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Ethanol and Polyphenolic Free Wine Matrix Stimulate the Differentiation of Human Intestinal Caco-2 Cells. Influence of Their Association with a Procyanidin-Rich Grape Seed Extract

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The effect of daily contact with ethanol on Caco-2 cell differentiation was investigated. Pure ethanol (1%) and a polyphenolic free wine matrix (polyphenol-free wine containing 1% ethanol) associated or not with a procyanidin-rich grape seed extract (GSE) were added to Caco-2 cells from confluency for 2 h a day after successive incubation in salivary, gastric, and pancreatic media. Treatment with 1% ethanol did not appear to be cytotoxic to cells, but it also stimulated Caco-2 cell differentiation, particularly in the first days following confluency, and this effect was more marked when associated with polyphenolic free wine matrix constituents. This activation resulted in an increase in microvillar density, organization, and elongation (+70%) and was associated with strong stimulation of sucrase-isomaltase (+780%) and a concomitant regular increase in cell protein content (+50−88%). While the presence of GSE in alcoholic solutions did not modify the morphological pattern observed in cells subjected to ethanol and polyphenolic free wine matrix alone, it had a clear reducing effect on their microvillus elongation (−30%). However, these stimulating effects of ethanol on morphological differentiation were attenuated from day 10 postconfluency, which could suggest cell cytoprotection against ethanol. These are the first results in support of the notion that moderate concentration of ethanol may stimulate the differentiation of Caco-2 cells, particularly when integrated with a polyphenolic free wine matrix.

KEYWORDS: Ethanol; polyphenolic free wine matrix; phenolic compounds; Caco-2 cells; differentiation

INTRODUCTION

Intestinal cell differentiation occurs in vivo as the cells migrate from the crypt to the villus tip. Previous studies have shown the effect of different compounds such as drugs, minerals, hormones (*1*−*6*), or phenolic compounds (*7*−*9*) on this differentiation. On the other hand, data are scarce concerning the possible impact of chronic alcohol or wine consumption on intestinal epithelium. In wine, alcohol is associated with phenolic compounds, and we recently demonstrated that daily contact of human intestinal Caco-2 cells with a procyanidin-rich grape seed extract (GSE) inhibited proliferation of these cells while stimulating their differentiation (*10*). In this work, we thus investigated the effect of ethanol and a polyphenolic free wine matrix, associated or not with GSE, on human intestinal cell differentiation. We worked with the Caco-2 cell line, which has been extensively used as a convenient in vitro model for investigating the maturation process (*11*).

Drinking excessive amounts of alcohol regularly for years is toxic for almost every tissue of the body. Many of the toxic effects of alcohol are due to the disturbance of a wide variety of metabolic functions and organ damage. Concerning the intestinal epithelium, some studies have shown that ethanol could have damaging effects, especially by disruption of barrier integrity (*12*, *13*), which could be correlated with increased tight junction permeability and precede the development of colon cancers (*14*). Most studies have shown that Caco-2 cell exposure to ethanol disrupted cytoskeleton components and particularly microtubules and could lead to increased cell permeability (Banan, *15*−*18*). Banan et al. (*19*) also demonstrated that 30 min exposure of Caco-2 cells to ethanol had damaging effects on the intestinal barrier by causing activation of nitric oxide synthase, NO overproduction, increasing oxidative stress and superoxide anion production. On the other hand, epidemiological and clinical evidence shows that light−moderate drinking (<2 drinks per day) is associated with a reduced risk of coronary heart disease and total mortality in middle-aged and elderly men and women (*20*, *21*). In a previous study, we thus showed that 12% ethanol improved the preventive effect of GSE on atherosclerosis in hamsters, involving a synergic effect between the two compounds (*22*). Data are scarce concerning the possible protective effect of ethanol on intestinal cells, but Malagolini et al. (*23*) have shown that ethanol (200 mM) could stimulate in vitro differentiation of Caco-2 cells over a 21-day period of

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constant treatment, leading to a dose-dependent increase in sucrase activity, i.e. a biochemical marker of differentiation.

Thus, in order to evaluate the in vitro effect of chronic moderate consumption of ethanol on intestinal epithelium, we studied the differentiation process of Caco-2 cells subjected to daily contact with ethanol or polyphenolic free wine matrix, with or without GSE. To mimic physiological conditions, treatment solutions were previously incubated successively in salivary, gastric, and pancreatic media before being added to Caco-2 cells for 2 h a day for a 30-day period.

MATERIALS AND METHODS

Materials. GSE was obtained from La Gardonnenque SCA (Cruviers Lascours, France) and contained approximately 67.4% procyanidins, with 13.5% of them being (+)-catechin (CAT), (−)-epicatechin (EC), and B1, B2, B3, and B4 dimers (according to the supplier).

