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TRAIL Up-Regulation Must be Accompanied by a Reciprocal PKCε Down-Regulation During Differentiation of Colonic Epithelial Cell: Implications for Colorectal Cancer Cell Differentiation

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PKC isoenzymes play central roles in various cellular signalling pathways, participating in a variety of protein phosphorylation cascades that regulate/modulate cellular structure and gene expression. It has been firmly established that several isoforms of PKC have a role in the regulation of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) activity. Our interest in probing the role of the epsilon isoform of PKC in the colonic cell differentiation stems from the discovery that PKCε and TRAIL are involved in the differentiation of other cell types like hematopoietic stem cells. Although the role of PKCε and TRAIL in the gastrointestinal system is unclear, it has been observed that PKCε has oncogenic activity in colon epithelial cells (CEC), while TRAIL increases the death of intestinal epithelial cells during inflammation. Here we demonstrate a reciprocal expression of PKCε and TRAIL in human colon mucosa: CECs at the bottom of the colonic crypts show high levels of PKCε, being negative for TRAIL expression. On the contrary, luminal CECs are positive for TRAIL, while negative for PKCε. Indeed, TRAIL- and butyrate-induced differentiation of the human colorectal cancer cell line HT29 requires the decrease of PKCε expression, whose absence in turn increases cell sensitivity to TRAIL-induced apoptosis. Moreover, TRAIL preferentially promotes HT29 differentiation into goblet cells. Taken together, this data demonstrate that TRAIL and PKCε must be reciprocally regulated to ensure physiological CEC differentiation starting from the stem cell pool, and that the down-regulation of PKCε is however critical for the differentiation and apoptosis of cancer cells.

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Cellular differentiation in the lower GI tract is a finely tuned and ordered process. In the small and large intestine, stem cells and progenitors cells are located near or at the crypt base while precursors proceed to migrate toward the luminal epithelium. In fact, the position of an intestinal/colonic epithelial cell within the crypt correlates with its differentiation and proliferative status. Butyrate, an inhibitor of histone deacetylases, is present in the colonic lumen after fermentation of dietary fibers and has been demonstrated to induce cell cycle arrest, differentiation and/or apoptosis of colon cancer cells (Bordonaro et al., 2008) but not of normal colonic epithelial cells (Roediger, 1980). Indeed HT29 cells, a human colon cancer-derived cell line with several biological and molecular similarities to small intestinal crypt cells (Hodin et al., 1996), treated in vitro with sodium butyrate (NaBu) show biochemical and morphological changes that are characteristic of well-differentiated enterocytes (Augeron and Laboisse, 1984).

Recombinant soluble tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a natural immune molecule expressed by cytotoxic lymphocytes and macrophages that selectively induces apoptosis of transformed or stressed cells but not of most normal cells (Takeda et al., 2007). TRAIL interacts with 4 cell surface receptors: TRAIL-R1 and -R2 activate caspases, while TRAIL-R3 and -R4 are decoy receptors able to sequester the ligand and to promote prosurvival signalling, like the activation of NF-kB (Sheridan et al., 1997). Although hepatotoxicity was observed as a side effect using agonistic antibodies against TRAIL-R2 (Mori et al., 2004), the emerging idea is that TRAIL will likely be used as a component of complex antitumor cocktails in therapeutic regimens. Normal colonic epithelial cells are TRAIL resistant (Sträter et al., 2002) but inflammation, in particular TNFα, sensitizes intestinal epithelial cells to TRAIL-induced apoptosis (Begue et al., 2006).

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Moreover, histone deacetylase inhibitors increase colon cancer cell sensitivity to TRAIL by the induction of TRAIL-R1 expression via Sp1 activation (Kim et al., 2004). Interestingly, it has been also reported that the treatment of normal intestinal and colon mucosa with cycloeximide makes enterocytes but not goblet cells sensitive to TRAIL (Gassler et al., 2007). However, soluble paracrine factors can protect colorectal cancer cells from TRAIL like, for instance, IL-4 that activates antiapoptotic signalling pathways (Todaro et al., 2008), or osteoprotegerin (OPG) that acts as a soluble decoy receptor for TRAIL ligand (LeBlanc and Ashkenazi, 2003). Although TRAIL was discovered and is commonly referred to as an apoptogenic ligand, mounting experimental evidence suggests that it is also involved in non-apoptotic functions. TRAIL has been described as a promoter of megakaryocyte and monocyte differentiation as well as an inhibitor of erythropoiesis (Zamai et al., 2000; Secchiero et al., 2002; Melloni et al., 2005; Mirandola et al., 2006; Gobbi et al., 2007; Gobbi et al., 2009). Moreover, it has been reported that TRAIL can also exert pro-differentiative effects on intestinal cells, inducing a more differentiated phenotype of the human tsFHI cell line (Rimondi et al., 2006).

Protein kinase C (PKC) isoenzymes play central roles in various cellular signalling pathways, participating in a variety of protein phosphorylation cascades that regulate/modulate cellular structure and gene expression (Bassini et al., 1999; Newton, 2003; Steinberg, 2008). It is well established that several isoforms of PKC have a role in the regulation of TRAIL activity (Harper et al., 2003; Shi et al., 2005; Mirandola et al., 2006; Gobbi et al., 2007). In particular, we have previously demonstrated that PKCε protects late erythroid precursors but also acute myeloid leukemia cells from the apoptogenic effects of TRAIL (Mirandola et al., 2006; Gobbi et al., 2007; Gobbi et al., 2009). PKCε expression has been reported in colon mucosa (Jiang et al., 1995) and intestinal cells (Saxon et al., 1994) where it was found associated with intermediate filaments (cytokeratins) of the belt desmosome. Furthermore, the development of experimental colitis, by 2,4,6-trinitrobenzenesulfonic acid treatment, increases PKCε expression in rat mucosa (Brown et al., 1999). Although PKCε is also known for its oncogenic activity in colon epithelial cells (Perletti et al., 1996; Perletti et al., 1998), the role of this kinase in intestinal epithelial cell differentiation is unclear: indeed, it has been reported that butyrate reduces the levels of PKCε in the colon cancer cell lines LIM1215 and Caco-2, but this effect is unlikely to be a consequence of the induction of differentiation (Rickard et al., 2000).

