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Apc mutation induces resistance of colonic cells to lipoperoxide-triggered apoptosis induced by faecal water from haem-fed rats

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Recent epidemiological studies suggest that high meat intake is associated with promotion of colon cancer linked with haem-iron intake. We previously reported that dietary haem, in the form of either haemoglobin or meat, promotes precancerous lesions in the colon of rats given a low-calcium diet. The mechanism of promotion by haem is not known, but is associated with increased lipid peroxidation in faecal water and strong cytotoxic activity of faecal water on a cancerous mouse colonic epithelial cell line. To better understand the involvement of faecal water components of haem-fed rats in colon-cancer promotion, we explored the effect of faecal water on normal [adenomatous polyposis coli (Apc) +/+] or premalignant cells (Apc +/-). Further, we tested if this effect was correlated to lipoperoxidation and 4-hydroxynonenal (HNE). We show here for the first time that heterozygote Apc mutation represents a strong selective advantage, via resistance to apoptosis induction (caspase 3 pathway), for colonic cells exposed to a haem-iron-induced lipoperoxidation. The fact that HNE treatment of the cells provoked the same effects as the faecal water of rats fed the haemrich diet suggests that this compound triggers apoptosis in those cells. We propose that this mechanism could be involved in the promotion of colon carcinogenesis by haem *in vivo*.

Abbreviations: Apc, adenomatous polyposis coli; CD, control diet; DMEM, Dulbecco-modified essential medium; HNE, 4-hydroxynonenal; PBS, phosphate-buffered saline; SD, standard deviation; TBARs, thiobarbituric acid reactive substances.

Introduction

Recent epidemiological studies suggest that intake of red and processed meat, loaded with haem iron, is associated with promotion of colon cancer (1–4). In agreement with these epidemiological studies, we previously reported that dietary haem, in the form of either haemoglobin or meat, promotes precancerous lesions, aberrant crypt foci (ACF) and mucindepleted foci in the colon of rats given a low-calcium diet (5,6). Further, we demonstrated that dietary calcium inhibits the haem-induced ACF promotion (5). The mechanism of promotion by haem is not known, but might be linked to oxidative stress and subsequent events such as peroxidation, increased proliferation (7) and genotoxicity (8). Indeed, this haem-induced promotion was associated with increased lipid peroxidation in faecal water and strong cytotoxic activity of faecal water on a cancerous mouse colonic epithelial cell line (5). Moreover, the protection induced by calcium supplementation was associated with a normalization of lipid peroxidation and cytotoxic activity of faecal water.

Lipid peroxidation, and more widely oxidative stress, have been linked to DNA damage in many physiopathological situations, among which is colon carcinogenesis (9). In recent years, numerous studies have emphasized the role of faecal water and lipid peroxidation in colon cancer as diet-related factors (10,11). Faecal water components can induce apoptosis in colon epithelial cells in vitro (12). In the same way, terminal aldehydes of lipid peroxidation [malondialdehyde and 4-hydroxynonenal (HNE)] or hydroperoxides (LOOH) present in faecal water can induce apoptosis and necrosis of human colon carcinoma cells (13). Simultaneously, both phenomenon induce DNA damage and are thus mutagenic to the surviving cells (14). Taken together, these results can at least partly explain the mechanism of haem promotion associated with high lipid peroxidation and cytotoxicity of faecal water. However, up to now, cell line studies have only been performed on human or mouse carcinoma cell lines. These transformed cell lines are poorly suited to investigate the biological effect of promoters in faecal water, since normal or premalignant epithelial cells are the physiological targets of these components (15). Hence, mechanistic conclusions for cancer promotion could not really be extrapolated from these in vitro studies. In colon carcinogenesis, mutations in the adenomatous polyposis coli (Apc) gene on chromosome 5q21 locus are considered to be one of the earliest events in the initiation and promotion of colorectal cancer (16). Apc mutation has been shown to reduce the level of caspases 3, 7 and 9 in mouse colonocytes, leading to a resistance to apoptosis (17). Thus, in vitro studies with normal and Apc-mutated epithelial cells seem more suitable than carcinoma cell lines for drawing conclusions on the involvement of faecal water components in colon-cancer promotion. We recently obtained a new intestinal cell line derived from C57BL/6J mice (Apc +/+) and Min mice (Apc -/+), which retains the heterozygous Apc genotype and the disordered actin cytoskeleton network for the Apc +/- cell lines (18,19). This cellular model can contribute to a better understanding of biological effects of promoters on normal (Apc +/+) or premalignant cells (Apc +/-). The importance of such cell culture systems modelling different stages of tumorigenesis in comprehension of mechanisms by which dietary components impact cancer progression has been recently described by Fenton et al. (20). The aim of this study was to investigate whether a single Apc mutation in normal colon cell lines could modulate the cytotoxicity of faecal water obtained from haem-fed rats (haemoglobin or different types of meat). Furthermore, we explored if this effect was correlated to lipoperoxidation. As HNE is a major by-product of peroxidation of omega-6 fatty acids, we tested its effect under the same conditions to check whether they were the same as those obtained with faecal water from haem-fed rats. We show here for the first time that heterozygote Apc mutation represents a strong selective advantage for normal colonic cells exposed to a lipoperoxidation-related genotoxic environment such as haem iron in excess or HNE.

