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Topoisomerase II Inhibition Differentially Modulates Caco-2 Intestinal Epithelial Cell Phenotype

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Caco-2 intestinal epithelial cells differentiate spontaneously after confluence when contact inhibition slows proliferation. We hypothesized that such reversible differentiation might be dependent on DNA synthesis and repair. We studied the effects of the topoisomerase II inhibitor etoposide on Caco-2 proliferation and on the differentiation markers alkaline phosphatase and dipeptidyl dipeptidase specific activity, as well cell motility. Etoposide (0.3–10 μM) dose-dependently inhibited proliferation and alkaline phosphatase activity. However, etoposide (0.7–3 μM) dose-dependently stimulated dipeptidyl dipeptidase activity. Above this concentration, dipeptidyl dipeptidase was also inhibited. Similar effects on enzyme activity were observed when proliferation was blocked with mitomycin C. Etoposide (1–10 μM) also dose-dependently inhibited cell motility. The selective stimulation of dipeptidyl dipeptidase activity by etoposide may offer a clue to the regulation of intestinal brush border enzyme expression at the molecular level.

The intestinal epithelium is a dynamic and heterogenous cell population requiring exquisite integration of proliferation and differentiation. The intestinal epithelial cell proliferates rapidly and modulates proliferation and phenotype during migration from crypt to villus tip and during healing (1,2). However, the link between intestinal epithelial differentiation and proliferation is incompletely understood.

Changes in intestinal epithelial differentiation may reflect alterations in the average maturity of the cells as in hematopoietic cells (3–8). Alternatively, the DNA synthesis and repair enzymes required for proliferative regulation might also modulate differentiation to maintain a functionally steady state over time. Topoisomerases are activated during both DNA repair (9) and normal proliferation (10). In particular, topoisomerase II is required for the segregation of daughter chromosomes and DNA replication, recombination as well as gene transcription (9). Many cellular phenomena have been linked to the topological state of the topoisomerase II molecule (10) including DNA replication, inhibition of RNA transcription by polymerases I and II, and DNA transposition (11–13). Inhibition of DNA topoisomerase II co-modulates proliferation and phenotype in several leukemic cell lines (3,5–7), and the proliferative component of the intestinal epithelium encompasses a mixture of totipotent and pluripotent cells similar to that which generates diversity in hematopoietic cell populations. We hypothesized that the regulation of topoisomerase II activity might link proliferation and differentiation in intestinal epithelial cells as well.

We sought to determine whether tonic topoisomerase II activity modulates intestinal epithelial differentiation using well differentiated human Caco-2 cells as a model and the topoisomerase II inhibitor etoposide as a probe. Caco-2 cells express many features of primary intestinal epithelial cells including morphological differentiation, brush border enzyme expression, and a variety of polarized transport processes and have frequently been used to model intestinal mucosal physiology (1,14–17). We have previously used Caco-2 cells to study the regulation of brush border enzyme activity and motility (15,16,18) and we now sought to study these phenomenon in the setting of topoisomerase II inhibition. We studied the effects of the topoisomerase II inhibitor etoposide in Caco-2 cells on the specific activity of brush border enzymes alkaline phosphatase and

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dipeptidyl dipeptidase, conventional markers of intestinal epithelial differentiation (18), as well as on Caco-2 motility and proliferation.

METHODS

Cells. The cells used for these studies represented a clonal Caco-2 subpopulation selected for their highly differentiated state. These cells were maintained at 37°C in 5% CO_2 in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum, 10 μg/ml transferrin (Boehringer Mannheim, Indianapolis, IN), 2 mM glutamine, 1 mM pyruvate, 10 mM HEPES, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. Experiments in each figure were performed on the same day using the same passage of cells.

Proliferation. Proliferation was quantitated directly by serial direct cell counts. 2.5×10^5 cells/ml were plated in quadruplicate in 35 mm bacteriologic plastic dishes precoated with type I collagen and allowed to adhere for one hour, resulting in 80–90% adhesion. Non-adherent cells were washed away with phosphate buffered saline. One set of dishes was immediately fixed in 10% formalin and stained with hematoxylin for counting of adherent cells. The remaining dishes were treated with media containing 0–10 μM etoposide for 24 hours and then washed, fixed, and stained similarly. The number of cells/high power field was counted in 20 random fields per dish and doubling time calculated by logarithmic transformation.