Therefore we studied the expression of PKCε in the differentiation of primary colonic cells using as a model system the butyrate- and TRAIL-induced differentiation of the human colon cancer cell line HT29.

Methods

Tissues

Colonic autoptic specimens were fixed in 10% neutral buffered formaldehyde and routinely processed (Gobbi et al., 2004; Vitale et al., 2005; Mirandola et al., 2006b; Gobbi et al., 2009b). Representative paraffin tissues blocks were selected from specimens and serially cut into 4 μm sections. Serial sections from each specimen were routinely stained with H&E for histological examination or processed for immunohystochemistry.

Cells and treatments

The human colon adenocarcinoma cell line HT29 and human melanoma cell line A375 were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in D-MEM medium (Euroclone, West York, UK) containing 10% of heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cell cultures were routinely assayed for mycoplasm contamination by Mycoalert mycoplasm detection kit (Lonza, Rockland, ME).

HT29TII cells were obtained by growing HT29 cells in the presence of high concentrations of TRAIL (200 ng/ml), added twice in 10 days. Surviving cells were expanded, and several clones were obtained by limiting dilution: HT29 TII cells were plated in 96 wells plate at one cell/well and grown for one week. For subsequent experiments, clones E3 and E7 were used.

Flow cytometry analysis

Aliquots of 5×10^5 cells/experimental point were labeled by a panel of anti-TRAIL-receptors monoclonal antibodies (Alexis Biochemical, San Diego, CA). Expression of TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 was analyzed by indirect staining using 1 μg HS101 anti-human TRAIL-R1, HS201 anti-human TRAIL-R2, HS301 anti-human TRAIL-R3, HS401 anti-humanTRAIL-R4 MoAbs, followed by PE-labeled goat anti-mouse IgG (Immunotech, Luminy, France) as a second reagent. Isotype-matched irrelevant mAbs were used to label control samples (Mirandola et al., 2006). The absolute number of surface antigens expressed/cell was calculated. For this goal, the flow cytometer was calibrated with a set of standardized beads (DAKO, Glostrup, Denmark) each with a known amount of fluorochrome (either FITC or RPE) expressed in units of MESF (Molecules of Equivalent Soluble Fluorescein). Thus, a standard curve was constructed by plotting the beads MESF values against the median channel in which the fluorescence sample peak was displayed (Schwartz et al., 1996). Analysis of the samples was performed by an Epics XL flow cytometer (Beckman Coulter Fullerton, CA) or with the Cytomix FC500 flow cytometer (Beckman Coulter), and the Expo ADC software (Beckman Coulter).

TRAIL neutralizing activity assay

Human melanoma A375 cells were seeded at 2.5×10^5 cells/well and grown for 24 h to allow cell adhesion. Then, medium was replaced either: (i) with A375 spent supernatant (control), or (ii) with HT29, HT29TII, E3, E7 or NaBu-treated HT29 spent supernatant. These cultures were treated for 24 h with 25 or 50 ng/ml of soluble TRAIL to induce cell death. The number of dead cells was finally measured in each sample.

Assessment of apoptosis

Cell culture viability was assessed by tripan blue exclusion. Apoptotic cells were identified by flow cytometry by Annexin V/propidium iodide (PI) staining. Specifically, cells were stained by FITC conjugate Annexin V (ACTIPLATE; Valter Occhiena, Torino) in Ca^{2+} and PI staining buffer, following manufacturer's protocol.

Semiquantitative reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA (1 μg) was reverse transcribed, and progressive dilutions (1/10, 1/100, 1/1000) were subjected to PCR amplification to detect β-actin, Osteoprotegerin (OPG), MUC-2 and Villin cDNA. PCR analysis of β-actin, OPG, MUC-2 cDNA was performed under the following reaction conditions: 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Villin cDNA was detected with 95°C for 1 min, 59°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Thirty-five cycles of amplification were used. The sequence of primers used for PCR was as follows: β-actin, 5′-TGACG GGGTC ACCCA CACTG TGCCC ATCTA-3′ (sense) and 5′-CTAGA AGCAT TTGCG GTGGA CGATG GAGGG-3′ (antisense) (Merighi et al., 2005); OPG, 5′ – GGAGG CATTC TTCAG GTTTG CTG- 3′ (sense) and 5′- AGTTA TAAGC AGCTT ATTTT TACTG– 3′ (antisense); MUC-2, 5′-CTGCA CCAAG ACCGT CCTCA TG-3′ (sense) and 5′-GCAAG GACTG AACAA AGACT CAGA-3′ (antisense) (Garg et al., 2007); Villin, 5′-CGAGG

CCATG CAGAT GGTG-3′ (sense) and 5′-GCGCT TGCCC AGCAC GTGG-3′ (antisense). We found that MUC-2 sense and antisense primers made two PCR products: one of about 400 bp, that has been reported by Garg et al. (2007) and a bigger product that had not been previously described. However, the analysis of the human MUC-2 gene sequence revealed that these primers encompass an intron (108 bp) of the *homo sapiens* chromosome 11 genomic sequence (NT_009237.18 GeneBank locus) located at the MUC-2 gene locus. Thus, the two PCR products obtained likely are the unspliced (immature 509 bp) and spliced (mature 401 bp) human MUC-2 mRNAs.

