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Grape Seed Extract Affects Proliferation and Differentiation of Human Intestinal Caco-2 Cells

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The effect of daily contact of a grape seed extract (GSE) on Caco-2 cell proliferation and differentiation was investigated. GSE at 400 mg/L was added to Caco-2 cells for 2 h a day after successive incubation in saliva, gastric, and pancreatic media. When applied at the beginning of the cell culture, GSE triggered inhibition of cell growth associated with a possible cytotoxic reaction. On the other hand, when the treatment was applied to confluent cells, treated cells displayed a higher protein content than control cells and a more developed brush border, with taller and denser microvilli. These observations were accompanied by stimulation of alkaline phosphatase activity, especially at day 5 postconfluency, with a 2.2-fold increase in comparison with the control. On the other hand, aminopeptidase N activity was inhibited throughout the differentiation period in GSE-treated cells to reach 28.8% of control cell activity on day 30. GSE did not affect either sucrase−isomaltase activity or cytoplasmic lactate dehydrogenase (LDH) activity, which otherwise appeared to be a good cellular marker. GSE treatment of Caco-2 cells thus inhibited their proliferation from seeding onward and stimulated both proliferation and differentiation after confluency.

KEYWORDS: Caco-2 cells; phenolic compounds; differentiation; proliferation

INTRODUCTION

The flavonoids found in vegetables, fruits, and certain beverages have recently attracted increasing attention because of their potent antioxidant and antimutagenic properties (*1*, *2*). For example, grape seed extracts, which are rich in catechins and procyanidins (*3*), display cardioprotective effects and antitumor-promoting activities (*4*, *5*). However, although there have been many studies on flavonoid absorption and bioavailability in vivo (*6*−*9*) and in vitro (*10*−*13*), little is known about the effect of such compounds on intestinal epithelium and particularly enterocytic differentiation. In vivo, intestinal cells arise from an undifferentiated proliferating stem cell population in the intestinal crypt region (*14*). As these cells migrate from the crypt to the villus tip, they differentiate into absorptive cells and exhibit well-developed brush borders, with maximum expression of their associated hydrolases and mature tight junctions. Originally derived from human colon adenocarcinoma, Caco-2 cells have been widely studied for their ability to differentiate into enterocyte-like cells, expressing some features characteristic of mature small intestinal cells (*15*). Caco-2 cells spontaneously differentiate over a period of 20−30 days of postconfluent culture, forming a polarized monolayer with a well-defined brush border on the apical cell membrane and enterocyte-like tight cellular junctions (*15*, *16*). Caco-2 monolayers not only morphologically resemble small intestinal absorptive cells but also express typical small intestinal microvillar hydrolases, including disaccharidases (*17*, *18*), peptidases (*17*, *19*) and alkaline phosphatase (*20*, *21*). Caco-2 cell differentiation is known to be affected by certain compounds such as hormones, drugs, and minerals. Such compounds can affect cell proliferation, enzyme activity, and/or morphological aspects (*20*, *22*−*26*). Other studies have shown the antiproliferative potency of certain flavonols such as quercetin or genistein (*27*), cocoa procyanidins and flavanols (*28*), and other phenolic compounds such as cinnamic acid (*29*) on Caco-2 cells. This growth inhibition may be caused by either an apoptotic (*27*) or a nonapoptotic (*28*) process. Such compounds also modulate the Caco-2 cell phenotype, inducing changes in enzyme expression. Cinnamic acid treatment stimulated sucrase and aminopeptidase activities while inhibiting alkaline phosphatase in postconfluent Caco-2 cells (*29*). Treatment of Caco-2 cells with flavonoids such as genistein increased antioxidant enzyme activity like metallothionein (*30*), while others (e.g., catechins), displayed an inhibiting effect on sulfotransferase (*31*).

In all these studies, Caco-2 cells were subjected to permanent contact with purified compounds. However, in vivo, intestinal cells are exposed to flavonoids for only few hours a day and after prior salivary, gastric, and pancreatic digestion. The aim of this study was to investigate the effect of daily exposure of a quantity of flavonoids, in the form of a grape seed extract

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(GSE), on Caco-2 cell proliferation and differentiation. Thus, to mimic physiological conditions, GSE was added to Caco-2 cells after being subjected to successive incubation in salivary, gastric, and pancreatic media. The treatment was performed on Caco-2 cells for 2 h a day for a 30-day period, and the effect on proliferation and Caco-2 cell differentiation was investigated.