Preparation of Polyphenolic Free Wine Matrix (WM). Polyphenolic free wine matrix was prepared from French white wine (Bordeaux Richaumont 2001) sold in the shops, according to the following procedure: white wine was stripped of phenolic compounds using a separation column consisting of polyvinylpolypyrrolidone on which polyphenols were adsorbed. The polyphenol-free wine obtained was then sterilized by filtration (0.2 μm) and called WM. We used white wine because of its low polyphenol content. Although no information about the production and composition of this wine are available, WM may also contain components such as minerals, glycerol, organic acids, residual sugars, or mannoproteins according to the literature (*24*, *25*).

Cell Culture. Caco-2 cells from human colorectal adenocarcinomas 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^2 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 reached confluence, they were harvested by treatment with a solution containing 0.25% trypsin and 1 mM 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^2 (*26*) 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 placed 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 (*27*−*29*). Incubations in salivary, gastric, and intestinal solutions were conducted on a rocking platform shaker (Rotomix, Bioblock, Illkirch, France) in an incubator at 37 °C. All enzymatic solutions, such as 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).

Artificial saliva was created with the main constituents of human saliva, as previously reported (*30*−*35*). It consisted of phosphate-buffered saline (PBS) (diluted 1:5), containing $CaCl_2$ 1.336 mM, $MgSO_4$ 0.174 mM, KH_2PO_4 12.8 mM, and $NaHCO_3$ 23.8 mM. 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 (*30*, *31*, *33*, *35*, *36*) and α-amylase (1000−1500 units/mL) (*31*, *36*, *37*).

The in vitro digestion is presented in **Figure 1**. 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 1 N HCl, and 0.05 mL of pepsin (25 mg/mL in 0.1 N HCl) was added per mL of sample to simulate a gastric juice. Incubation was performed for 60 min at 55 oscillations/min. Finally, to reproduce

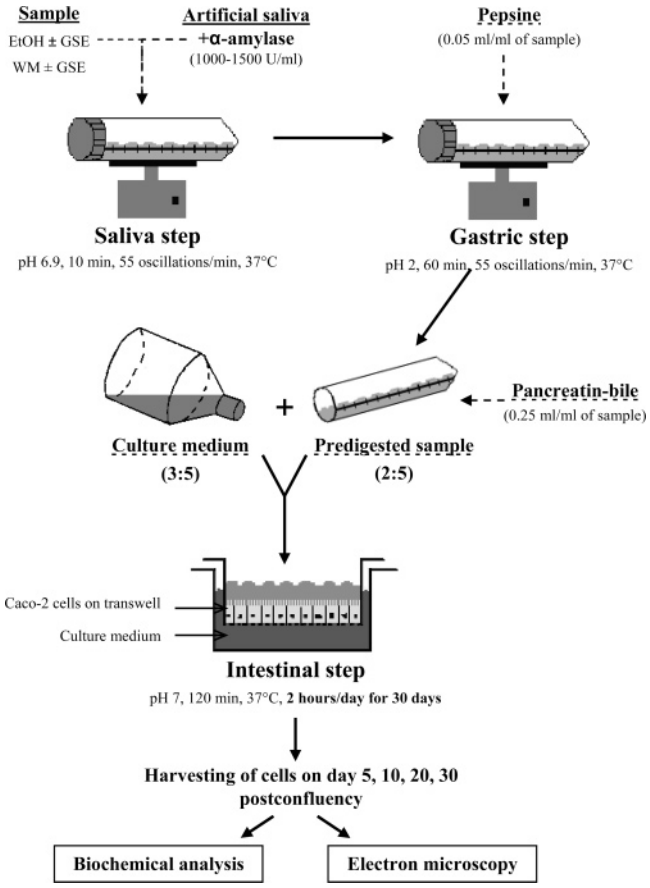

Figure 1. In vitro digestion scheme and experimental design.

the intestinal medium, the pH was raised to 6 through 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 mL 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.

Experimental Design. The chronic effect of ethanol and WM, with or without GSE, on the Caco-2 cell differentiation process was investigated by studying both biochemical and morphological cell characteristics. The treatment started at confluency. Treatment solutions were first subjected to successive incubations in saliva and gastric media according to the in vitro simulated digestion model developed previously. 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 treatment solution diluted with culture medium (3:5). Incubations were performed for 2 h at 37 °C. Culture medium was also added (2 mL) in the lower compartment. The treatment solutions were removed upon completion of the 2 h incubation 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.