Western blot

Cultured cells were counted and 1.5×10^6 cells were collected at specific time points, washed in phosphate-buffered saline (PBS) and centrifuged at 200 g for 10 min. Pellets were resuspended in a cell lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, and 1 mM NaF) supplemented with fresh protease inhibitors, and protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). A total of 30 μg of proteins from each sample were then migrated in 5% SDS-acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were blocked and incubated with specific primary antibodies diluted as described in the manufacturers' protocols. Specifically, rabbit polyclonal anti-PKCε (Upstate, Lake Placid, NY) antibody was used at the concentration of 1 μg/mL. The moAb anti β-actin (Sigma-Aldrich S.r.l., Milan, Italy) was diluted 1:5000.

Filters were washed and further incubated for 1.5 h at room temperature with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase-conjugated anti-mouse IgG (Pierce) in the primary antibody working solution at room temperature. Specific reactions were revealed with the ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce) (Mirandola et al., 2006; Gobbi et al., 2007).

Histochemistry

Alcian Blue staining. Cells were fixed in 40% formalin, washed and stained with 1% Alcian Blue (AB) stain (pH 2.5). After rinsing with distilled water, samples were counterstained with nuclear fast red.

PAS staining. Cells were fixed in 40% formalin, washed and oxidized with periodic acid (1% in distilled water). After rinsing with distilled water, samples were incubated with Shiff's reactive, washed and stained with hematoxylin.

Alkaline phosphatase (ALP) staining. Cells were fixed in 10% cold formalin, washed and incubated with a solution of sodium ß-glicerophosphate 2%, calcium chloride 2% and $Na_2B_4O_7$ 2%. Cells were then washed and incubated with calcium chloride and cobalt nitrate 2%, rinsed again, stained with ammonium sulfide 1%, washed and finally incubated with nuclear fast red. At the end of the AB, PAS, and ALP staining, cells were washed and dehydrated by serial passages through increasing concentrations of ethanol and xylene (Gobbi et al., 2004; Vitale et al., 2005).

Immunohistochemistry. Paraffin blocks were serially cut into 4 μm sections, that were independently stained to detect TRAIL ligand and PKCε. All sections were dewaxed with xylene and rehydrated by passages through decreasing concentrations of ethanol (from 100 to 80%). Endogenous peroxidase activity was blocked by a 30 min incubation at room temperature with distilled water containing 3% H_2O_2 (Mirandola et al., 2006b; Gobbi et al., 2009b). After washing with PBS 10 mM pH 7.4 sections were preincubated for 30 min at room temperature with normal serum (Vectastain Elite ABC Kit, Vector Laboratories) and then incubated in a dark and wet box for 90 min at room temperature with primary antibodies: monoclonal anti-human TRAIL (R&D System, 1:100) and polyclonal anti-PKCε (Genetex, undiluted). Sections were then washed twice in the buffer and incubated for 30 min with diluted biotinylated secondary antibody solution (Vectastain Elite ABC kit). At the end of incubation, sections were then washed in PBS and incubated again for 30 min at room temperature with ABC reagent (Vectastain Elite ABC Kit). After washing, peroxidase activity was detected by incubating tissue sections for 10–15 min with a solution of 3-3-diaminobenzidine (DAB) (500 μl DAB (2.5%) in 50 ml PBS; Sigma-Aldrich S.r.l., Milan, Italy) in the presence of 50 μl (30%) H_2O_2. Sections were finally counterstained with Mayer's haemalum (Sigma-Aldrich S.r.l., Milan, Italy). Negative controls were treated in parallel, incubating sections in the absence of the primary antibodies.

Small interfering RNA design and cell transfection

PKCε expression levels were downregulated in HT29 cells by transfection of double-stranded small interfering (si)RNAs designed to target sequences corresponding to nucleotides 223–244, 429–450, 942–963, and 1,158–1,179 on human PKCε mRNA (NM005400). The target sequences were 5′- AAGAT CAAAA TCTGC GAGGCC-3′, 5′-AAGAT CGAGC TGGCTG TCTTT-3′, 5′-AACTA CAAGG TCCCT ACCTTC-3′, and 5′-AAAAA GCTCA TTGCT GGTGCC-3′. The respective sense and antisense RNA sequences were synthesized by Silencer siRNA Construction Kit (Ambion, Austin, TX). Nonspecific siRNA duplexes containing the same nucleotides, but in irregular sequence (i.e., scrambled PKCε siRNA), were prepared according to manufacturer's protocol and used as controls (Mirandola et al., 2006; Gobbi et al., 2007; Gobbi et al., 2010). The green fluorescent protein (GFP)-PKCε expression and control plasmids were kindly provided by Professor Peter Parker (Cancer Research UK, London Research Institute) (Ivaska et al., 2005). To maximize transfection efficiency, siRNAs (100 nM each) and GFP-PKCε plasmids (1 μg per transfection) were delivered using liposomes transfection with Superfect solution (Qiagen, Hilden, Germany).

Morphological analysis by scanning electron microscopy

The surface characteristics of HT29 cells were examined by Scanning Electron Microscopy (SEM). Samples were rinsed and vigorously washed with phosphate-buffered saline and fixed with 2% glutaraldeyde in 0.1 M sodium cacodylate buffer at pH 7.4. After fixation, samples were dehydrated, critical-point dried, coated with a gold sputtering device (Balzer), and observed under a Philips 501 SEM (Azzali et al., 2002).

Results

PKCε and TRAIL expression in human colon

We first examined the expression levels of PKCε and TRAIL in fixed specimens of human colon. Figure 1 shows that TRAIL and PKCε have a reciprocal distribution in colon epithelium: while luminal epithelial cells show a stronger immunoreactivity to TRAIL than the cells at the base of the crypts, PKCε is more expressed in the basal cells than in the luminal epithelium. For a more immediate visualization of the different distribution of PKCε and TRAIL expression, histological images in Figure 1 have been modified (lower panels) masking all colors except the brown of DAB precipitates, that appear as white dots in a black background. Given the colonocyte differentiation gradient present from the bottom to the top of the crypts, both PKCε and TRAIL expression appear modulated during in vivo colonocyte differentiation. Therefore, subsequent studies were performed in vitro to functionally correlate the modulation of PKCε and TRAIL expression with intestinal cell differentiation.