Materials and methods

Cell lines

Apc +/+ and Apc +/- colon epithelial cells were established as described previously (18,19). Cells harbour a temperature-sensitive mutation of the simian virus 40 large tumour antigen gene (tsA58), under the control of interferon γ . All cells of these mice are 'immortalized', that is, they express active SV40 at the permissive temperature (33°C). Cells were cultured at permissive temperature of 33°C in Dulbecco-modified essential medium (DMEM) supplemented with 10% fetal calf sera, 2% penicillin/streptomycin, 2% glutamin and 10 U/ml interferon γ . The experiments were performed at non-permissive temperature of 37°C, and without interferon γ , to inhibit the SV40 transgene and limit proliferation. Indeed, S-phase (data not shown) decreased respectively from 37.6 and 46.1% at permissive temperature to

6.8 and 9.4% after 24 h at non-permissive temperature for Apc $+/+$ and Apc $-/+$ cells. At non-permissive temperature cell lines can be maintained in culture for 8 days, which is comparable with that of normal epithelial cells.

Animals

Fisher 344 female rats were purchased at 4 weeks of age from Iffa Credo. Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. They were distributed randomly in pairs in stainless-steel wire-bottom cages. The room was kept at a temperature of 22°C on a 12 h light–dark cycle. The rats were allowed 7 days of acclimatization to the room and to a control diet (CD) before being injected intraperitoneally with the carcinogen azoxymethane (Sigma Chemical, 20 mg/kg body wt) in NaCl (9 g/l). Seven days after the injection the rats were allowed free access to their respective diets for 100 days. Three nutritional studies were designed to investigate the effect of diets containing respectively haemoglobin, meats or meats and calcium.

Nutritional experimentations

First study. In a previously published study (5), we tested the effect of haem in the form of haemoglobin on the promotion of colon carcinogenesis. A control group was given a low-calcium (20 mmol/g), low-fat diet. The experimental diets were based on this CD and were balanced for fat (5% safflower oil, rich in omega-6 fatty acids), protein and iron.

Experimental diets were formulated to contain 0.36 and 0.72 mmol/g haemoglobin. Second study. This published study (6) was designed to test the hypothesis that haem in the food matrix can promote colon carcinogenesis. All groups were fed low-calcium (20 mmol/g), high-fat diets balanced for fat (of which 5% safflower oil), protein and iron. Three meat-based diets were formulated to contain varying concentrations of haem by the addition of raw chicken (low haem), beef (medium haem) or blood sausage (high haem). The control (no-haem, CD) diet was supplemented with ferric citrate to match the iron content of beef diet.

Third study. In agreement with previous results (5) demonstrating that calcium prevented the cancer-promoting effect of haem in the form of haemin, we tested the effect of calcium on beef-based diet. Control (CD) and beef diets were identical to diets used in the second study (low-calcium diets). A third beef diet was formulated to contain 250 mmol/g of calcium.

Preparation of faecal water for thiobarbituric acid reactive substances (TBARs) and cytotoxicity assay For assay of TBARs, and cytotoxic activity on epithelial cells, faecal water was prepared from faeces collected for 24 h under each cage of two rats, as described previously (6), but black pudding samples were diluted twice more than the other samples. TBARs were measured in faecal water according to Ohkawa et al. (21), exactly as described previously (6).

Cytotoxicity assay

Cells used for cytotoxicity assay of faecal water were seeded into 96-well culture plates at a seeding density of $3.2 \cdot 10^4$ cells per well in DMEM culture medium. The cells were cultured at 33°C with interferon γ until subconfluence, and transferred at 37°C without interferon γ for 24 h. After 24 h, cells were at confluence and the culture medium was replaced with diluted faecal water in DMEM culture medium (1/10 for all faecal waters and 1/50 for the blood sausage group of Study 2). After adding faecal water, cells were incubated at 37°C for another 24 h. Cells were then washed, 100 μ l of MTT solution [0.45 mg/ml in phosphate-buffered saline (PBS)] was added to each well and the cells were incubated at 37°C for another 4 h. The reaction product was solubilized in 100 μ l lyse buffer (SDS 10%, NaOH 0.1 M) before the colour of reaction product was quantified using a plate reader at 570 nm.

4-Hydroxynonenal quantification in faeces

Pooled 24 h faeces from two rats were homogenized in water (30 ml) and centrifuged twice. The supernatants were pooled and extracted twice with methylene chloride. After evaporation under vacuum, the residue was extracted using isoctane/acetonitrile (1/4, v/v) and the

acetonitrile phase was evaporated under vacuum and then dissolved in phase A of the HPLC system used for analysis. HPLC system (Hewlett-Packard series 1100 with low-pressure quaternary gradient, Hewlett Packard, Labe`ge, France) was equipped with a Spherisorb ODS2 reverse-phase column (25mm · 4.6mm, 5m) and a 100 ml loop and the oven was set at 35°C. UV detection was set at 221 nm and the following elution gradient was used: 100% A for 18 min, then 100% A to 100% B from 18 to 25 min and then 100% B from 25 to 30 min, where A was water/acetonitrile (70/30, v/v) and B was water/ acetonitrile (45/55, v/v), at a flow rate of 1 ml/min. Retention time of HNE was 17.2 min. HNE quantification was done by using a calibration curve. Cell treatment with vitamin E and sodium selenite To document the role of oxidative stress in the cytotoxic effect, cells were cultured at 33°C with interferon g until subconfluence, and transferred in medium without interferon g at 37°C, with or without vitamin E (300 mmol/l) and sodium selenite (500 nmol/l) for 24 h (22). Subsequently, culture medium was replaced with dilutions of faecal water (1/10) with or without vitamin E and sodium selenite. The cytotoxicity assay was used with faecal waters of the control and beef group of the second study.