The treatment solutions were as follows: WM with or without GSE (WM-GSE and WM, respectively) and ethyl alcohol with or without GSE (EtOH-GSE and EtOH, respectively). The GSE concentration used in this study was the equivalent to 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 g to 1.25 g. The volume of total digestive secretions in humans is estimated to be 10 L per day, thus around 3 L per 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. The EtOH and WM solutions could be likened to red wine, so their consumption could be estimated at 250

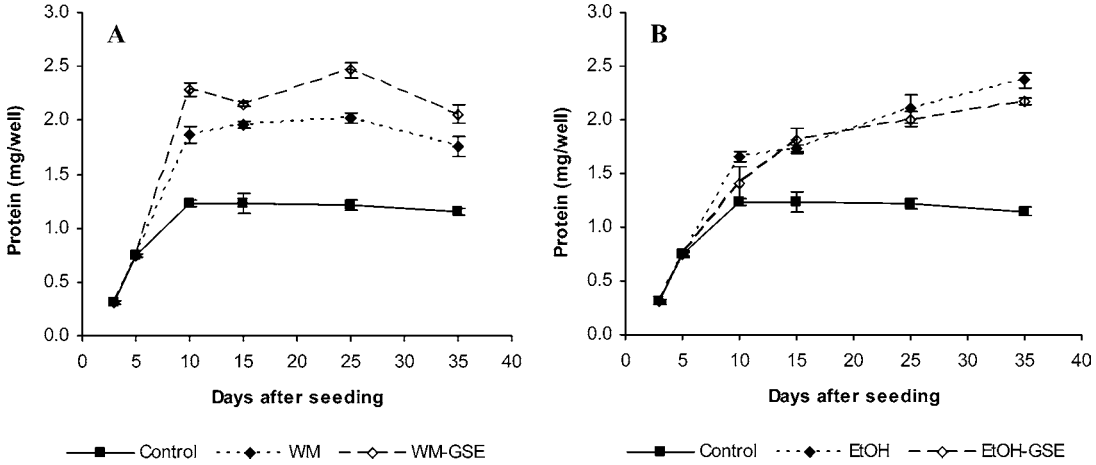

Figure 2. Protein content of both control and treated Caco-2 cells over a 30-day period of postconfluency. (**A**) Protein content in cells subjected to 80 mL/L of polyphenolic free wine matrix (1% ethyl alcohol) with or without 0.4 g/L GSE (WM-GSE and WM, respectively). (**B**) Protein content in cells subjected to 1% ethanol with or without 0.4 g/L GSE (EtOH-GSE and EtOH, respectively). Solutions were added to Caco-2 cells from confluency for 2 h a day after being subjected to in vitro digestion. Protein contents are expressed in milligrams per well and are means ± SEM ($n = 9$).

mL per meal. Diluted in a 3 L volume of digestive secretions, their final proportion at the intestinal level was estimated as 80 mL/L of secretions. Initial EtOH and WM solutions both contained 12% (v/v) ethyl alcohol, so the final diluted solutions applied on cells contained 1% (v/v) ethyl alcohol (174 mM).

Cell Protein Content. The Caco-2 cell protein content was assayed by the bicinchoninic acid (BCA) method (*38*) with bovine serum albumin as standard.

Cytotoxicity Assay. To assess the possibility of a cell-damaging effect by the different treatments, cell monolayer integrity was investigated by measurement of lactate dehydrogenase (LDH) release into the culture medium of the upper compartment from treated and control cells. LDH was assayed using a standard ultraviolet spectrophotometric kit (SIGMA, method 228-UV).

Enzymatic Assays. Some brush border membrane enzyme activities typical of a cell differentiation state, such as alkaline phosphatase, aminopeptidase N, and a disaccharidase complex (sucrase-isomaltase), were 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 (*39*), alkaline phosphatase according to Bessey et al. (*40*), and aminopeptidase N according to Maroux et al. (*41*) using l-leucine-*p*-nitroanilide as substrate. Enzyme activities were expressed as milliunits or units per mg of protein: one unit is defined as the activity that hydrolyzes 1 μmol of substrate per minute under the experimental conditions.

Electron Microscopy. Cells were fixed for 30 min at room temperature with a solution of 2% glutaraldehyde in 100 mM Na-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; then the cells were 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), which was 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. For microvillus length determination, data were means from 80 measurements per sample ($n = 80$). Enzymatic and morphologic analysis data were presented as mean ± SEM. and analyzed by one-way or two-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) method used for group comparisons, using Stat View 4.5 (Abacus concepts, Inc, Berkeley, CA). A significance level of $P < 0.05$ was used for all comparisons.