PKCε expression and sensitivity to TRAIL in butyrate- and TRAIL-induced differentiation

As a model system for colonocyte differentiation in vitro, we used the HT29 cell line. We first analyzed the expression levels of PKCε in HT29 cells treated with the differentiation agent sodium butyrate (NaBu). Figure 2A,B shows that 3 days of treatment impair PKCε expression up to 20%. NaBu-treated cells also show an increased sensitivity to TRAIL-induced apoptosis: a non-toxic dose (1 mM) of NaBu significantly increased the percentage of apoptotic cells induced by 100 ng/

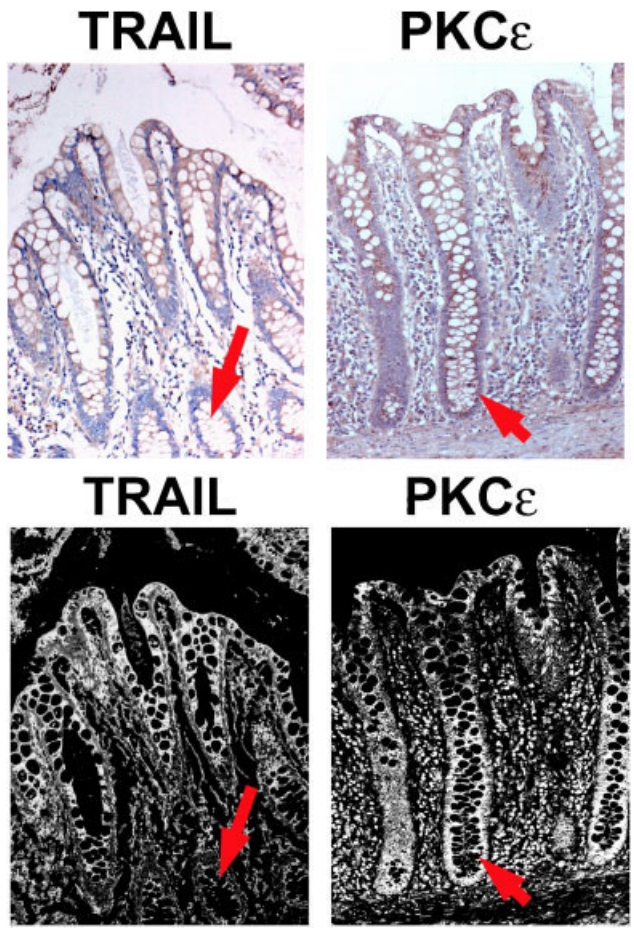

Fig. 1. Expression of TRAIL and PKCε in human colon mucosa. Upper parts: Immunohistochemical detection of TRAIL and PKCε in the normal human colon mucosa (original magnification 200×). Note the weak expression of TRAIL and the strong expression of PKCε at the bottom of the crypt (red arrows). On the contrary, a strong expression of TRAIL and a weak expression of PKCε can be observed in luminal epithelial cells. Lower parts: Images have been modified masking all colors except the brown of DAB precipitates, that appear as white dots in a black background.

ml of TRAIL ($P < 0.05$ vs. untreated cells) (Fig. 2C). The flow cytometric analysis of TRAIL receptors surface expression levels revealed that NaBu did not increase TRAIL-R1 and -R2 expression (Fig. 2D and E).

Similar experiments were performed using TRAIL as a differentiation agent. For this goal, HT29 cell cultures were preliminarily treated with soluble TRAIL at relatively high concentrations (200 ng/ml) twice in 10 days. Residual cells were amplified (HT29TII) and several clones were obtained, two of which (clone E3 and clone E7) were thoroughly studied, together with the parental cell line. Like NaBu, TRAIL treatment reduced the expression levels of PKCε in HT29 cells and also in E3 and E7 clones (Fig. 2F). Of note, although a TRAIL-mediated selection had been preliminarily performed, and thus TRAIL resistant cells were expected, both HT29TII cells and E3 and E7 clones showed an increased sensitivity to TRAIL-induced apoptosis (Fig. 2H) than HT29 parental cells. We then analyzed the cell surface expression of TRAIL-Rs in E3 and E7 clones: again there was no difference with respect to the parental cells line HT29 (Fig. 2I and J).

Secretion of TRAIL-neutralizing agents

It is recognized that colon carcinoma cells are resistant to TRAIL thanks to the secretion of TRAIL-neutralizing agents like OPG (Pettersen et al., 2005). Given the increased sensitivity to TRAIL-induced apoptosis observed in HT29TII and the clones, we did not expect an upregulation of its expression. The analysis of OPG mRNA levels by RT-PCR revealed in fact that both NaBu and TRAIL did not modify the transcription rate of *OPG* gene in HT29 cells (Fig. 3A). We therefore focused on other known TRAIL-neutralizing agents, studying their concentration in the spent medium of TRAIL- or NaBU-treated HT29 cell cultures (Todaro et al., 2008). In a functional cytotoxic assay with A375 cells, supernatants of HT29 cell cultures were used to neutralize 25 and 50 ng/ml of TRAIL. Figure 3B shows that HT29 spent medium reduced the apoptogenic activity of TRAIL with no significant difference between HT29TII, E3, E7 or NaBU-treated HT29 cell cultures respect to the parental cell line.

Role of PKCε in HT29 resistance to apoptosis

Since differentiated HT29 cells constitutively express PKCε at low levels, we studied its role in the sensitivity to TRAIL of HT29 cells.

The inhibition of PKCε expression by siRNA transfection in HT29 cells increased TRAIL-induced apoptosis while – at the opposite - the upregulation of PKCε expression impaired their sensitivity to TRAIL (Fig. 3C).