Cell treatment with 4-hydroxynonenal

To investigate the role of terminal aldehydes of lipid peroxidation in the cytotoxic effect, cells were cultured at 33°C with interferon g until subconfluence and transferred in medium without interferon g at 37°C. Subsequently, culture medium was replaced by different concentrations of HNE (from 10 to 250 mM) diluted in faecal water of the control group of the third study (1/10). Cytotoxicity assays were then performed. Measurement of caspase 3 activity Caspase 3 activity was measured with a caspase 3 colorimetric assay (Sigma). Cells were seeded in 6-well culture plates. After culture at 33°C and transfer at 37°C for 24 h, the medium was replaced with a dilution of faecal water (1/10 in DMEM medium for faecal waters of third study) for 30 min of contact. Cells ($0.5 \cdot 10^6$) were scraped and incubated in 50 ml of lysis buffer (provided by Sigma) for 20 min on ice. After centrifugation, 10 ml of cell lysates were mixed with buffer and caspase 3 substrate (Ac-DEVD-pNA) and incubated at 37°C overnight. Induction of caspase 3 was determined by reading at 405 nm. Caspase 3 activity was determined as mmol pNA released per minute per milligram of protein. Total protein in cell lysates was determined by Bradford assay (Sigma).

Quantification of apoptotic cells

Cells were seeded in F25 culture flask. After culture at 33°C and transfer at 37°C for 24 h, the medium was replaced with a dilution of faecal water (1/10 in DMEM medium for faecal waters of third study) for 12 h of contact. Apoptosis was assessed by flow cytometry with an original method adapted to adherent cells. This method is based on the selective thermal denaturation of apoptotic DNA and detection of denatured DNA with a monoclonal antibody (F7-26) (23). The characteristic of antibody F7-26 is the binding to single-stranded DNA and the absence of reactivity with DNA in doublestranded conformation. The assay is highly specific for apoptotic cells as demonstrated by the absence of reactivity with necrotic cells (24). Formamide-induced DNA denaturation combined with detection of denatured DNA with F7-26 antibody made possible the identification of the apoptotic cells. Cells were resuspended in 250 ml formamide for 5 min at room temperature, heated for 10 min in a water bath at 75°C and cooled for 15 min in a water bath at room temperature. The cell suspension was then incubated with 2 ml 1% non-fat dry milk in PBS for 15 min. After centrifugation, cells were exposed to 100 ml anti-ssDNA Mab (10 mg/ml in PBS containing 5% FCS) for 15 min, rinsed and stained with fluorescein-conjugated anti-mouse IgM (20 mg/ml in PBS containing 1% non-fat dry milk) for another 15 min. After rinsing with PBS, cells were resuspended in 0.5 ml PBS containing 1 mg/ml propidium iodide. Negative controls were treated with mouse IgM instead of the specific primary antibody. Data were analysed with the Expo32 software (Beckman Coulter, France).

Statistics

The results were analysed using Systat 5 software for Windows. Differences between groups were analysed by one-way analysis of variance (ANOVA). When ANOVA showed a statistically significant effect ($P < 0.05$), comparison was made using Dunnett's test, which corrects for multiple comparisons. For viability and caspase 3 test, the effect on Apc $-/+$ cell line was compared with the effect on Apc $+/+$ cell line; for TBARs test, groups were compared with the control group (CD) fed a diet without haem.

Results

To test whether the haem-induced lipid peroxidation affects differentially the viability of normal (Apc $+/+$) or premalignant cells (Apc $-/+$), we analysed the response of these genetically defined cells to faecal water of haem-fed rats. Addition of haem in the form of haemoglobin in the diet of rats induced a strong dose-dependent increase in lipid peroxidation of faecal water indicated by TBARs (Figure 1B). Whereas faecal water of the no-haem diet (CD) did not affect cell viability in spite of an identical iron concentration, normal and premalignant cells displayed different sensitivity to faecal water of haemoglobin-fed rats. The haem-induced lipid peroxidation was associated with specific cytotoxic activity against normal cells (Figure 1A). To test the effect of haem in a food matrix, we supplemented the diet with no-haem meat (chicken) and with haem-rich meat (beef and blood sausage). As for haemoglobin, addition of haem in the form of meat induced specific sensitivity of normal cells (Figure 2A), correlated to increased lipid peroxidation of faecal water (Figure 2B). For the cytotoxicity assay, faecal water of blood sausage group was diluted five times more than other samples, which explains the weakest cytotoxic activity observed in vitro for this group (Figure 2A). As previously observed with haemin (5), we demonstrated that calcium abolished the formation of lipid peroxides in faecal water of beef-fed rats (Figure 3B). In the second study, faecal water of beef-fed rats was cytotoxic only against normal cells. In that study we showed that the supplementation of diets with calcium, which decreased formation of peroxides, abolished the cytotoxic activity against normal cells with no difference in the sensitivity of the two cell lines (Figure 3A). To further explore the impact of lipid peroxidation on cell viability, we attempted to inhibit the oxidative stress induced by supplementation of the culture medium with protective antioxidants (vitamin E and selenium). As expected, without supplementation of the culture medium, faecal water of control-fed rats (no-haem diet, CD) had no differential effect on cellular viability, while faecal water of beef-fed rats was cytotoxic against normal cells (Figure 4A). The significant difference between normal and Apc-mutated cells was abolished by the addition of antioxidant agents to the culture medium: indeed the protection against oxidative stress by the supplementation of culture medium with selenium and vitamin E water showed moderated effect of beef-fed rats' faecal water on cell loss, specifically on normal cells (Figure 4B).