RESULTS

Effect of the Different Treatments on Caco-2 Cell Protein Content. The results concerning protein content are presented in **Figure 2**. For each experiment, confluency was reached on day 5 postseeding. In control cells, the protein content pattern was characterized by two phases, namely, an exponential phase from day 3 until day 10 postseeding, followed by a stationary phase until day 30 postconfluency. When the polyphenolic free wine matrix treatment was applied to cells from confluency (**Figure 2A**), the exponential phase was enhanced relative to control and more markedly in the presence of GSE (WM-GSE). At day 10, cell protein contents were 1.5- to 1.85-fold higher in WM- and WM-GSE-treated cells, respectively, than in control. A stationary phase was observed from day 10 postseeding until the end of the culture period in both WM- and WM-GSE treated cells. When cells were subjected to ethanol (**Figure 2B**), some differences appeared relative to control cells since no stationary phase was observed. Protein content per well exponentially increased over the culture period to reach, at day 30 postconfluency, 1.88- and 1.55-fold higher than in control cells, in the presence of EtOH and EtOH-GSE, respectively.

Cytotoxicity Assay. LDH release in the apical culture medium was measured to assess the possible cytotoxicity of chronic treatment with WM, WM-GSE, EtOH, or EtOH-GSE (**Figure 3**). In the presence of polyphenolic free wine matrix (**Figure 3A**), LDH release was around 1.7-fold lower than in control cells, from day 10 to day 30 postconfluency, and independently of the presence of GSE. In contrast, the extent of LDH release in the presence of ethanol (**Figure 3B**) was similar to that of control throughout the differentiation process, independently of the presence of GSE, except at day 10 postconfluency, when the values were 1.4-fold lower than in control cells.

Effect of WM and EtOH on Caco-2 Cell Morphological Differentiation. Caco-2 cells in culture undergo morphologic

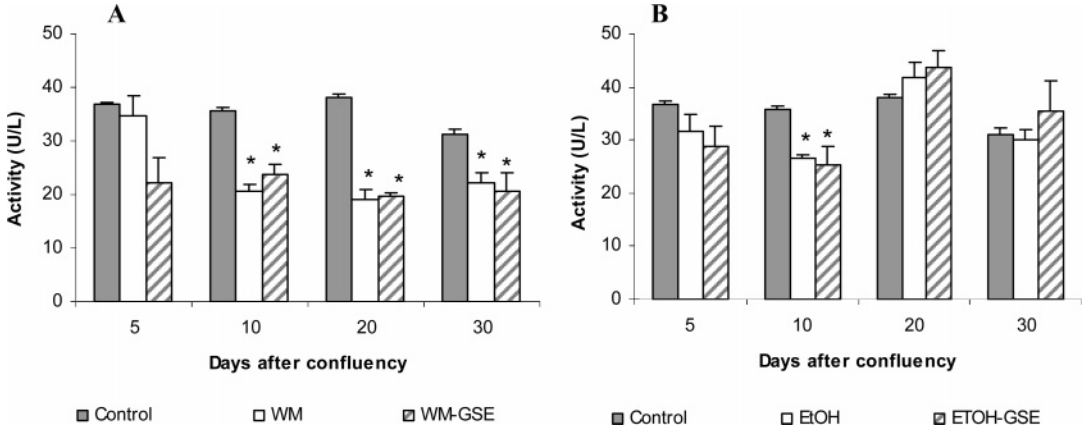

Figure 3. LDH release in apical medium, in treated and control Caco-2 cells over a 30 day period of postconfluency. (**A**) LDH release by cells subjected to 80 mL/L of polyphenolic free wine matrix (1% ethyl alcohol) with or without 0.4 g/L GSE (WM-GSE and WM, respectively). (**B**) LDH release by cells subjected to 1% ethanol with or without 0.4 g/L GSE (EtOH-GSE and EtOH, respectively). Solutions were added to Caco-2 cells from confluency 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.

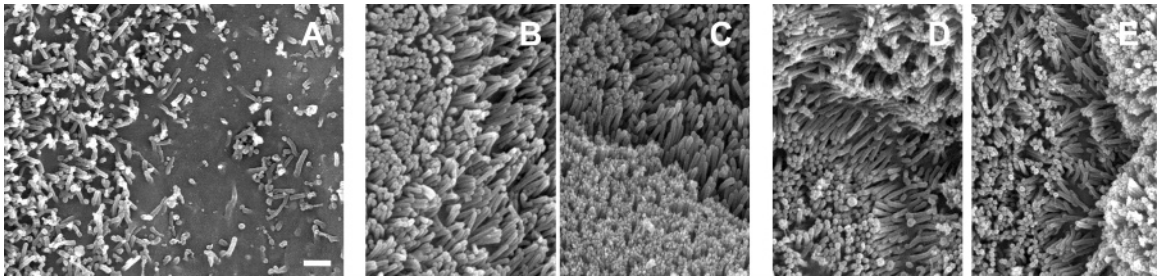

Figure 4. Scanning electron microscopy of Caco-2 cells in culture after 5 days of postconfluency. (**A**) Control cells. (**B**) WM-treated cells. (**C**) WM-GSE-treated cells. (**D**) EtOH-treated cells. (**E**) EtOH-GSE-treated cells. The different solutions were added to Caco-2 cells from confluency for 2 h a day after being subjected to in vitro digestion. Bars, 500 nm.