Assays. For enzyme activity assays, confluent monolayers on bacteriologic plastic dishes (Falcon, Oxnard CA) pre-coated with type I collagen were treated with 0.1–10 μM etoposide for 24 hours and lysed on ice for one hour in phosphate buffered saline supplemented with 9 mM calcium, 4.9 mM magnesium, and 0.5% Triton X-100 (Sigma, St. Louis, MO). After separation of insoluble lipid components by centrifugation, the supernatants were assayed for protein (BCA Assay, Pierce, Rockford IL) and diluted to equal protein content. Enzyme assays were then performed by digestion of synthetic substrates (12).

Alkaline phosphatase was assayed by digestion of p-nitrophenyl phosphate hexahydrate (Sigma, St. Louis, MO) in 100 mM glycine buffer (pH 10.0). After incubation at 37°C for one hour the reaction was stopped with 1M NaoH. The reaction product in each sample was measured with an extinction coefficient of 568.5 against a reagent blank and enzyme activity was calculated by interpolation against a simultaneously assayed enzyme standard. Dipeptidyl dipeptidase was similarly quantitated by the digestion of Ala-p-nitroanilide (Sigma, St. Louis, MO) in 114 mM acetate buffer (pH 8.0) for four hours at 37°C. The reaction was stopped with 1M acetate (pH 4.2) and reaction products in the samples were measured with an extinction coefficient of 532. Standard dilution curves for alkaline phosphatase and dipeptidyl dipeptidase were performed simultaneously with each assay using purified enzymes (Sigma, St. Louis, MO). All assays were performed within the linear range. Results were standardized during each experiment against these standards and expressed as enzyme activity in international units per microgram of protein. In parallel experiments designed to determine whether topoisomerase inhibition exerted a direct effect on cell phenotype independent of its effect on proliferation, cells were pre-treated for two hours with 20 μg/ml mitomycin C (Sigma, St. Louis, MO) to block cell proliferation (19) after which they were washed with phosphate buffered saline and incubated with etoposide in conventional media.

Migration. Cell motility was quantitated by monolayer expansion over type I collagen as previously described (15). Briefly cells were plated to confluence within stainless steel 'fences' on collagen I precoated bacteriological plastic substrata. After the cells had achieved confluence, the fences were removed, permitting outward migration for 6 days. The media were supplemented with 0–10 μM etoposide and changed every 2 days. Cells were fixed in situ and hematoxylin-stained. The monolayer area was measured and the area of the original fence subtracted. Monolayer expansion in the absence of proliferation was studied by pretreating the cells with 20 μg/ml mitomycin C for 2 hours as described above.

Statistics. Quantitative data were assessed by unpaired t test or ANOVA at 95% confidence. Numerical data are represented as mean ± SE. For migration studies, areas of monolayer expansion are represented graphically after normalization to the area over which control Caco-2 monolayers expanded during the same experiment. The area over which each monolayer in each dish had expanded was treated as a separate data point in analysis of migration assays, so that n represents the number of dishes studied in a given experiment, but at least three parallel experiments were performed with similar results. Data from at least 3 similar experiments were pooled for purposes of data analysis, where n represents the total number of dishes studied. For proliferation studies, doubling time (hours) was calculated by logarithmic transformation of serial cell counts. Data from one of at least three typical studies are shown.

RESULTS

Etoposide inhibits proliferation. Increasing etoposide concentrations (0.1–10 μM) in the culture medium dose-dependently prolonged the Caco-2 doubling time (Figure 1). This effect achieved statistical significance at 0.3 μM etoposide, which increased the doubling time by 111.0 ± 3.4% ($n = 20$, $p < 0.001$), while 10 μM etoposide further increased the Caco-2 doubling time to 319.4 ± 21.8% ($n = 20$, $p < 0.001$).