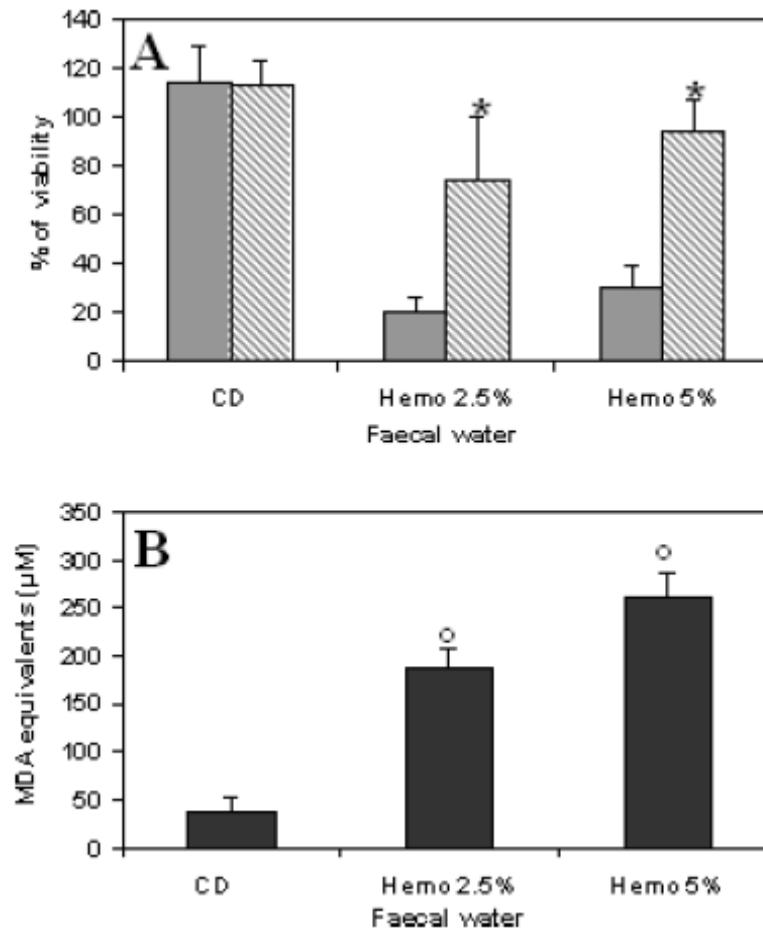


Fig. 1. Effect of haemoglobin diets on cytotoxicity and TBARs of faecal water. (A) Cytotoxic effect of faecal waters on cellular viability of Apc +/+ (solid bars) and Apc +/- (hatched bars) epithelial cell lines, after 24 h of incubation. *Significantly different from Apc +/+ cell line for the same faecal water ($P < 0.01$, by Dunnett's test). (B) TBARs (MDA equivalents) in faecal waters as marker for lipid luminal peroxidation. °Significantly different from CD ($P < 0.01$, by Dunnett's test). Data are mean \pm standard deviation (SD) ($n = 3$).

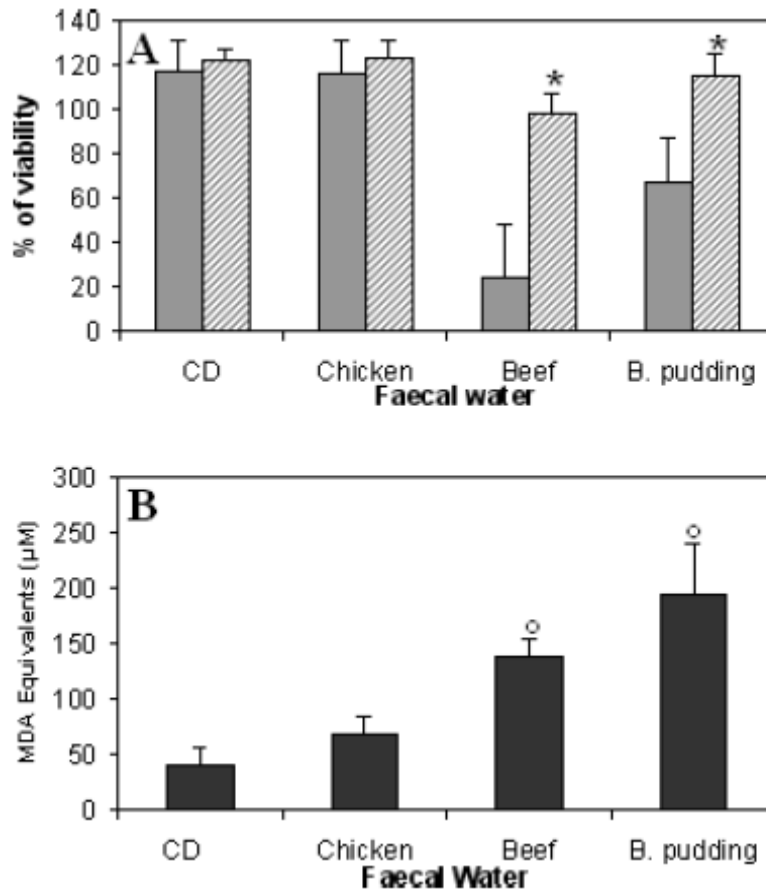


Fig. 2. Effect of meat diets on cytotoxicity and TBARs of faecal water. (A) Cytotoxic effect of faecal waters on cellular viability of Apc +/+ (solid bars) and Apc +/- (hatched bars) epithelial cell lines, after 24 h of incubation. *Significantly different from Apc +/+ cell line for the same faecal water ($P < 0.05$, by Dunnett's test) (B) TBARs (MDA equivalents) in faecal waters as marker for lipid luminal peroxidation. ^oSignificantly different from CD ($P < 0.05$, by Dunnett's test) Data are mean \pm SD (n $\frac{1}{4}$ 3).