MATERIALS AND METHODS

Materials. Grape seed extract (GSE) was obtained from La Gardonnenque SCA (Cruviers Lascours, France) and contained approximately 67.4% procyanidins, 13.5% of these being (+)-catechin, (−)-epicatechin, and dimers B1, B2, B3, and B4 (according to the supplier). The GSE concentration used in this study was the equivalent of the consumption of 250 mL of red wine (1−5 g/L total polyphenols) per meal for an adult subject, corresponding to a polyphenol intake of 0.25−1.25 g. The volume of total digestive secretions in man can be estimated to be 10 L/day, thus around 3 L/meal. Ingested polyphenols may thus be diluted in this secretion volume and reach intestinal cells at a concentration range of 85−400 mg/L. A final GSE concentration of 400 mg/L was therefore chosen for the experiment.

Cell Culture. Caco-2 cells from human colorectal adenocarcinoma were obtained from the American Type Culture Collection and used in experiments between passages 36 and 48. Caco-2 cells were maintained and expanded in 75 cm^3 flasks at 37 °C in an atmosphere of 5% CO_2/95% air at constant humidity and in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 15% heat-inactivated fetal calf serum, 2% L-glutamine, 1% antibiotic antimycotic solution, and 1% nonessential amino acids and was changed daily. When the cells were confluent, namely, when they formed a monolayer, they were harvested by treatment with a solution containing 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA), thoroughly washed, and resuspended in supplemented growth medium. All products were purchased from Invitrogen SARL (Cergy Pontoise, France). For the experiments, Caco-2 cells were seeded at a density of 50 000 cells/cm^3 (*32*) in permeable collagen-coated transwell cell culture inserts (pore size 0.4 μm; Corning Costar Science Products, Brumath, France) to mimic the physiological conditions of an intestinal barrier. The filters were located in 6-well plates separating an apical from a basolateral side. The cultures were run for 30 days postconfluency.

In Vitro Simulated Digestion. This was performed as previously reported (*33*−*35*). Incubations in salivary, gastric, and intestinal solutions were conducted on a rocking platform shaker (Rotomix, Bioblock, Illkirch, France) in an incubator at 37 °C with a 5% CO_2/95% air atmosphere maintained at constant humidity. α-Amylase from human saliva (1000−1500 units/mg of protein), porcine pepsin (800−1000 units/mg of protein), pancreatin (activity, 4 × USP specifications), and bile extract (glycine and taurine conjugates of hyodeoxycholic acids and other bile salts) were purchased from Sigma (Saint Quentin Fallavier, France).

An artificial saliva was created with the main constituents of human saliva as previously reported (*36*−*41*). It consisted of phosphate-buffered saline (PBS) (diluted 1:5), containing 1.336 mM $CaCl_2$, 0.174 mM $MgSO_4$, 12.8 mM KH_2PO_4, and 23.8 mM $NaHCO_3$. Apart from minerals, the main constituents used were food casein, known to be a proline-rich protein at 2 g/L, according to the total protein concentration in human saliva (*36*, *37*, *39*, *41*, *42*), and α-amylase (1000−1500 units/mL) (*37*, *42*, *43*).

Samples were added to artificial saliva to reproduce the salivary step. The pH was adjusted to 6.9 with 1 N HCl and α-amylase was added. The samples were incubated for 10 min at 55 oscillations/min. The pH was adjusted to pH 2 with 1N HCl and 0.05 mL of pepsin (25 mg/mL in 0.1 N HCl) was added per milliliter of sample to simulate a gastric juice. Incubation was performed for 60 min at 55 oscillations/min. Finally, to reproduce the intestinal medium, the pH was raised to 6 by the dropwise addition of 1 M $NaHCO_3$, and 0.25 mL of pancreatin−bile extract solution (0.05 g of pancreatin and 0.3 g of bile extract in 25 mL of 0.1 M $NaHCO_3$) was added per milliliter of sample. The pH was adjusted to 7 with NaOH and the sample was diluted (2:3) with a 120 mM NaCl/5 mM KCl solution. The intestinal digestion step was subsequently performed in the presence of Caco-2 cells.

Cell Proliferation. Two chronic treatments with 400 mg/L GSE were performed to investigate the effect of GSE on cell proliferation. In experiment 1, treatment began 24 h after seeding and was applied to cells daily. In experiment 2, GSE was added to cells daily from confluency until day 30 postconfluency. Cell growth was monitored by assays of cellular proteins and electron microscopy.

Cell Differentiation. The biochemical and morphological aspects of differentiation in both treated and control cells were examined to investigate the effect of a chronic treatment with GSE on the Caco-2 cell differentiation process. The treatment started at confluency. GSE was first subjected to successive incubations in saliva and gastric media according to the in vitro simulated digestion model previously developed. Pancreatic incubation was conducted on Caco-2 cells for 2 h a day to mimic the intestinal digestion step. The culture medium was removed from each well just before the experiment and replaced in the upper compartment by 1.5 mL of predigested GSE solution diluted with culture medium (3:5). Incubation took place for 2 h at 37 °C. Culture medium was also added (2 mL) in the lower compartment. The GSE solution was removed on completion of the 2-h period and 1 mL of culture medium was added to each well. Treated and control cells were harvested on days 5, 10, 20, and 30 postconfluency for biochemical analysis and electron microscopy.

