Red meat and colorectal cancer: Nrf2-dependent antioxidant response contributes to the resistance of preneoplastic colon cells to fecal water of hemoglobin- and beef-fed rats

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Abstract

Epidemiological studies have associated red meat intake with risk of colorectal cancer. Experimental studies explain this positive association by the oxidative properties of heme iron released in the colon. This latter is a potent catalyst for lipid peroxidation, resulting in the neoformation of deleterious aldehydes in the fecal water of heme-fed rats. The toxicity of fecal water of heme-fed rats was associated to such lipid peroxidation. This study demonstrated that fecal water of hemoglobin- and beef-fed rats preferentially induced apoptosis in mouse normal colon epithelial cells than in those carrying mutation on Apc (Adenomatous polyposis coli) gene, considered as preneoplastic. Highlighting the importance of lipid peroxidation and neoformation of secondary aldehydes like 4-hydroxy-2-nonenal (HNE), we optimized the depletion of carbonyl compounds in the fecal water which turned out to abolish the differential apoptosis in both cell lines. To explain the resistance of preneoplastic cells towards fecal water toxicity, we focused on Nrf2, known to be activated by aldehydes, including HNE. Fecal water activated Nrf2 in both cell lines, associated with the induction of Nrf2-target genes related to aldehydes detoxification. However, the antioxidant defense appeared to be higher in preneoplastic cells, favoring their survival, as evidenced by Nrf2 inactivation. Taken together, our results suggest that Nrf2-dependent antioxidant response was involved in the resistance of preneoplastic cells upon exposure to fecal water of hemoglobin- and beef-fed rats. This difference could explain the promoting effect of red meat and heme-enriched diet on colorectal cancer, by initiating positive selection of preneoplastic cells.

Introduction

Colorectal cancer is the first cause of global mortality by cancer in non-smokers in affluent countries (1). Epidemiological studies proposed that physical activity and diet are important factors for colorectal cancer development (2–4). Concerning the diet, based on epidemiological and experimental studies, the International Agency for Research on Cancer (IARC) monograph...
cer (which is a frequent and early event in human colorectal cancer) by guest

ized mouse colonic epithelial cells derived from wild-type and Hb- and Bf-fed rats in normal and preneoplastic immortalized cell lines (21). We previously tested that different resistance between normal and preneoplastic cells upon exposure to HNE could be due to higher basal expression of HNE detoxification enzymes in preneoplastic cells compared with normal cells (27), giving preneoplastic cells a better antioxidant shield to tackle toxic effects of HNE. As the cytotoxicity of fecal water of heme-fed rats was associated to lipid peroxidation, Nr2f could be involved in the resistance of preneoplastic cells upon exposure to fecal water.

In this study, we firstly characterized cell death type as apoptosis and compared its induction in normal and preneoplastic cells regarding fecal water of Hb- and Bf-fed rats, in comparison to HNE. After treatment with fecal water or HNE, preneoplastic cells appeared to exhibit lower level of apoptosis compared with normal cells. Moreover, HNE and fecal water activated Nr2f in both cell lines and the depletion of carbonyl compounds in fecal water prevented this event. The resistance of preneoplastic cell was correlated to a higher Nr2f-dependent response compared with normal cells, as evidenced by Nr2f invalidation. Thus, this study demonstrated the importance of fecal carbonylated secondary lipoperoxidation products in explaining the differential apoptosis between normal and preneoplastic cells following exposure to fecal water of Hb- and Bf-fed rats.

Materials and methods

Cell culture

Normal wild-type and Apc-mutated colon epithelial cells (later called normal and preneoplastic cells) were established as described previously (21). Both cell lines express the heat-labile simian virus 40 large tumor antigen gene (AgT tsAS8) under the control of interferon-γ and are ‘immortalized’, that is, they expressed active SV40 at permissive temperature (33°C). They have been tested (as published in 2014 (10)) through the characterization of Apc genotype (presence of Min mutation in preneoplastic cells), Apc phenotype (destructured actin network in preneoplastic cells) and epithelial phenotype (expression of cytokeratin 18 in normal and preneoplastic cells).

Cells were cultured (approximate density 6.0 x 10^5 cells/cm²) at permissive temperature of 33°C in Dulbecco-modified essential medium (DMEM) supplemented with 10% fetal calf sera, 1% penicillin/streptomycin, 2% glutamine, 100U/ml interferon γ and 10U/ml epidermal growth factor. All experiments were performed at non-permissive temperature of 37°C without interferon γ and epidermal growth factor to inhibit the SV40 transgene and to reach differentiated state. The treatments with HNE and fecal water were performed in serum-free DMEM containing 2% glutamine to avoid any reaction between serum and fecal water or HNE.

HNE synthesis

HNE was synthesized as described previously (28). Briefly, Grignard reaction between fumaraldehyde monoacetate and 1-pentylmagnesium bromide afforded HNE-dimethylacetal. HNE was obtained by acid hydrolysis.