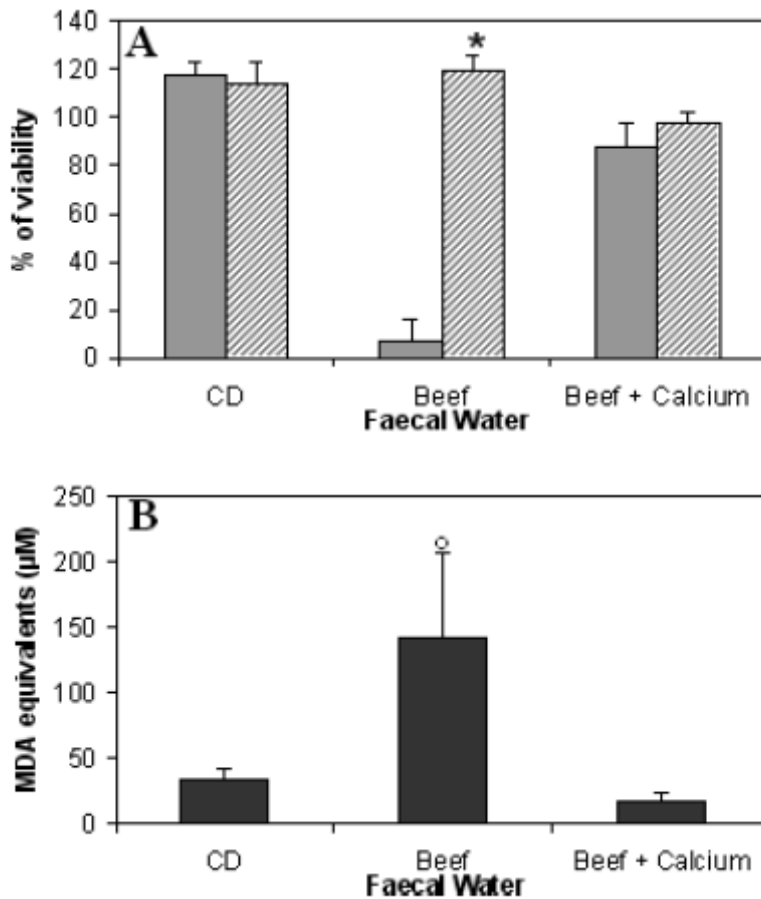


Fig. 3. Effect of beef supplemented with calcium diets on cytotoxicity and TBARs of faecal water (A) Cytotoxic effect of faecal waters on cellular viability of Apc +/+ (solid bars) and Apc +/- (hatched bars) epithelial cell lines, after 24 h of incubation. *Significantly different from Apc +/+ cell line for the same faecal water ($P < 0.05$, by Dunnett's test). (B) TBARs (equivalent MDA) in faecal waters as marker for lipid luminal peroxidation. °Significantly different from CD ($P < 0.05$, by Dunnett's test). Data are mean \pm SD (n $\frac{1}{4}$ 3).

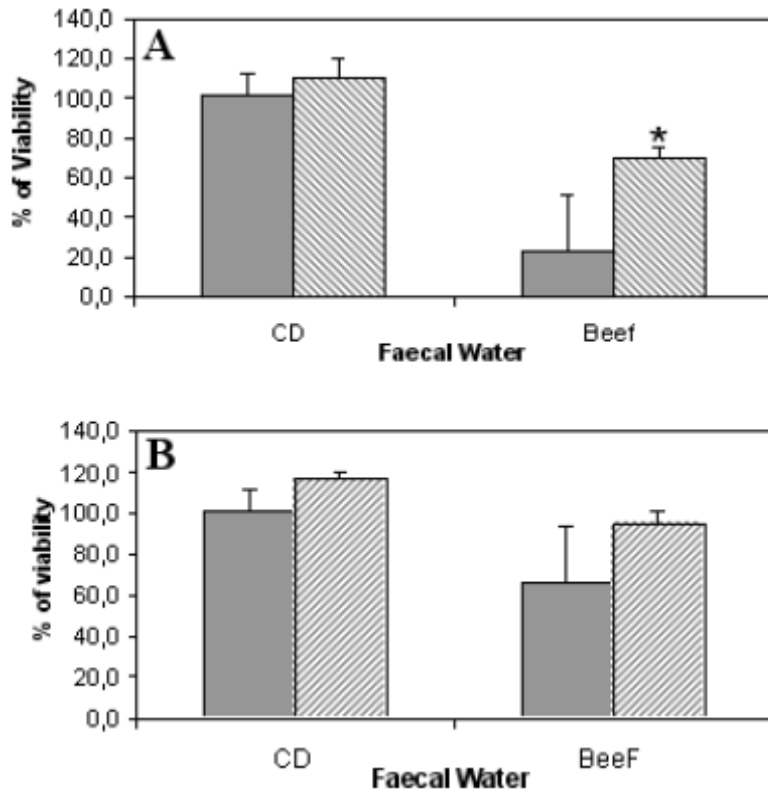


Fig. 4. Effect of treatment of epithelial cells with vitamin E (300 mmol/l) and sodium selenite (500 nmol/l) on cytotoxicity of control and beef faecal waters on cellular viability of Apc +/+ (solid bars) and Apc +/- (hatched bars) epithelial cell lines, after 24 h of incubation. (A) Without vitamin E and sodium selenite. (B) With vitamin E and sodium selenite. *Significantly different from Apc +/+ cell line for the same faecal water ($P < 0.05$, by Dunnett's test). Data are mean \pm SD (n $\frac{1}{4}$ 3).