changes from confluency and develop, during 30 days of differentiation, a typical brush border with increasingly regular, tall and uniformly distributed microvilli. Treatments with polyphenolic free wine matrix or ethanol led to some differences, particularly during the first days of differentiation. As shown in **Figure 4**, at day 5 postconfluency, control cells exhibited some sparce, short and irregular microvilli, while all treated cells presented rather long microvilli, with higher density, organization, and regularity, independently of the presence of GSE. This difference between control cells and WM-treated cells persisted until day 30, with a higher microvillus density and homogeneity. On the other hand, after 20 and 30 days postconfluency, cells exposed to WM-GSE, EtOH, and EtOH-GSE exhibited morphological features similar to those of control cells and presented a complete differentiation process, with a regular and organized brush border (data not shown).

Effect on Microvillus Increase. Both polyphenolic free wine matrix and ethanol chronic treatments led to a higher increase in microvillus length than control, in the first days following confluency and more markedly with WM (**Figure 5**). Indeed, at day 10 postconfluency, the average microvillus lengths were 1.7- and 1.4-fold higher in WM- and EtOH-treated cells, respectively, than in control cells (**Table 1**). Regardless of the liquid matrix used, the addition of GSE significantly attenuated the increase in microvillus length ($P < 0.0001$). Nevertheless these values were 1.4- and 1.2-fold higher than in control cells, for WM-GSE and EtOH-GSE, respectively. At day 20 post-confluency, microvillus length in WM-treated cells did not significantly differ from that of control cells ($P = 0.083$), but in the presence of GSE (WM-GSE), the lengths significantly

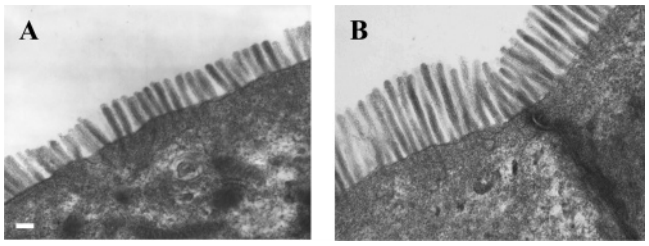

Figure 5. Transmission electron microscopy of Caco-2 cells in culture after 10 days of postconfluency. (**A**) Control cells. (**B**) WM-treated cells, in which WM was applied daily from confluency for 2 h a day over a 30 day period and after being subjected to in vitro digestion. Bars, 200 nm.

decreased ($P < 0.0001$). When EtOH treatment was applied, microvilli at day 20 were clearly smaller than in control (429.3 and 534.8 nm, respectively), and this effect was significantly more marked in the presence of GSE (EtOH-GSE) (335.3 nm, $P < 0.0001$). Last, at day 30, while control cells presented long microvilli (886.6 nm), treated cell microvilli were 1.72- to 2.24-fold smaller, according to the treatment. In this case, the nature of the liquid matrix did not differently affect the microvillus size, whereas the presence of GSE did ($P < 0.0001$), and the greatest effect on this decreased size was observed with EtOH-GSE (395.2 nm).

Hence, regarding variations in microvillus length between day 10 and day 30 postconfluency, we observed a regular marked increase in control cells, which did not occur in treated cells, since the microvillus length remained rather stable from day 10 of differentiation, despite the stronger increase observed in the first 5 days of treatment.

Table 1. Effect of Daily Exposure of Caco-2 Cells to Polyphenolic Free Wine Matrix (1% Ethanol) with (WM-GSE) or without 0.4 g/L GSE (WM) and 1% Ethanol with (EtOH-GSE) or without 0.4 g/L GSE (EtOH) on Microvillar Length after 10, 20, and 30 Days Postconfluency[a]

		experimental groups[b]					
						ANOVA *P* value	
day	control[c]	WM[c]	WM-GSE[c]	EtOH[c]	EtOH-GSE[c]	liquid matrix[d]	GSE
10	361.2 ± 13.5[a]	608.9 ± 20.1[a]	501.0 ± 23.3[a]	507.8 ± 13.8[a]	447.8 ± 9.9[a]	<0.0001	<0.0001
20	534.8 ± 21.3[b]	575.4 ± 8.4[a]	403.3 ± 16.5[b]	429.3 ± 9.5[b]	335.3 ± 5.7[b]	<0.0001	<0.0001
30	886.6 ± 26.1[c]	516.0 ± 9.7[b]	461.5 ± 12.9[a]	490.2 ± 9.8[a]	395.2 ± 9.5[c]	NS	<0.0001