Etoposide addition affects brush border enzyme activity in a dichotomous pattern. Etoposide

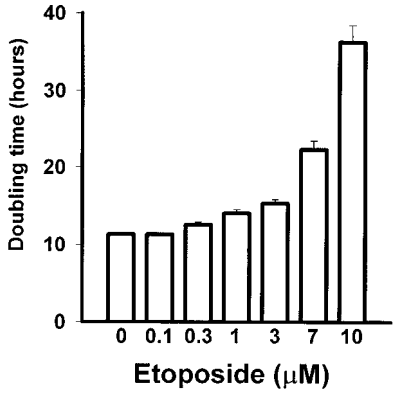

FIG. 1. Effect of etoposide on Caco-2 proliferation. Caco-2 cell numbers were counted 1 hour after seeding and then after 24 hours after etoposide treatment. Doubling time was calculated by logarithmic transformation of the cell counts. Cells cultured without etoposide served as controls. Doubling time was significantly different from control at etoposide levels above 0.3 μM (mean ± SE, n = 20, $p < 0.001$).

dose-dependently inhibited alkaline phosphatase specific activity. (Figure 2) This effect achieved statistical significance at 0.3 μM etoposide which reduced alkaline phosphatase to 89.1 ± 1.1% of control levels. (n = 9, $p < 0.001$) Alkaline phosphatase was maximally inhibited at 10 μM etoposide to 19.0 ± 2% of basal levels. (n = 9, $p < 0.001$) Since etoposide had also been observed to modulate cell proliferation, we also assessed the effects of etoposide on alkaline phosphatase when proliferation was prevented by pretreatment with 20 μg/ml mitomycin C. In particular, alkaline phosphatase was dose-dependently inhibited by etoposide even after mitomycin C blockade. (Figure 3) The etoposide effect after mitomycin C blockade also achieved statistical significance at 0.3 μM (89.5 ± 1.2% of control, n = 9, $p < 0.001$) and was maximal at 10μM etoposide (60 ± 3% of control, n = 9, $p < 0.001$).

In contrast, etoposide stimulated dipeptidyl dipeptidase specific activity. (Figure 2) At 0.7 μM etoposide, dipeptidyl dipeptidase was increased to 115.0 ± 3.2% (n = 9, $p < 0.001$) while 3 μM etoposide further stimulated dipeptidyl dipeptidase to 133.6 ± 2.5% of control (n = 9, $p < 0.001$). At concentrations in excess of 3 μM, etoposide did not stimulate dipeptidyl dipeptidase. Indeed, at 10 μM concentrations, etoposide inhibited dipeptidyl dipeptidase to 62.6 ± 4.4% of basal (n = 9,

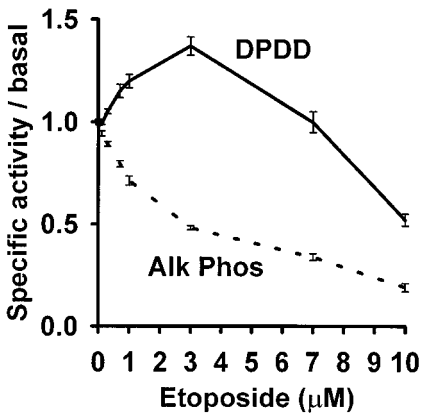

FIG. 2. Effects of etoposide on Caco-2 brush border enzyme specific activity. Caco-2 cells were cultured for 24 hours in 0-10 μM etoposide, lysed and assayed for alkaline phosphatase (lower dashed line) and dipeptidyl dipeptidase (upper solid line). Alkaline phosphatase activity differed for all etoposide concentrations above 0.3 μM and dipeptidyl dipeptidase activity differed significantly from control at 0.7 μM etoposide (mean ± SE, n = 9, $p < 0.001$ by ANOVA for each).

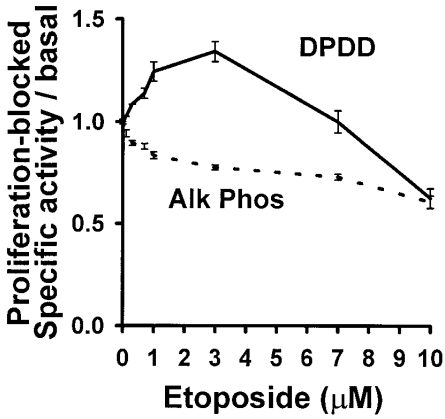

FIG. 3. Effect of inhibition of proliferative activity with mitomycin C (20 μg/ml) on etoposide inhibition of Caco-2 brush border enzyme specific activity. The specific activity of alkaline phosphatase (lower dashed line) and dipeptidyl dipeptidase (upper solid line) was statistically significantly different from control at etoposide concentrations of 0.3 μM for alkaline phosphatase and 0.7 μM for dipeptidyl dipeptidase (mean ± SE, n = 9, $p < 0.001$ by ANOVA).

