tested by incubating T84 epithelial cells with DHCB. The ensuing actin cytoskeleton disruption produced the activation of caspase-9, -2, and -3, as well as DNA fragmentation, which generally follows the same profile as those observed during PMNL transmigration. Our study points out the importance of the actin cytoskeleton depolymerization in the onset of epithelial apoptosis. This hypothesis has been verified by inhibiting procaspase-9 and PARP cleavage by pretreating T84 cells with phallacidin before PMNL transmigrations assays. The fact that phallacidin pretreatment abrogates the onset of epithelial apoptosis strengthens the idea that the status of actin cytoskeleton polymerization is crucial for the commitment of cells toward apoptosis.

It is well established that activation of focal adhesion kinase, following integrin attachment, stimulates phosphatidylinositol 3-kinase and the serine/threonine protein kinase AKT/PKB. These enzymes could participate in, or be responsible for, the generation of an anoikis-suppressive signal (Frisch and Ruoslahti, 1997). Due to the fact that actin depolymerization could be linked to inhibition of Fas phosphorylation (Delcommenne et al., 1998), it is tempting to suggest that prolonged remodeling of actin cytoskeleton inhibits survival signaling cascades (Frisch et al., 1996; Frisch and Ruoslahti, 1997).

In summary, we provide evidence that IEC apoptosis could occur during sustained PMNL transmigration. Sequential and selective caspase activation is observed in T84 cells that are subjected to the paracellular passage of PMNLs. We propose that intensive transmigration of PMNLs may play a crucial role in the commitment of IECs to apoptosis occurring during IBD.

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