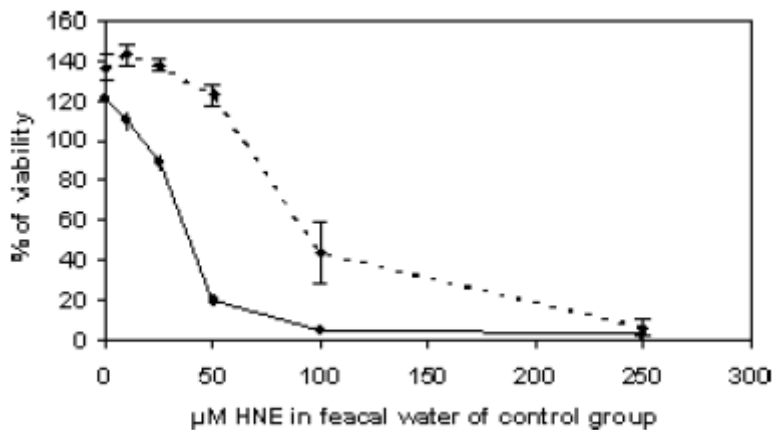


Fig. 5. Effect of treatment of epithelial cells with different concentrations of HNE (from 10 to 250 mM) on cellular viability of Apc +/+ (solid line) and Apc +/- (hatched line) epithelial cell lines, after 24 h of incubation. Data are mean \pm SD (n $\frac{1}{4}$ 3).

Table I. Quantification of haem and HNE in faeces

	Control diet	Beef + calcium diet	Beef diet
HNE in faeces (ng/24 h)	94.4 ± 96.7%	132.1 ± 70.2%	346.4 ± 5.6%* ^o

Values are mean of three determinations of 24 h pooled faeces from two rats. *Different from CD using Dunnett's test ($P < 0.05$). ^oDifferent from CD and beef + calcium diet using Tukey's test ($P < 0.05$).

Lipid peroxidation yields end-products such as alkanes, aldehydes and isoprostanes. One of the major end-products of lipid peroxidation is HNE. We thus explored the dose effect of HNE in culture medium on normal and premalignant cells. As shown in Figure 5, the treatment of epithelial cells with HNE reduced the cellular viability of the two cell lines, but with a particularly strong effect on normal cells. As shown in Table I, HNE has been found in the faeces of rats, almost three times more in beef-fed rats as compared with beef + calcium-fed rats. As faecal water can induce apoptosis in colon carcinoma cells with an induction of caspase 3 and Apc mutation has been shown to reduce the caspase 3 level, we tested the inducibility of caspase 3 in normal and premalignant cells treated with faecal water of control (CD), beef and beef + calcium-fed rats. Figure 6 shows the induction of caspase 3 on the normal cells treated with faecal water rich in lipid peroxides (beef-fed rats). For the faecal water with little lipid peroxidation (control and beef + calcium), no induction of caspase 3 was observed. For the premalignant cells, a similar lack of induction of caspase 3 was observed for control and beef + calcium-fed rats' faecal waters. Interestingly, no induction was observed for the treatment with beef-fed rats' faecal water in premalignant cells.

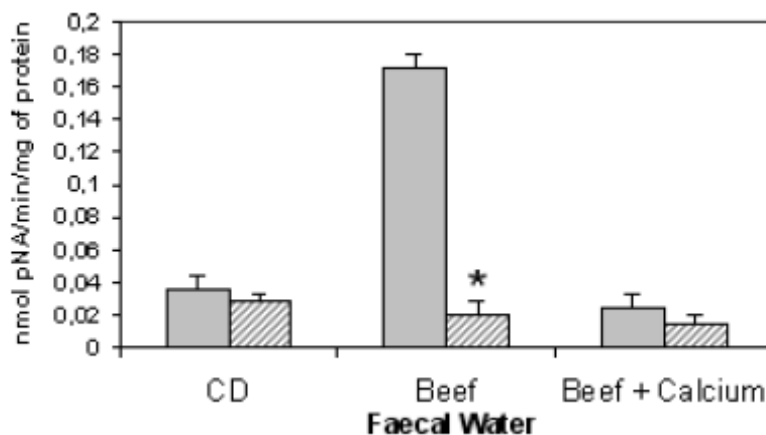


Fig. 6. Caspase 3 activity of Apc +/+ (solid bars) and Apc +/- (hatched bars) epithelial cell lines, after 30 mn of incubation with control, beef and beef supplemented with calcium faecal waters. □ Significantly different from Apc +/+ cell line for the same faecal water ($P < 0.05$, by Dunnett's test). Data are mean ± SD ($n = 3$).