$p < 0.001$). Similar stimulation of dipeptidyl dipeptidase activity by etoposide was observed in cells in which proliferation had been blocked with mitomycin C. (Figure 3) In cells in which proliferation had been prevented by mitomycin C pretreatment, etoposide (0.7–3.0 μM) dose-dependently stimulated dipeptidyl dipeptidase activity, achieving a maximal 134.6 ± 4.6% of basal levels at 3 μM etoposide (n = 9, $p < 0.001$) As for proliferating cells, Caco-2 cells pretreated with mitomycin C also exhibited an inhibition of dipeptidyl dipeptidase specific activity upon treatment with 10 μM etoposide, which inhibited dipeptidyl dipeptidase activity in mitomycin-pretreated cells to 62.5 ± 4.5% of basal levels. Despite these biochemical changes, no light level morphological changes were demonstrable after etoposide treatment.

Etoposide inhibits Caco-2 motility. Etoposide (1–10 μM) dose-dependently inhibited Caco-2 monolayer expansion across a type I collagen matrix. For instance, inhibition of monolayer expansion to 82.8 ± 0.1% of control was observed after treatment with 1 μM etoposide (n = 12, $p < 0.001$) while 7 μM etoposide further decreased monolayer expansion to 69.5 ± 0.1% of control (n = 12, $p < 0.001$). (Figure 4, open bars) Since the observed inhibition of monolayer expansion might have reflected only decreased cell number caused by etoposide inhibition of proliferation, these studies were repeated after pretreatment with mitomycin C to block proliferation. Etoposide

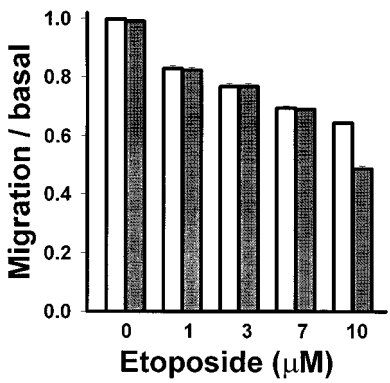

FIG. 4. Etoposide dose dependently inhibits Caco-2 sheet migration. Monolayer expansion was substantially inhibited with etoposide at all concentrations studied both in proliferating cells (open bars) and cells in which proliferation had been blocked by mitomycin C pretreatment (shaded bars) (mean ± SE, n = 12, $p < 0.001$ by ANOVA for each).

also inhibited motility after proliferation had been blocked to virtually the identical extent observed for cells without mitomycin pretreatment (Figure 4, shaded bars). For instance, 1 μM etoposide inhibited motility to 82.2 ± 0.1% ($n = 12$, $p < 0.001$) of control (ie. of motility of mitomycin C-pretreated cells not exposed to etoposide) while 7 μM etoposide inhibited motility to 69.2 ± 0.1% of control ($n = 12$, $p < 0.001$).

DISCUSSION

The anti-cancer drug etoposide (VP-16) is a semi-synthetic derivative of the anti-mitotic agent podophyllotoxin which specifically inhibits topoisomerase II activity (20,21). This study demonstrates that etoposide independently modulates both the proliferation and the phenotype of Caco-2 cells, a common model for intestinal epithelium. The effects of etoposide on Caco-2 differentiation were reflected both in conventional parameters of differentiation (brush border enzyme specific activity) and in modulation of cell motility. Although etoposide could have other targets in the cell, these data suggest that topoisomerase II inhibition may modulate Caco-2 differentiation as well as proliferation.

Etoposide dose-dependently prolonged the Caco-2 doubling time, as measured by logarithmic transformation of serial cell counts. Although proliferation is often assessed by manual hemocytometer or automated cell counting in cell suspensions, cell-cell adhesion and clumping may impede such techniques in Caco-2 cells which adhere tightly to the underlying matrix and to each other, unless the cells are triturated or enzymatically digested sufficiently to risk some cell lysis. We have therefore previously counted adherent cells in multiple random fields at serial time points in order to assess Caco-2 proliferation (18,22,23). The finding that etoposide inhibits Caco-2 intestinal epithelial cell proliferation is consistent with previous reports in small-cell lung cancer cells (24) and human leukemia cells (5,23).