TRAIL promotes goblet cell differentiation

HT29 cell line can differentiate toward the two major intestinal cell types: colonocytes (absorbent cell, AC) and goblet cell (secernent cell, SC). Thus, we have studied the differentiation pathways of HT29 cell cultures after NaBu and TRAIL treatment, using a morphological approach. At the SEM analysis, HT29 cells show fine blebbings of secretion material at the cell surface and small digital and filiform expansions.
Figure 4A shows a typical monolayer of HT29 cells with relatively short surface microvilli-like structures, whose length was calculated (0.62 ± 0.19 μ) (Fig. 4B). Differently, E3 and E7 clones showed significantly bigger microvilli (1.06 ± 0.36 and 1.09 ± 0.26 μ, respectively; $P < 0.05$ vs. parental HT29 cells). NaBu-induced differentiation of HT29 cells was able to increase the length of microvilli as well, that were even significantly bigger than those observed in TRAIL-induced clones (1.31 ± 0.54 μ; $P < 0.05$ vs. E3 and E7 clones).

To better define the differentiation pathways of NaBu- and TRAIL-treated cells, we stained them for: (i) glycoproteins and neutral mucins (PAS staining); (ii) sialoproteins and acid mucins (AB staining); and, (iii) ALP. We observed that TRAIL-differentiated cell cultures showed a higher number of AB positive cells, while only NaBu was able to increase the number of ALP and PAS positive cells (Fig. 4C).

We finally analyzed by RT-PCR the expression levels of villin and Muc-2, respectively, AC and SC molecular markers. After 48 h treatment, TRAIL (200 ng/ml) promoted the expression of Muc-2 at higer levels than those observed with NaBu, while villin mRNA levels did not increase (Fig. 5A). On the contrary, NaBu promoted the expression of both villin and Muc-2 differentiation markers.

PKCε role in HT29 differentiation

To finally test whether the downregulation of PKCε expression had a role in HT29 cell differentiation, we forced PKCε expression in NaBu-treated HT29 cells. The prevention of PKCε down-regulation (Fig. 5B) impaired NaBu-induced up-regulation of Muc-2, without effects on villin transcription.