In order to conclude on the contribution of apoptosis to cytotoxic activity of faecal water of beef-fed rats against normal cells and its contribution to difference in the sensitivity of the two cell lines, apoptosis was assessed by flow cytometry with the anti-ssDNA Mab. Figure 7B shows a strong induction of apoptosis ($76.90 \pm 2.33\%$) on the normal cells treated with faecal water of beef-fed rats. Interestingly, a significantly weaker induction was observed for the treatment of premalignant cells ($27.79 \pm 5.19\%$, $P < 0.05$). Figure 7C shows that the

supplementation of diets with calcium abolished significantly apoptosis of normal cells ($4.75 \pm 0.92\%$ for beef + calcium diet versus $76.90 \pm 2.33\%$ for beef diet, $P < 0.05$).

Supplementation of diets with calcium has no significant effect on apoptosis of premalignant cells (Figure 7D).

Discussion

The aim of this work was to document the action of faecal water prepared from meat-fed rats on normal and Apcmutated heterozygote mouse colonocytes. We showed here that normal and early premalignant colonocytes gave results that correlate well with previous in vivo studies in which meat promoted pre-neoplastic lesions (5,6). We confirmed the previously described cytotoxicity of faecal water prepared from haemoglobin-treated rats against normal cells. This cytotoxicity was correlated with the amount of haem in the diet, and was reproducible using different meats with different haem contents, chicken being the least effective and blood sausage the most (Figures 1 and 2). All the diets were equilibrated in iron, and white meat gave negative results: this being a specific effect of haem iron rather than free iron. A direct correlation was observed between the cytotoxicity of the faecal water and the production of TBARs (Figures 1 and 2). Moreover, TBARs were normalized and the cells correlatively protected by calcium (Figure 3) inactivating haem-induced lipoperoxidation by haem complexation (5,25). In the same way, limitation of haem-induced oxidative stress by antioxidant supplementation in medium culture (vitamin E and sodium selenite) protected the cells (Figure 4). This showed that a strong oxidative stress occurring in the faecal water of haem-fed animals was the main origin of the cytotoxic effects observed on normal cultured cells.

Excess haem-iron intake and promotion of colon carcinogenesis

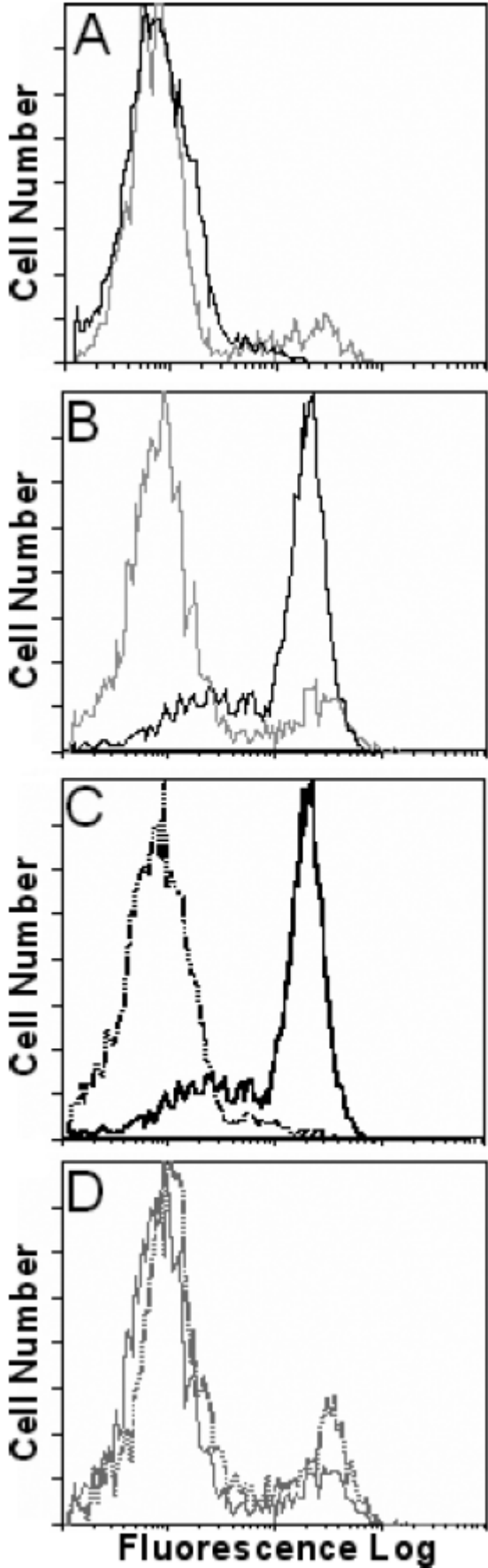


Fig. 7. Apoptosis of Apc +/+ (black lines) and Apc +/- (grey lines) epithelial cell lines, after 12 h of incubation with beef and beef supplemented with calcium faecal waters. Apoptosis was detected by flow cytometry using anti-ssDNA antibody. (A) Effect of Apc mutation on apoptosis of colon epithelial cells upon control faecal water exposure (Apc +/+ : black line/ Apc +/- : grey line). (B) Effect of Apc mutation on apoptosis of colon epithelial cells upon beef faecal water exposure (Apc +/+ : black line/ Apc +/- : grey line). (C) Effect of beef and beef supplemented with calcium diets on apoptotic effect of faecal waters on Apc +/+ epithelial cells (beef: black line/beef + calcium: black hatched line). (D) Effect of beef and beef supplemented with calcium diets on apoptotic effect of faecal waters on Apc +/- epithelial cells (beef: grey line/beef + calcium: grey hatched line). Data are mean ± SD (n = 3).