Caco-2 cells express increasing amounts of both alkaline phosphatase and dipeptidyl dipeptidase as they differentiate spontaneously. Etoposide altered the specific activity of these two brush border enzymes in a divergent pattern. Alkaline phosphatase was downregulated and dipeptidyl dipeptidase upregulated by etoposide. The magnitude of the changes seen in these marker enzymes is similar to that observed with spontaneous differentiation, and differentiation induced by neuropeptides, luminal nutrients and matrix proteins. (1,22,25,18) Since Caco-2 cells spontaneously increase dipeptidyl dipeptidase activity as they differentiate, it might be speculated that the decreased dipeptidyl dipeptidase activity in cells treated with 0.7–3.0 μM etoposide might simply reflect the increased average maturity of the cell population under study after inhibition of proliferation (2). However, etoposide also modulated Caco-2 differentiation in cells pre-treated with mitomycin C to inhibit proliferation, suggesting that the effects of etoposide on dipeptidyl dipeptidase activity are not an epiphenomenon of the proliferative effects of the drug. We have previously demonstrated mitomycin-pretreatment in the manner used here completely ablates Caco-2 proliferation, measured by thymidine autoradiographic labelling (19). This is also consistent with previous reports that etoposide modulates hematopoietic cell differentiation independently of proliferation (3,24). Although the effect of etoposide on dipeptidyl dipeptidase was essentially identical in proliferating and mitomycin-treated cells, etoposide inhibition of alkaline phosphatase was less substantial in mitomycin-treated cells than in proliferating cells. This may reflect mitomycin toxicity since the concentration required for complete inhibition of replication is also likely to interfere with translation. We have previously observed such an attenuation of other proliferation-independent effects in Caco-2 cells after mitomycin treatment (16,19).

The divergent effects of etoposide on alkaline phosphatase and dipeptidyl dipeptidase may reflect differences in susceptibility to etoposide or different regulatory roles of topoisomerase II isozymes (3,26). The α and β topoisomerase II isozymes are localized differently within the cell (27) and expressed differently as NIH-3T3 cells pass through the cell cycle (10). It thus seems

plausible that they might have different activities as well. Alternatively, the opposing effects of etoposide on alkaline phosphatase and dipeptidyl dipeptidase may reflect two different intracellular regulatory pathways for these brush border enzymes. Indeed, we have previously observed that these markers are also differently modulated by protein kinase C inhibition (1,15,23) and by physical deformation of Caco-2 monolayers (28).

Cell motility is another important functional aspect of the intestinal epithelial cell phenotype (1). Etoposide dose-dependently inhibited Caco-2 monolayer expansion. This effect was maintained after mitomycin blockade, suggesting a true effect on motility by this agent. We have previously reported (16) that the tyrosine kinase inhibitor genistein, which also inhibits topoisomerase II activity by blocking the homologous ATP binding site, inhibits Caco-2 motility to a greater extent than the tyrosine kinase inhibitor 2,5 dihydroxymethylcinnamide, which competes at the tyrosine kinase substrate binding site and does not affect topoisomerase II activity. We had hypothesized that this difference might reflect variations in the selectivity of these two tyrosine kinase inhibitors, but the present data suggest that at least some of the additional inhibition of motility by genistein may reflect the topoisomerase II inhibition by genistein.

Intestinal epithelial phenotype changes both during normal proliferation and with alterations in proliferation during healing or oncogenesis. These data suggest the possibility that topoisomerase II might be involved in the linkage between intestinal epithelial cell phenotype and proliferation. Although topoisomerase II is required for proliferation, its activity is also closely regulated in amount and stability during passage through the cell cycle in many cell lineages.(11,14,24,29,30) Intracellular topoisomerase II protein and activity are higher in proliferating cells than in quiescent cells.(10) Topoisomerase II α protein is not detectable in Go cells by quantitative immunoblotting but increases during S phase, peaks in G2-M, and then declines.(11) Levels of the β isoform, by contrast, do not change during the cell cycle.(4)

Clearly extrapolation from the Caco-2 cell model to the primary intestinal epithelial cell must be cautious. Furthermore the interpretation of this data awaits investigation into the activity of other inhibitors of topoisomerase II activity and other DNA repair enzymes during intestinal epithelial cell proliferation and differentiation. Nevertheless, this study raises the possibility that the relationship between cell proliferation and differentiation in the human intestinal epithelial cell may reflect modulation of topoisomerase II activity in addition to a change in the average maturity of the intestinal epithelial cell population. It is likely that topoisomerase II exerts control over intricate intestinal epithelial cellular processes in a differential manner and that the topoisomerase II inhibitor etoposide may interfere with this regulation.

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