Cytotoxicity Assay. To evaluate the possibility of a cell-damaging effect by GSE treatment, cell monolayer integrity was investigated by measurement of lactate dehydrogenase (LDH) release into culture medium of the upper compartment from treated and control cells. LDH assay was performed on day 5 after seeding in experiment 1 and from confluency to day 30 postconfluency in experiment 2, by use of a standard ultraviolet spectrophotometric kit (Sigma, method 228-UV).

Enzymatic Assays. Some brush border membrane enzyme activities typical of differentiation state, such as alkaline phosphatase, aminopeptidase N, and a disaccharidase (sucrase−isomaltase), were measured. Cytoplasmic LDH activity was also measured. After removal of the apical solution, the cell monolayer was washed twice with prewarmed (37 °C) PBS, scraped, harvested in 2 mL of water, and sonicated for 30 s. All assays were performed with a microplate reader (Dynex Technologies, Grafton, OH).

Sucrase−isomaltase was assayed according to Messer and Dahlqvist (*44*), alkaline phosphatase according to Bessey et al. (*45*), and aminopeptidase N according to Maroux et al. (*46*) with l-leucine-*p*-nitroanilide as substrate. Cellular LDH activity was measured according to the method previously described. Enzyme activities were expressed as milliunits or units per milligram of protein: one unit is defined as the activity that hydrolyzes 1 μmol of substrate per minute under the experimental conditions. Caco-2 cell protein content was assayed by the bicinchoninic acid (BCA) method (*47*) with bovine serum albumin as a standard.

Electron Microscopy. Cells were fixed for 30 min at room temperature with a solution of 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, containing 12 mM $CaCl_2$. After washing, cells were postfixed for 2 h with 2 mL of the same buffer with 1% OsO_4 and 150 mM sucrose added; the cells were then dehydrated in a graded series of ethanol (30%−100%).

Scanning Electron Microscopy (SEM). After the dehydration stage, cells were dried with a CO_2 critical point apparatus (Bal-Tec SCD 0.30), coated with a gold and palladium mixture (Bal-Tec SCD 0.50), and examined with a scanning electron microscope (JEOL JFM 6300 F).

Transmission Electron Microscopy (TEM). After dehydration, cells were embedded in Spurr (Sigma, Saint Guentin Fallavier, France) and polymerized at 60 °C overnight. Ultrathin sections were cut with a diamond knife on a Reichert OM-U3 microtome. The sections were contrasted by uranyl acetate and lead citrate and examined with a transmission electron microscope (JEOL 1200 EX2).

Statistical Analysis. For the enzymatic assays, three wells were run and each assay was repeated three times. Data from each experiment were averaged ($n = 9$) and this average value was the data point used in the statistical analysis. Data were given as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) method for comparing groups by use of Stat View 4.5 (Abacus Concepts, Inc., Berkeley, CA). A significance level of $P < 0.05$ was used for all comparisons.