Our results compared for the first time the action of haem-induced oxidative stress on normal and single allele Apc-mutated colonocytes. The single Apc mutation rendered the cells strongly resistant to haem-induced cytotoxicity. This gave a clear survival advantage to these cells when they were subjected to the action of an oxidative stress, which at the same time can act as a selective factor. Indeed, only faecal water of haem-fed rats with high TBARs presented cytotoxic activity against Apc +/+ cells (Figures 1 and 2) but not against Apc +/- cells. Furthermore, inhibition of this oxidative stress by calcium (Figure 3) in diet, or antioxidants in medium culture (Figure 4), normalized this difference in sensitivity. When we correlated this difference in sensitivity with in vivo results on pre-neoplastic lesions, we observed that only promoting diets (haemoglobin and red meats) were associated with this difference in sensitivity while non-promoting diets (control, chicken and beef supplemented with calcium) were not. Hence, oxidative stress was most probably the main initial event leading to early promotion of pre-tumoural lesions in vivo in rats receiving a haem-iron-rich diet (5,6). In agreement with a recent work (17), we suggest that the main cause of this Apc-linked resistance was a lack of caspase induction, unpairing apoptosis. Our results show that caspase 3 induction was triggered by beef-fed rats' faecal water only in wild cells but not in Apc +/- cells (Figure 6). The assessment of apoptosis by flow cytometry with the anti-ssDNA Mab indicated that this phenomenon is sufficient to account for the decrease in viability in wild cells (Figure 7C) and for the difference of sensitivity between the two cell lines (Figure 7B). The suppression of haem-induced oxidative stress by calcium supplementation also rendered faecal water inefficient, with respect to apoptosis and caspase induction. As the assessment of apoptosis was carried out with a procedure that specifically stains apoptotic cells and thus distinguishes apoptosis from necrosis, and as we obtained, in the present work, $76.90 \pm 2.33\%$ of staining (for normal cells with beef faecal water), we can exclude a role of necrosis in the cytotoxic effect observed. As our experimental diets were rich in haem and omega-6 fatty acids, we explored the effect of HNE, a major by-product of omega-6 fatty acid peroxidation. We showed in a previous work that HNE was present in large quantities in the diets used for the present study. We also showed that the urinary excretion of its major end-metabolite (the 1,4-dihydroxynonane mercapturic acid) was dramatically increased in the rats fed the haem-rich diets, indicating that HNE was present in large amounts (F.Pierre, G.Peiro, S.Tache, A.J.Cross, S.Bingham, N.Gasc, D.E.Corpet, and F.Gue'raud, manuscript in preparation). In the present work, we show that HNE concentration is increased in faeces from beef-fed rats but not from beef + calcium-fed rats as compared with faeces from rats fed the CD. The fact that HNE treatment of the cells provoked the same effects as the faecal water of rats fed the haem-rich diet suggests that this compound triggers apoptosis in wild cells. Indeed, this compound has previously been shown to induce apoptosis in many cell lines, and some authors have shown that this induction was through caspase 3 activation (13). HNE has been suggested to be the key-mediator of oxidative stress-induced cell death (14). All these data suggest that this compound, or other hydroxyalkenals coming from lipid peroxidation process, could be the link between oxidative stress and selection of Apc-mutated cells. Taken together, our present data strongly suggest that the main cytotoxicity of the faecal water leads to apoptosis, and that apoptosis is suppressed in Apc +/- cells. In addition to its cytotoxic consequences, oxidative stress has been described as a major event of mutagenic DNA damage in colon cancer (26). Variations in diet are also a modulating factor of oxidative DNA damage in colonocytes (27–29). Thus, oxidative stress produced in faecal water by consumption of meat and meat-related products can be regarded as a cytotoxic event for normal cells, a selective event for premalignant cells and also a genotoxic, mutation-prone event.

By grouping the results of present study with former in vivo studies on rats (5,6) by our group and with other results published in the literature on epidemiological studies (1,3), we are now

able to propose a model explaining the increase in the risk of colonic cancer due to excess consumption of meat products in humans:

(i) Red meat and related products induce an oxidative stress in faecal water, at least in rats. The richer the diet in haemoglobin, the stronger the stress and the cellular consequences.

(ii) The cellular effects of this oxidative stress are lipid peroxidation, genotoxicity and apoptosis in most cells. Apoptosis may be triggered by HNE, suggesting that omega-6 fatty acids play a key role.

(iii) Some colonic cells mutated in Apc are resistant to apoptosis and can survive the contact of the cytotoxic faecal water, enabling them to undergo further mutations and follow a tumoural pathway.

(iv) The consequences of these regimens have been observed in rats as early precancerous damages (6) and in humans as an increased colon-cancer risk factor (1).

One could argue that, owing to its specific physiological diet, the rat represents a poorly fitted model to test an overloaded meat diet. Excess of meat in species such as rodents could indeed give rise to some specific toxic pathways that are possibly not reproducible in other species. However, epidemiological studies in humans and the universal mechanism of the generation of oxidative stress by haem-iron suggest that the consequences could be very similar in humans, which remains to be addressed. In conclusion, accumulated evidence allows us to propose that excess haem-iron intake is a risk factor for colon carcinogenesis in human diets and that this risk is highly synergized in some previously Apc-mutated colonocytes, even on a single allele.

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Conflict of Interest Statement: None declared.

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