[a] Measurements were made at high magnification (×20000) and are expressed in nanometers. [b] Values are means ± SEM, $n = 80$. Data were analyzed by two-way ANOVA followed by the least significant difference test. A difference of $P < 0.005$ was considered significant. NS, not significant, $P > 0.005$. [c] Values in each column are means ± SEM ($n = 80$). For each column, data were analyzed by one-way ANOVA followed by the least significant difference test. Data in the same column with not the same letters are significantly different ($P < 0.005$). [d] Liquid matrix is 1% ethanol or wine matrix.

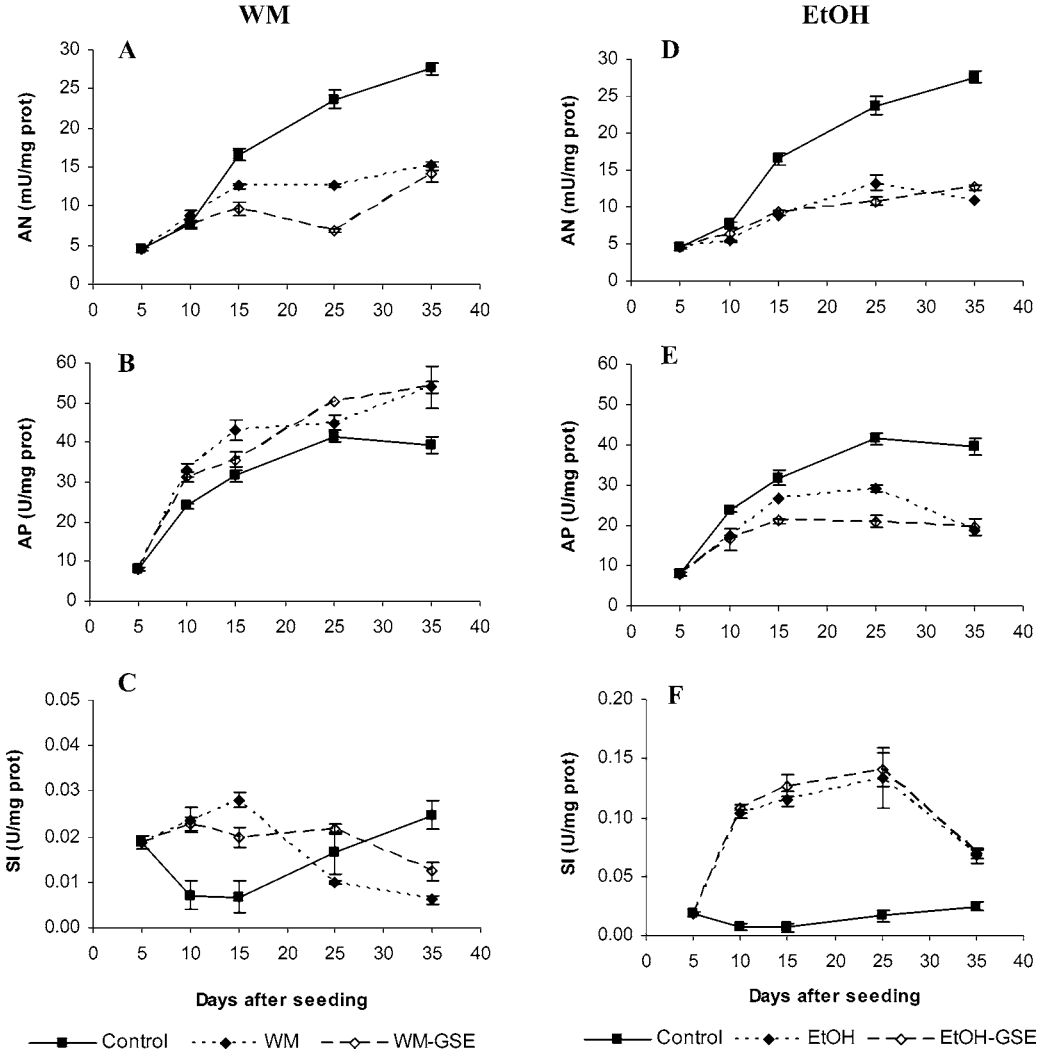

Figure 6. Activity of some hydrolases in both control and treated cells. (**A**–**C**) Caco-2 cells subjected to 80 mL/mL of polyphenolic free wine matrix (1% ethyl alcohol) with or without 0.4 g/L GSE (WM-GSE and WM, respectively). (**D**–**F**) Caco-2 cells subjected to 1% ethanol with or without 0.4 g/L GSE (EtOH-GSE and EtOH, respectively). Solutions were added to Caco-2 cells from confluency for 2 h a day after being subjected to in vitro digestion. Specific panels are defined as follows: (**A**, **D**) aminopeptidase N (AN); (**B**, **E**) alkaline phosphatase (AP); (**C**, **F**) sucrase-isomaltase (SI). Activities are expressed in milliunits or units per milligram of protein and are means ± SEM ($n = 9$).

Effect of the Different Treatments on Caco-2 Cell Hydrolase Activities. The activities of membrane-bound sucrase-isomaltase (SI), aminopeptidase N (AN), and alkaline phosphatase (AP) in Caco-2 cells subjected to polyphenolic free wine matrix or ethanol are presented in **Figure 6**. As shown in **Figure 6A,D**, the specific activity of AN regularly increased in control cells over the 30 day period. Irrespective of the treatment applied, this activity was reduced more effectively with EtOH, and reached about 41.7 and 46.4% of the control cell activity on day 30, with or without 0.4 g/L GSE, respectively. While the presence of GSE did not modulate the effect of EtOH on AN activity over the treatment period, it was found to significantly accentuate the inhibitory effect of WM (**Figure 6A**). Concerning AP, we observed opposite effects of polyphenolic free wine matrix and alcohol (**Figure 6B,E**). While WM significantly increased AP activity independently of the presence

of GSE, EtOH decreased it with a more marked effect when associated with GSE. At day 30, AP activities in WM- and EtOH-treated cells were, respectively, 1.37-fold higher and 2-fold lower than control cell activity. Last, regarding **Figure 6C**, sucrase-isomaltase activity in WM-treated cells increased at the beginning of differentiation and then decreased to reach 40% and 24% of the control activity, with and without GSE, respectively, at day 30 postconfluency. In the presence of EtOH (**Figure 6F**), we observed a marked stimulation of sucrase-isomaltase activity until day 20 (8.8-fold higher than control). However, a decrease was noted from day 20 to day 30.

DISCUSSION