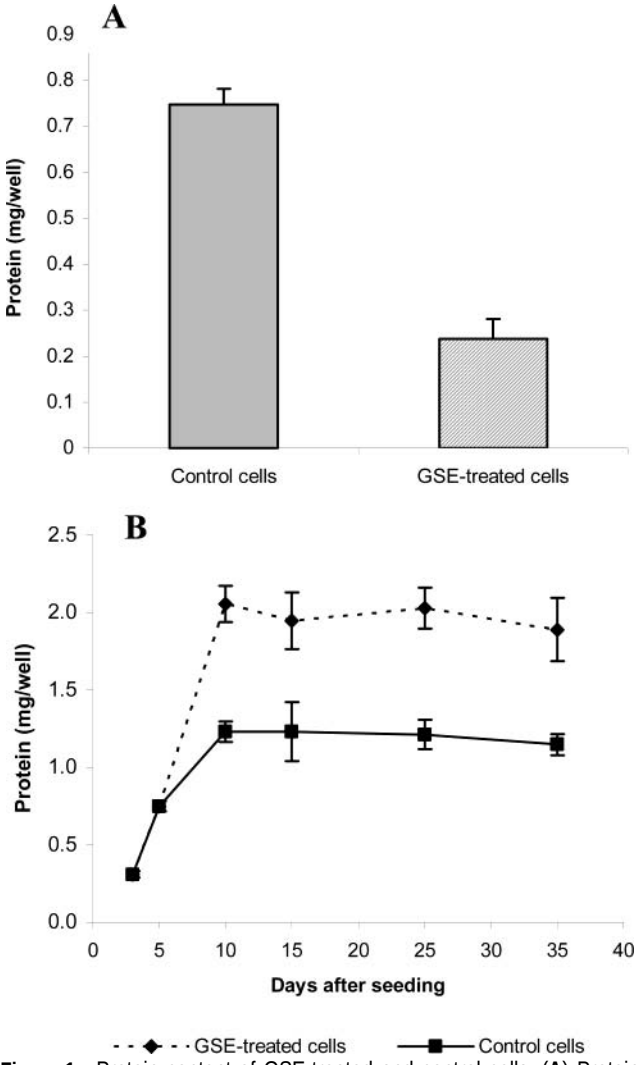

Figure 1. Protein content of GSE-treated and control cells. (**A**) Protein content was assayed on day 5 after seeding, when GSE treatment was applied to Caco-2 cells 24 h after seeding (experiment 1). (**B**) Protein content was assayed over a 30-day period, when GSE treatment was daily applied to cells from confluency (5 days after seeding) (experiment 2). In both experiments, GSE was added to Caco-2 cells for 2 h a day, after being subjected to in vitro digestion. Protein contents are expressed in milligrams per well and are means ± SEM ($n = 9$).

RESULTS

Effect of GSE Treatment on Caco-2 Cell Growth. When Caco-2 cells were subjected to GSE treatment from seeding (experiment 1), cell growth was inhibited and cell monolayer or confluency was never reached. At day 5 after seeding, the protein content of treated cells was 68.2% lower than that of control cells (**Figure 1A**). Moreover, morphological observation by SEM showed clear differences between treated and untreated cells (**Figure 2**); while control cells exhibited some short, sparsely distributed microvilli, treated cells displayed severely damaged apical membrane. When treatment with GSE began after confluency (experiment 2), growth evolution was similar to that of control (**Figure 1B**). Two growth phases were observed, with an exponential growth phase from the beginning of culture until day 5 postconfluency, followed by a stationary phase until day 30 postconfluency. However, the amount of protein per well during the 5 days following confluency increased faster in treated cells than in control cells and remained 40% higher during the stationary phase.

Cytotoxicity Assay. LDH release in the apical culture medium was measured for evaluation of the possible cytotoxicity of a chronic treatment with GSE at 400 mg/L. In experiment 1, LDH activity in apical medium on day 5 postseeding was 73.1% higher in treated cells than in control cells (**Figure 3A**). In contrast, LDH release in experiment 2 during the differentiation period was 1.5−1.6-fold lower in treated cells than in control cells, except on day 30, where values were not significantly different (**Figure 3B**).

Effect of GSE on Caco-2 Cell Morphological Differentiation. Caco-2 cells in culture undergo morphologic changes from confluency, characterized by the development of a typical brush border that, during 30 days of differentiation, gradually develops increasingly regular, tall, uniformly distributed microvilli. Some differences appeared in the presence of GSE (experiment 2), especially at the beginning of the differentiation process. As shown in **Figure 4B**, treated cells displayed numerous microvilli—more abundant and also better organized—as early as day 5 of postconfluency. Some cells exhibited a fully developed brush border of regular microvilli typical of differentiated cells. These differences remained in the days that followed, since on day 10 of postconfluency, the brush border was still denser and better organized than in the control (**Figure 5A**), with taller microvilli (**Figure 5B**). On day 30, the differentiation process was fully completed in both treated and control cells.

Effect of GSE on Caco-2 Cell Hydrolase Activities. The activities of membrane-bound sucrase−isomaltase, aminopeptidase N, alkaline phosphatase, and cellular LDH are shown in **Figure 6**. The results were very different according to the enzyme. The most striking effect was observed for aminopeptidase N. As shown in **Figure 6A**, the specific activity of aminopeptidase N increased regularly in control cells whereas

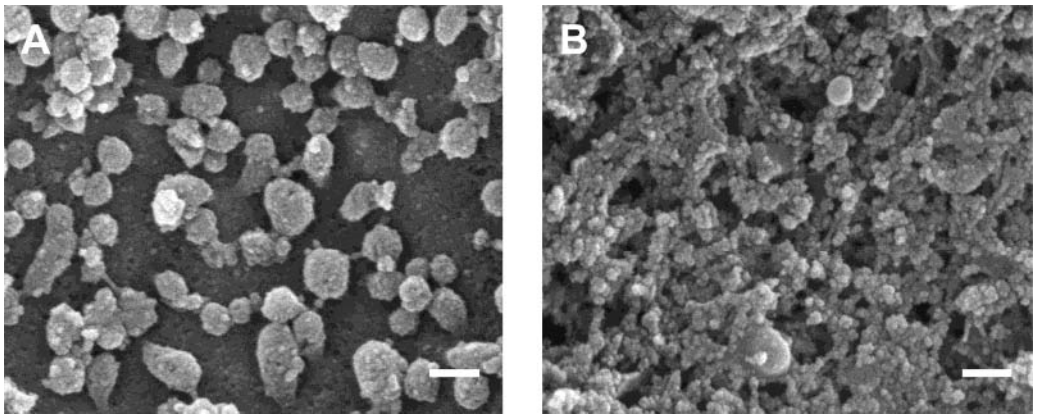

Figure 2. Scanning electron microscopy of Caco-2 cells in culture on day 5 postseeding. (**A**) Control cells; (**B**) GSE-treated cells, in which GSE was daily applied 24 h after seeding for 2 h a day, after being subjected to in vitro digestion. Bars, 200 nm.