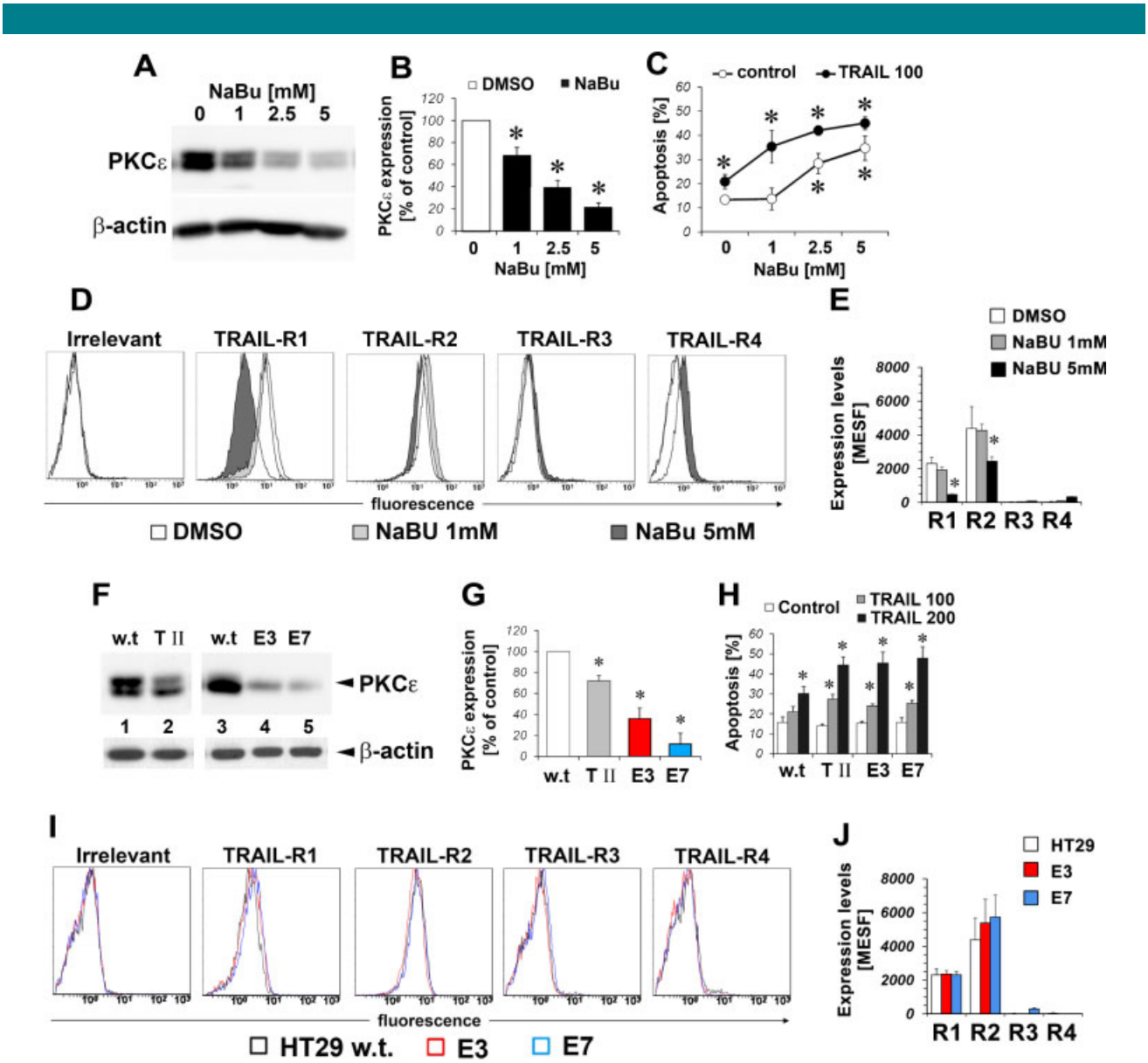

Fig. 2. NaBu and TRAIL impair PKCε expression and increase HT29 sensitivity to TRAIL-induced apoptosis. Part A: HT29 were treated for 48 h with the indicated concentration of NaBu and the expression of PKCε was detected by western blot. Part B: PKCε expression is reported as mean of three independent Western Blot experiments ± SD; $P < 0.05$ Anova, Dunet's test (vs. DMSO treated cells). Part C: apoptosis of HT29 cells treated with TRAIL and NaBu. Cells were treated for 24 h with the indicated concentrations of NaBu and for additional 24 h with 100 ng/ml TRAIL. Apoptosis is reported as mean of three indepenent experiments ± SD. $P < 0.05$ Anova and Dunnet's test (vs. untreated control cells). Part D: TRAIL-R immunophenotyping by flow cytometry of HT29 cells treated for 48 h with NaBu (1 and 5 mM) or with DMSO alone (control). Fluorescence emitted by isotype matched irrelevant control IgG is also reported. Part E: Flow cytometry quantification of cell surface expression of TRAIL-Rs on HT29 cells treated with (1 or 5 mM) NaBU or with DMSO. MESF values (means ± SD of three independent experiments), are reported as percentages of control values (empty bar). $^*P < 0.05$ Anova-Dunnet's test (vs DMSO). Part F: representative PKCε immunoblotting of HT29 cells treated with TRAIL (TII) and of HT29 clones E3 and E7. Part G: PKCε expression is reported as densitometric means of three independent Western Blot experiments ± SD; $P < 0.05$ Anova, Dunet's test (vs HT29 parental cell line). Part H: sensitivity of clones E3, E7 and of HT29TII cells to TRAIL. Means of three independent experiments are shown ± SD. $P < 0.05$ Anova and Dunnet's test (vs HT29 untreated cells). Part I: TRAIL-Rs immunophenotyping by flow cytometry of clone E3 (red line) and E7 (blue line) and of HT29 cells (black line). Part J: Flow cytometry quantification of cell surface expression of TRAIL-Rs on clones E3 (red bars) and E7 (blue bars) and on HT29 cells (empty bars). MESF values (means ± SD of three independent experiments), are reported as percentages of control values (empty bar). $^*P < 0.05$ Anova-Dunnet's test (vs black bars).

Discussion

TRAIL is expressed in virtually all tissues and also in the intestinal mucosa (Sträter et al., 2002; Koornstra et al., 2003; Begue et al., 2006; Rimondi et al., 2006). Soluble TRAIL is known to promote the differentiation of the conditionally immortalized human fetal intestinal cell line tsFHI (Rimondi et al., 2006). According to previous reports (Rimondi et al., 2006), we here show that TRAIL is upregulated during intestinal differentiation. We demonstrate that TRAIL is progressively expressed in human colon mucosa from the bottom of the crypts to the luminal epithelium; this observation is also in agreement with previous data showing a similar gradient of expression in duodenum (Begue et al., 2006; Rimondi et al., 2006) and in colon itself (Sträter et al., 2002). Intestinal epithelium is composed of two principal cells: goblet secernent