For many years, alcohol was described as a toxic agent for almost every tissue of the body when consumed regularly in excessive amounts. More recently, epidemiological and clinical evidence has shown that moderate alcohol consumption could have beneficial effects on health and particularly on prevention of coronary heart disease (*20*, *22*). However, despite direct exposure of intestinal cells to ethanol, few studies have focused on the possible impact of such compounds on intestinal epithelium. This study showed that chronic treatment of Caco-2 cells with ethanol (1%) and polyphenolic free wine matrix (1% ethanol) with or without GSE (400 mg/L) modulated cell differentiation and led to morphological and functional changes in the cell phenotype.

First, the concentration of ethanol used (1%) did not appear to be cytotoxic for Caco-2 cells in regard to LDH release into the apical medium. In this way, Diebel et al. (*42*) have also shown that 1% ethanol exposure to MDCK cells for 1 h did not induce cell death, whereas 5% ethanol did. On the other hand, it was shown that acute low concentrations (2.5%, 5%) of ethanol decreased Caco-2 cell viability by induction of apoptosis and disrupted epithelial barrier function (*16*, *43*). Otherwise, in our work, EtOH appeared to be nontoxic for cells but it also had a protective effect on cell lysis when associated with WM constituents and independently of the presence of GSE.

Hence, regarding this work, Caco-2 cell morphological differentiation was strongly enhanced by daily treatment with 1% ethanol, promoting the expression and growth of microvilli as early as day 5 postconfluency and independently of the presence of GSE. Thus, treated cells observed after 5 days of treatment had morphological characteristics similar to those of control cells after 20 days of culture, namely, a fully developed brush border with regular microvilli, typical of differentiated cells. This higher differentiation state was observed throughout treatment regarding the higher density and homogeneity of the brush border at day 30 compared to control cells. These results suggest that alcohol stimulated the differentiation process of Caco-2 cells, especially when associated with WM components. Moreover, EtOH and WM enhanced microvillus expression while also increasing their growth, leading to microvillus lengths which were up to 1.7-fold greater than in control cells as early as day 10 postconfluency. However, this phenomenon seemed to be attenuated during the differentiation process, since the lengths reached at day 10 were not modified until day 30 in treated cells. Alcohol enhanced microvillus elongation in the beginning of differentiation, but the causal mechanism is not yet known. It was previously shown that an increase in microvillus elongation could occur in various cell types, in response to some stress, like hydrogen peroxide stress, via perturbations in intracellular calcium and actin polymerization (*44*). Nevertheless, in our study, this phenomenon seems to be attenuated from day 10 since microvillus sizes were no longer modified. This could be the result of protection mechanisms against ethanol injury. Indeed, according to previous studies, intestinal cells are able to protect themselves from alcohol injury, involving prostaglandins (*15*, *17*) or growth factor such as EGF (*16*, *45*). It was also shown that adaptative cytoprotection induced by ethanol might exist in Caco-2 cells, involving processes whereby intracellular calcium accumulation is prevented (*46*).