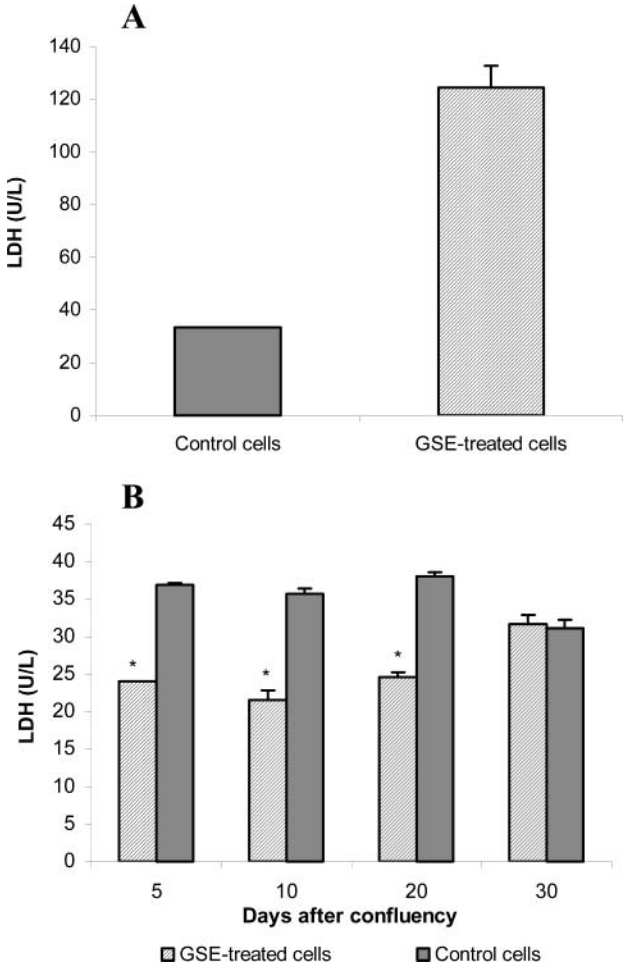

Figure 3. LDH release in apical medium in GSE-treated and control cells. (**A**) LDH was assayed on day 5 after seeding, when GSE treatment was applied to Caco-2 cells 24 h after seeding (experiment 1). (**B**) LDH was assayed over a 30-day period, when GSE treatment was applied daily to cells from confluency (5 days after seeding) (experiment 2). In both experiments, GSE was added to Caco-2 cells for 2 h a day after being subjected to in vitro digestion. LDH activities are expressed in units per liter and are means ± SEM ($n = 9$). Asterisks indicate a significant difference compared to control cells.

activity increased very slightly in the presence of GSE; on day 30 it reached only 28.8% of the activity observed in control cells. In contrast, GSE treatment led to a faster increase of alkaline phosphatase activity in the first days following confluency in comparison with control cells, since activity in treated cells was 52.2% higher than in control cells on day 5 (**Figure 6B**). However, this activity decreased from 49.4 to 27.1 units/mg from day 10 to day 30 postconfluency, whereas in control cells it continued to increase until day 20 and then remained stable. Control cells displayed decreasing sucrase−isomaltase activity from confluency to day 5, followed by a slight increase until the end of the differentiation period (**Figure 6C**). A similar activity pattern was observed in GSE-treated cells throughout the 30-day period. However, in this case cells seemed to exhibit higher activity levels than control cells although no significant differences were recorded. Finally, as shown in **Figure 6D**, GSE treatment had no effect on cellular LDH activity.

DISCUSSION

Since flavonoids are consumed daily and are known to possess beneficial health effects, numerous studies have been performed on their bioavailability and absorption mechanisms. However, because of their direct exposure to the intestinal tract, such compounds may have certain effects on intestinal epithelium. This study showed that chronic treatment of Caco-2 cells with GSE (400 mg/L) modulated cell proliferation and differentiation and led to morphological and functional changes in the cell phenotype.