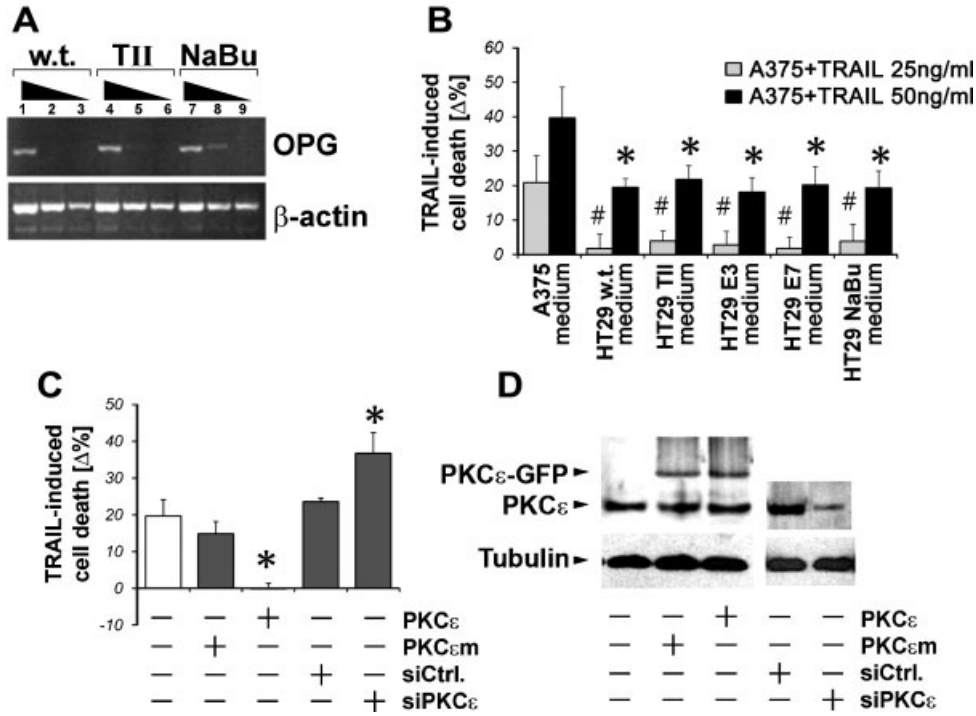

Fig. 3. TRAIL neutralizing activity of HT29 supernatants. Part A: RT-PCR analysis of OPG expression before (w.t.) and after NaBu (2 mM, 48 h) and TRAIL (TII cells) treatment. 1/10 (lanes 1, 4, and 7), 1/100 (lanes 2, 5, and 8) and 1/1000 (lanes 3, 6, and 9) of total cDNA was amplified to detect cDNA of OPG and β-actin. Part B: HT29 culture supernatants neutralize TRAIL-induced apoptosis of A375 cells. TRAIL sensitive cell line A375 was treated for 24 h with the indicated concentrations of TRAIL dissolved in A375 conditioned medium (A375) and in HT29 w.t., HT29TII, HT29 clones E3 and E7, and NaBu-treated HT29 cells cultures spent medium. Apoptosis is reported as mean value of three independent experiments ± SD. * $P < 0.05$ Anova and Dunnet's test (vs A375 cells treated with 50 ng/ml TRAIL); # $P < 0.05$ Anova and Dunnet's test (vs A375 treated with 25 ng/ml TRAIL). Part C: PKCε impairs TRAIL-induced apoptosis of HT29 cells. Cell cultures were transfected with control siRNA (siCtrl), with PKCε-specific siRNAs (siPKCε), with the vector expressing wild type PKCε (PKCε) or its mutated isoform (PKCεm). After 24 hours, cell cultures were treated with 200 ng/ml of TRAIL for additional 24 h. Apoptosis is reported as mean value of three independent experiments ± SD. * $P < 0.05$ Anova and Dunnet's test (vs A375 untransfected cultures). Part D: Modulation of PKCε expression. Endogenous and exogenous recombinant GFP-PKCε expression was analyzed by western blot in HT29 transfected cells.

cell and absorbent enterocytes. Small intestinal goblet cells secrete predominantly neutral mucins (PAS+ cells), while large intestinal goblet cells secrete acid mucins (AB+ cells). Furthermore, AB/PAS double positive cells are present in the small intestine and caecum, where they can be seen more frequently at the base of the crypts (Subbuswamy, 1997). Recently, immunohistological staining of middle and distal colon specimens revealed that the AB/PAS-positive goblet cells were strongly positive for Muc-2 protein, in contrast to the solely AB-positive cells at the crypt base, which did express Muc-2 mRNA (Makkink et al., 2002). However, no clear data on the lineage differentiation promoted by TRAIL are currently available. We show here that TRAIL induces SC differentiation of HT29 cells with acid mucins production, therefore promoting goblet cell differentiation.

We cannot exclude that differences between TRAIL and NaBu in differentiation commitment could be related to a lower efficacy of TRAIL vs. NaBu; nevertheless, the capacity of TRAIL to induce AB positivity but not PAS staining hints at a more restricted lineage commitment induced by TRAIL as compared to NaBu. This is strongly suggested by the analysis of Muc-2 expression, that is more robustly upregulated by TRAIL than by NaBu, and by the ability of NaBu to promote a stronger enterocytitc differentiation than TRAIL, as demonstrated by morphological analysis, ALP staining and mRNA levels of villin. Hence, TRAIL appears as a more goblet-cell oriented differentiation agent than NaBu, preferentially promoting the formation of acid mucins goblet cells, located in the colon and in the sigma epithelium.

The gastrointestinal epithelium is covered by a protective mucus gel composed predominantly of mucins secreted by goblet cells. The mucus layer of the intestinal tract functions as a barrier against pathogens and inflammatory stimuli (Deplancke and Gaskins, 2001; Linden et al., 2008). Muc-2 is the most abundant intestinal mucin in the mucus gel produced by goblet cells (Johansson et al., 2008). Muc-2-deficient mice spontaneously develop colitis (Van der Sluis et al., 2006), and frequently develop adenomas in the small intestine that progress to invasive adenocarcinoma and rectal tumors (Velcich et al., 2002). Moreover, the sulfation of the mucins is significantly reduced in patients with ulcerative colitis (Raouf et al., 1992), indicating that Muc2 production and the sulfation of colonic mucins are important for intestinal protection.

TRAIL, while promoting the production and secretion of acid mucins and Muc-2 transcription, also induces the cell death of adenocarcinoma cells. The upregulation of its expression, that has been observed in ileum and colon during inflammation (Begue et al., 2006), may therefore not only enhance, by an autocrine / paracrine mechanism, the elimination of intestinal epithelial cells via apoptosis, but can also promote SC differentiation and acid mucins production.

In parallel to TRAIL expression, we also describe an unprecedented inverse correlation between colonocyte differentiation and PKCε expression in vitro and in vivo. PKCε is