Otherwise, all these experiments were carried out without previous digestion of treatment solutions 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 therefore demonstrate for the first time a stimulating effect of daily exposure to ethanol and polyphenolic free wine matrix on Caco-2 cell morphological differentiation and microvillus elongation, particularly in the first days following confluency and independently of GSE presence.

In addition to these morphological changes, it was shown that chronic treatment with ethanol also increased the activity of sucrase-isomaltase, which is located at the brush border of enterocytes and is a marker of cell differentiation. However, this increase was sligthly attenuated in the presence of WM constituents. In the same way, Malagolini et al. (*23*) had already shown a dose-dependent increase in sucrase activity in Caco-2 cells exposed to ethanol (50−200 mM) for 21 days. Concerning the other brush border markers of differentiation, ethanol presented modulated effects according to the treatment applied. While alkaline phosphatase activity was slightly reduced by ethanol, it was significantly increased in the presence of WM. These results partly corroborated those of Malagolini et al. (*23*), who showed that permanent exposure of Caco-2 cells to 200 mM ethanol for 21 days did not significantly change the activity of alkaline phosphatase. On the other hand, we showed a reducing effect of both EtOH and WM treatments on aminopeptidase N activity. As this activity was expressed as specific activity (units per milligram of protein), we first thought that this decrease was the consequence of the concomitant increase in protein content, but when considering the activity expressed in units per milliliter of cell homogenate (data not shown), we also observed a reduction in activity with both treatments, suggesting that this was induced by alcohol itself. We have therefore shown for the first time that ethanol or polyphenolic free wine matrix could modulate the activities of brush border hydrolases during differentiation, but the mechanism by which they act is not yet well established, and further studies are then needed. Indeed, such compounds could act at the transcriptional or traductional level of enzyme synthesis or on transcellular transport.

Moreover, the stimulatory effect of ethanol on Caco-2 cells was also shown at the cell protein content level. Ethanol regularly increased the protein content of Caco-2 cells over the differentiation period, independently of the presence of GSE. No stationary phase was thus observed, unlike in control cells (*47*−*49*). This clear increase in protein content may have been associated with the activation of the differentiation process described previously, leading to higher expression of microvilli and some hydrolases, but it could also have been the result of the stimulation of cell proliferation multiplying cell number and/or enhanced expression of cytoplasmic proteins. The mechanism has not yet been elucidated, but it was shown in a previous study that 0.1% ethanol induced overexpression of the EGF (epidermal growth factor) receptor in Caco-2 cells (present

mainly in basolateral cell membranes), leading to increased mRNA and protein expression and increased mitotic fate (*50*). The regular increase in proteins observed during the first days of treatment with ethanol in the present study was significantly accentuated with WM, particularly in the presence of GSE. Nevertheless, from day 10 of treatment, and contrary to the pattern noted with ethanol, a stationary phase was observed, suggesting that WM components may reduce the ethanol-induced increase in protein content.

Finally, our results are the first indication that daily exposure of Caco-2 cells to a low concentration of ethanol (1%) could stimulate morphological differentiation, particularly in the first days following confluency. This effect appeared unchanged in the presence of GSE but more marked when ethanol was associated with polyphenolic free wine matrix constituents. This activation, resulting in an increase in microvillus density, organization, and elongation, was associated with stimulation of some hydrolase activities and a concomitant regular increase in protein content. However, these stimulating effects were attenuated from day 10 postconfluency, and we could suggest a cytoprotection of Caco-2 cells to ethanol exposure. Further investigations are required for the understanding of the cell mechanisms involved in this modulation of hydrolase activities taking into account the hypothesis of transcriptional, traductional, or intracellular transport regulations. It appeared therefore that each wine component, i.e. ethanol, polyphenolic free wine matrix, and GSE fraction (*10*), could have a stimulating effect on Caco-2 cell differentiation when applied daily. The possible interactions and synergistic effects between such components will be the aim of further studies.

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