GSE thus had contrasting effects on cell growth according to whether the treatment began just after seeding or once confluency had been reached. In the first case, Caco-2 cell proliferation was inhibited since confluency was never reached, even after 10 days of culture, whereas control cells were confluent on day 5 postseeding. This inhibition was associated with a cytotoxicity reaction on cell membrane as showed by the 73.1% higher release of LDH into the apical medium than in control cells after 5 days of culture. This cytotoxic effect was confirmed by the damaged cell apical membranes observed in the presence of GSE. Carnésecchi et al. (*28*) showed that a procyanidin-enriched cocoa extract (50 mg/L) caused 75% growth inhibition of Caco-2 cells, associated with an increased number of lysed cells, and ruled out the possibility of apoptotic cell death. Thus, although the experimental conditions were different, we also showed that GSE applied at the beginning of culture could have an antiproliferative effect by possible alteration of apical membrane. However, the mechanisms resulting in Caco-2 cell growth inhibition by flavonoids are not completely clear at the moment and may differ according to the phenolic compound. Some, such as cinnamic acid (*29*) or quercetin (*27*), may act directly on DNA, while others, such as procyanidins or resveratrol (found in grapes and wine), may induce inhibition of progress of the cell cycle, blocking cells at

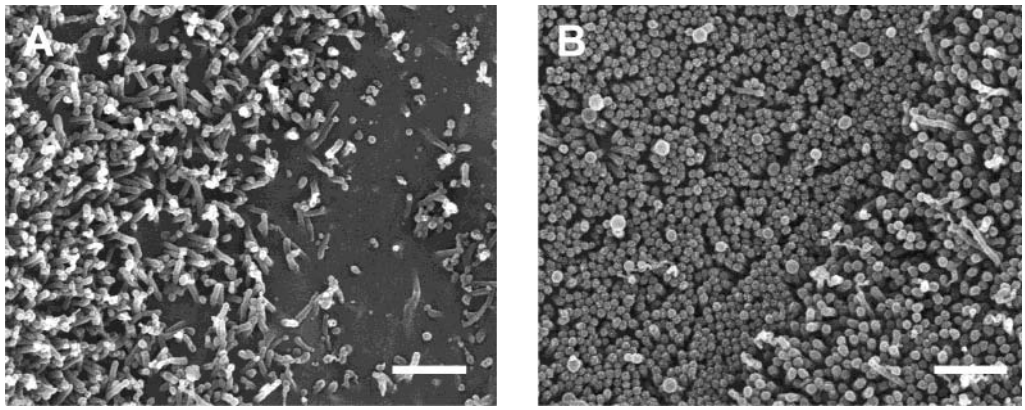

Figure 4. Scanning electron microscopy of Caco-2 cells in culture after 5 days of postconfluency. (**A**) Control cells; (**B**) GSE-treated cells, in which GSE was applied daily from confluency for 2 h a day over a 30-day period and after being subjected to in vitro digestion. Bars, 1 μm.

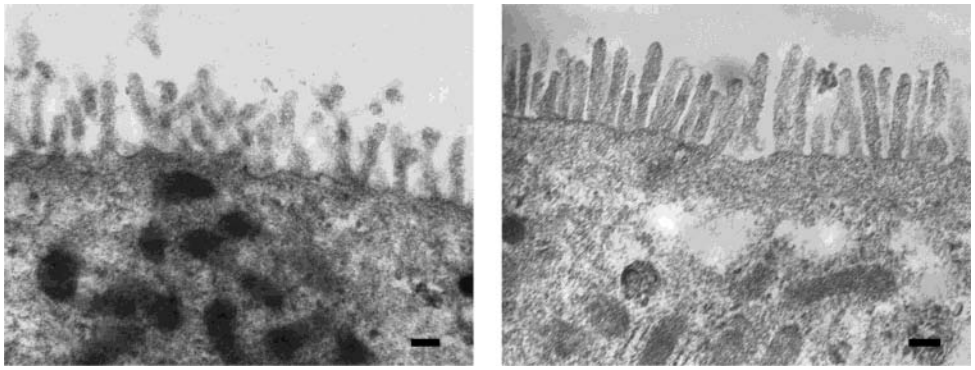

Figure 5. Transmission electron microscopy of Caco-2 cells in culture after 10 days of postconfluency. (**A**) Control cells; (**B**) GSE-treated cells, in which GSE was applied daily from confluency for 2 h a day over a 30-day period and after being subjected to in vitro digestion. Bars, 200 nm.