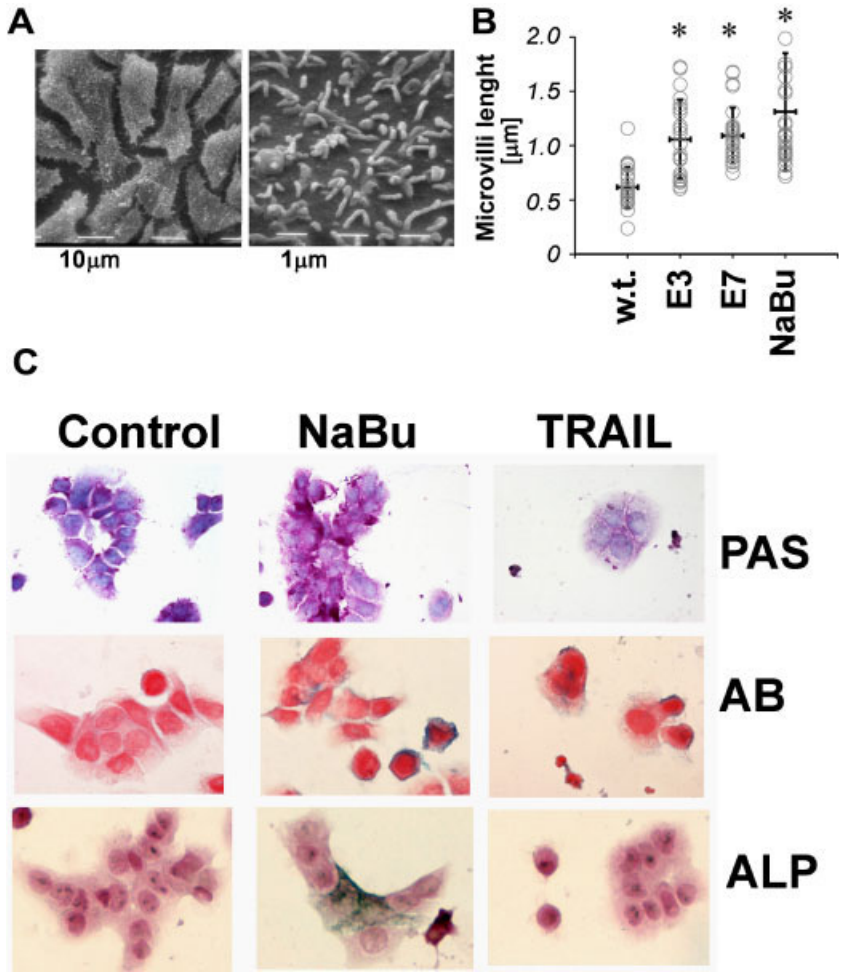

Fig. 4. NaBu- and TRAIL-induced differentiation of HT29 cells. Part A: SEM analysis of cell surface morphology of HT29 cells (dots: 10 μm and 1 μm). Part B: microvilli length of HT29, E3 and E7 clones and of NaBu-treated HT29 cells (2.5 mM, 3 days). Means ± SD are reported; #$P < 0.05$ Anova and Dunnet's test (vs untreated parental cell line). Part C: histochemical characterization of HT29 cell differentiation by: periodic acidic Shift (PAS) staining of neutral mucins; Acian Bleu (AB, pH 2.5) staining for acidic mucins; and alkaline phosphatase (ALP) staining. Cells were treated for 48 h with or without 2.5 mM NaBu or with 100 ng/ml TRAIL.

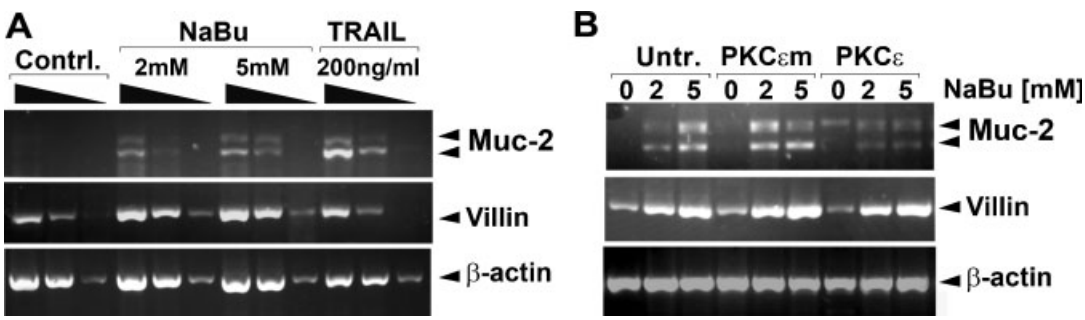

Fig. 5. PKCε overexpression impairs NaBu-induced HT29 cell differentiation Part A: RT-PCR analysis of Muc-2 and Villin before (Control) and after (48 h) treatment with NaBu and TRAIL. 1/10, 1/100 and 1/1000 of total cDNA was amplified to detect cDNA of Muc-2, Villin and β-actin. Part B. RT-PCR analysis of Villin and spliced/unspliced Muc-2 mRNA expression in PKCε (PKCε) and mutated PKCε (PKCεm) transfected cells. Twenty-four hours after transfection, cells were treated with NaBu (2 and 5 mM) for additional 48 h. The induction of cell differentiation was analyzed by RT-PCR amplifying equal amounts of total cDNA from transfected and control cell cultures (Untr).

known for its oncogenic activity in colon epithelial cells [29,30], but the role of this kinase in intestinal epithelial cell differentiation is unclear. PKCε expression is upregulated in colon mucosa during experimental colitis (Brown et al., 1999); it also plays a role in the control of chloride secretion across epithelium, whose deregulation is involved in significant pathological events, such as secretory diarrhea or cystic fibrosis (Chow et al., 2000). Moreover, it has been reported that PKCε mediates enterocyte migration in sepsis (Bu et al., 2007) and regulates thrombin-induced migration and adhesion of HT29 cells (Heider et al., 2004).

Distribution and expression of PKCε has been studied in colon mucosa both at the RNA and protein level, being detected along the entire colonic crypt (Jiang et al., 1995). Here we describe a decreasing gradient of PKCε expression from the bottom of the cryptps, where intestinal stem cells are located, to the luminal surface. Since the over-expression of PKCε stimulates proliferation in colon cancer cells (Perletti et al., 1996; Perletti et al., 1998), the expression of PKCε at the base of the crypts likely maintains the undifferentiated phenotype of intestinal stem cell pool, promoting cell proliferation and preventing cell commitment. Our data, in agreement with Rickard et al. (2000), that had previously observed similar effects in LIM1215 colon carcinoma cells, support this hypothesis. Of note, we additionally show that the down-regulation of PKCε preferentially promotes SC differentiation of HT29 cells, according to the findings of Hong DH et al., who showed that PKCε mediates the expression of two the major gastrointestinal mucins, MUC2 and MUC5AC, in HT29-derived mucin-producing colonic cell lines after PMA stimulation (Hong et al., 1999).

In summary, PKCε and TRAIL show a reciprocal modulation during physiological colon epithelial cell differentiation, as well acting as regulators of lineage commitment in multipotent human colorectal cancer cells. High levels of PKCε impair cell differentiation and rescue cells from TRAIL-induced apoptosis, preserving the pool of proliferating cells at the bottom of the intestinal crypt. Decreasing levels of PKCε, accompanied by increasing levels of TRAIL, indeed, allow progressive colon epithelial cell differentiation toward the lumen. TRAIL, acting both as a pro-differentiative and pro-apoptotic agent, exerts the numeric control of the expansion of differentiating goblet cells.

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