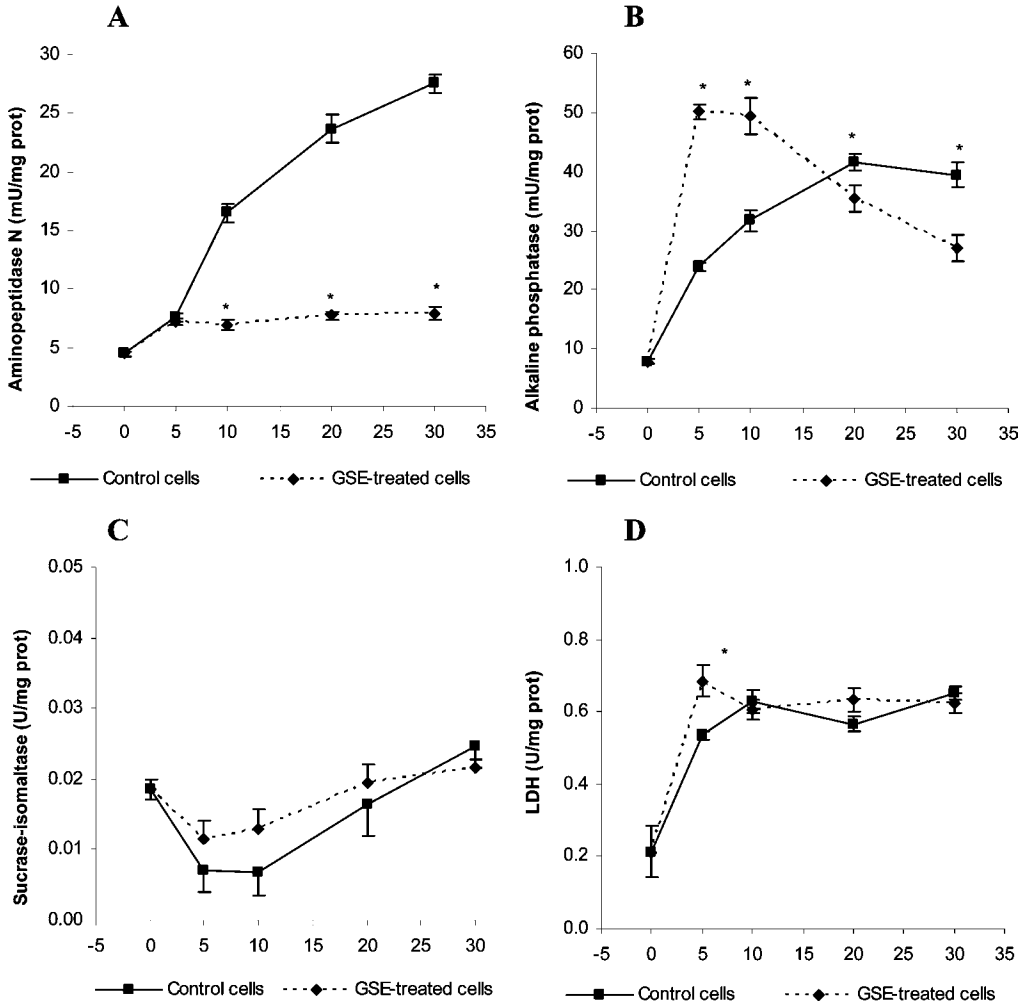

Figure 6. Activity of Caco-2 cell hydrolases in both control and GSE-treated cells during differentiation. GSE was applied from confluency for 2 h a day over a 30-day period and after being subjected to in vitro digestion. Specific panels are defined as follows: (**A**) aminopeptidase N, (**B**) alkaline phosphatase, (**C**) sucrase–isomaltase, and (**D**) LDH. Activities are expressed in milliunits or units per milligram of protein and are means ± SEM ($n = 9$). Asterisks indicate a significant difference compared to control cells.

S/G2 or G2/M transition phases (*28*, *48*−*50*). According to Schneider et al. (*50*), inhibition of ornithine decarboxylase expression might be a possible cause of this cell cycle modulation.

On the other hand, we showed that cell growth was not inhibited when GSE was applied once confluency had been reached. In both treated and control cells, the stationary phase of the growth curve was reached around day 5 after confluency, namely, 10 days after seeding, which agrees with previous studies (*15*, *20*, *24*). Moreover, compared with control cells, GSE treatment increased the protein content per well during the 5 days following confluency. This phenomenon was associated with morphological differences in the presence of GSE. The cell differentiation process was enhanced by daily GSE treatment, promoting expression of microvilli, but the causal mechanism is not yet known. Thus, chronic treatment with GSE at 400 mg/L did not lead to morphological abnormalities of confluent cells by cytotoxicity, as shown by the constant LDH release into apical medium throughout differentiation. Moreover, the same experiments were carried

without previous digestion of GSE in order to rule out the effect of the simulated digestion itself in morphological evolution of cells during differentiation and the results were similar to those presented here (data not shown). We have therefore demonstrated for the first time a stimulating effect of daily exposure to a grape seed extract on growth and morphological differentiation of confluent Caco-2 cells.

Since flavonoids have been shown to penetrate intestinal cells (*13*, *51*−*53*), it can be put forward that these effects are likely to occur in vivo as well. Intestinal cells that migrate from crypt to villus tip might therefore undergo more rapid differentiation into absorptive cells when exposed daily to grape seed flavonoids. However, it remains difficult to establish a direct correlation between the in vivo and in vitro models, as conditions are different and Caco-2 cells display certain phenotypic differences with intestinal cells (*54*).

In addition, we have shown that daily treatment with GSE also modified the confluent Caco-2 cell phenotype by stimulating alkaline phosphatase activity and inhibiting aminopeptidase N activity during the differentiation process. Sucrase−isomaltase and cellular LDH activities were unchanged elsewhere. With regard to LDH, the comparison between cell proliferation curve and LDH activity in control cells revealed that LDH may be considered as a good cellular marker of Caco-2 cell evolution since activity displayed the same development as protein content, with a stationary phase reached on day 5 after confluency.

Alkaline phosphatase, usually used as a marker for cell differentiation in the Caco-2 model (*15*, *17*, *21*), displayed steadily increasing activity from confluency. Our study showed that a chronic GSE treatment enhanced the specific activity of the enzyme (units per milligram of protein), suggesting that this stimulation resulted not only from the increase in the number of cells but also from a distinct effect of GSE on enzyme expression. Previous work showed that the effects of phenolic compounds on alkaline phosphatase activity might be dependent on the compound. Thus resveratrol did not induce any change in alkaline phosphatase activity when applied alone to Caco-2 cells, but in combination with butyrate it triggered 3.3-fold increased activity (*49*). Piceatannol was also reported to have no effect on alkaline phosphatase activity when applied to Caco-2 cells (*48*), whereas cinnamic acid had a negative effect on this enzyme, inhibiting activity in postconfluent cells (*29*). However, these authors used different experimental conditions from ours, since their concentration of cinnamic acid was 7-fold higher than the GSE dose and their treatment was applied over a 48-h period. Otherwise, it has been proved that flavonoids such as flavone could lead to a 6-fold increase in alkaline phosphatase activity in HT-29 human colon cancer cells (*55*). We have therefore shown for the first time that GSE flavonoids have an activating effect on alkaline phosphatase expression in confluent Caco-2 cells, but the mechanism by which they act is not yet well established. Indeed, such compounds could act at the transcriptional or traductional level of enzyme synthesis or on transcellular transport. Some authors have suggested that the effects of flavonoids on enzyme activities might be mediated through transcriptional changes by modulation of mRNA formation (*30*). Others have suggested that phenolic compounds such as cinnamic acid induced their effect on enzyme activity partly by modulating the intracellular cAMP signaling pathway (*29*). Further studies are needed to determine which compounds in GSE could exert this effect and to identify the mechanism involved.

On the other hand, while GSE phenolics enhanced alkaline phosphatase expression, they inhibited aminopeptidase N activity. Indeed, the specific activity reached at confluency remained unchanged throughout differentiation despite an increase in protein content. This lack of evolution may result from two distinct mechanisms: either an inhibition of enzyme expression or a direct interaction between GSE and enzyme phenolic compounds. Although no data are available about flavonoid affinities for aminopeptidase N, such interactions between flavonoids and other intestinal brush border enzymes have already been demonstrated (*56*). Tebib et al. (*57*) suggested an inhibitive effect of a tannin diet on dipeptidyl peptidase IV activity by direct interaction between the enzyme and the phenolic compounds. GSE flavonoids applied daily to Caco-2 cells thus caused a break in the increase of aminopeptidase N activity 5 days after seeding. Further investigation will be necessary to determine the cellular mechanism involved.

No effect of phenolic compounds from GSE was observed on sucrase−isomaltase and LDH activities since the latter were unchanged throughout differentiation in comparison with control cells. In contrast with previous studies (*15*, *58*), we noted a very slight increase in sucrase−isomaltase activity in control cells from confluency to day 30 postconfluence and low activity levels, whereas sucrase−isomaltase is usually considered to be a good marker of cell differentiation (*58*). However, it has been shown that sucrase−isomaltase expression in Caco-2 cells could display substantial variation depending on the clone (*18*) or the cell passage used (*59*).

Finally, our results are the first indication that daily exposure of Caco-2 cells to flavonoids from GSE (400 mg/L) affects the proliferation and differentiation of intestinal Caco-2 cells, inducing morphological and functional changes. When applied to confluent cells, GSE activated the formation of cell brush border, leading to longer and better organized microvilli, and this was corroborated by a stimulation of alkaline phosphatase, a cell differentiation marker. Moreover, whereas sucrase−isomaltase and intracellular LDH were not affected by GSE, we showed for the first time that aminopeptidase N activity was inhibited during Caco-2 cell differentiation, either by direct interaction with GSE compounds or by modulation of enzyme expression. Further studies are required for the identification of the compounds from GSE that may be able to act on these hydrolase activities and understanding of the cell mechanisms involved, taking into account the hypothesis of transcriptional, traductional, or intracellular transport regulations, as suggested in previous studies.

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