Fecal samples

Feces used to generate the fecal water were obtained from two animal studies set up to explore, respectively, the effect of dietary Hb (10) and Bf on colorectal carcinogenesis (not published). Briefly, 7 days following

| Abbreviations | Description |
|---------------|-------------|
| ALDH3A1       | aldehyde dehydrogenase 3A1 |
| Apc           | adenomatous polyposis coli |
| ARE           | antioxidant response element |
| Bf            | beef |
| CTR           | carbonyl-trapping resin |
| GSTA4         | glutathione S-transferase A4 |
| Hb            | hemoglobin |
| HNE           | 4-hydroxy-2-nonenal |
| HO1           | heme oxygenase 1 |
| Keap1         | Kelch-like ECH-associated protein 1 |
| MDF           | mucin-depleted foci |
| Nr2f          | nuclear factor (erythroid derived 2)-like 2 |
| PBS           | phosphate-buffered saline |
| TBARS         | thiobarbituric acid reactive species |
| xCT           | x-C-type cystine/glutamante antiporter |
an injection with the carcinogen azoxymethane (Sigma Aldrich, 20 mg/kg body wt in NaCl (9/0.9)). F344 male rats were allowed free access to their respective diets for 100 days. In the first experiment, rats were fed with modified AIN-76 diet [low calcium with addition of 5% (w/v) safflower oil; control group] or with modified AIN-76 diet containing 1% (w/w) HB (Hb-fed group) [10]. In the second experiment, rats were fed with modified AIN-76 diet (control group) or with 50% modified AIN-76 diet containing 50% raw Bf sirln. Feces were collected on the 88th and 95th day of experiment for Hb-fed and Bf-fed rats, respectively. The two studies resulted in four groups of collected feces: standard diet for Hb-fed rats, Hb-fed rats, standard diet for Bf-fed rats and Bf-fed rats. All experiment protocols were approved by the Local Animal Care and Use Committee of Instituto Nacional de la Recherche Agronomique (TOXCOM), and were conducted in accordance with the European directive 2010/63/UE and ARRIVE guidelines.

**Fecal water preparation and depletion of carbonyl compounds in fecal water**

Fecal water was prepared as described previously [10]. Briefly, 2 g of feces was diluted with 5 ml of distilled water, ground and centrifuged. The supernatant was collected, diluted 10 times in DMEM containing glutamine 2% and sterilized by microfiltration (pore size 0.2 μm).

4-Fmoc-hydrazinobenzoyl AM NovaGel™ (Novabiochem®) is a polymeric resin grafted with fomic-protected hydrazine functionality. Carbonyl compounds are known to readily react with hydrazine groups to yield hydrazones. Therefore, this resin, named carbonyl-trapping resin (CTR) in this work was used for carbonyl compounds depletion as described previously [10]. Briefly, following activation of the resin using 500 μl of piperidine 20% (v/v) in DMSO for 5 min of resin and repeated washes, fecal water was treated with the activated resin at 4°C for 2 h under agitation.

**TBARS and free HNE assay**

TBARS value was used as a global analysis of lipid peroxidation endproducts. TBARS in fecal water were analyzed as described previously [29]. For free HNE assay, sample preparation was realized as described previously [30] and adapted for free HNE in fecal water. Briefly, HNE present in fecal water (400 μl) was labeled and reduced into [H][DHN] by NaBH₄ (0.2 mmol) in EDTA (50 mM)/Hepes (2 mM). [H][DHN] (0.8 nmol) was added as internal standard. Following reduction of double bonds in the presence of Raney nickel, HNE was extracted with ethyl acetate and dried with Na₂SO₄. Analyses [31,32] were carried out on a triple quadrupole Quattro Micro mass spectrometer (Waters Corporation, UK) coupled with a gas chromatography Agilent 6890N system (Agilent Technologies, USA).

**Apoptosis detection: nuclei fragmentation, caspase activation by time-lapse microscopy and annexin V-propidium iodide (PI) staining by flow cytometry**

For nuclei fragmentation assay, cells seeded in 24-well plates were incubated for 6 h of contact with HNE 40 μM and fecal water (dilution 10x) at 37°C. Cells were then fixed in paraformaldehyde 4% and stained using fluorescent dye Hoechst 33342 (Life Technologies, 0.5 ng/ml in phosphate-buffered saline (PBS)). Apoptotic (fragmented and/or condensed) and alive nuclei were counted using fluorescence microscope (EvaS fluorescent inverted microscope, AMG) and expressed as percentage of total population (n > 500 nuclei).

For time-lapse microscopy, cells seeded in Labtek 8-well chamber 1.5 borosilicate coverglass slides were incubated under confocal microscopy Leica SP8 (with micro-incubator at 37°C, 5% CO₂) with HNE 40 μM and fecal water (dilution 10x) containing Hoechst 33342 (5 μg/ml), CellEvent™ Green 3-Iodide Detection Reagent (Life Technologies, 1 μM) and TO-PRO-3 iodide (Life Technologies, 1 μM), Images were acquired using lasers 405, 488 and 638 nm under 20x magnification every 30 min for 12 h. Image analysis was performed using ImageJ software.

For flow cytometry, cells seeded in 12-well plates were incubated for 6 and 24 h in the presence of HNE 40 μM or fecal water (dilution 10x) at 37°C. Cells were then harvested and stained using APC Annexin V apoptosis detection kit with PI (Biolegend, 50 ng/ml) according to manufacturer’s procedure. The flow cytometry was done using VenturiOne software (Applied Cytometry).

**siRNA transfection**

siRNAs were provided by Thermo Scientific Dharmacon (Courtabeuf, France). At 37°C, medium was replaced by OptiMEM and cells were transfected overnight with 100 pmole Nrf2-specific siRNA (ON-TARGET plus SMART pool, mix of four siRNA, 100 pmole lamin-specific siRNA (siGLO Lamina/C control siRNA) as control of transfection rate or 100 pmole negative control siRNA (ON-TARGET plus siRNA Smart pool Non targeting control pool) according to manufacturer’s instructions. The transfection rate was more than 75% in each cell type after 18h of transfection. The medium was then replaced by non-permissive medium and the cells were incubated at 37°C for 24 h prior to treatments.

**Immunofluorescence staining of Nrf2**

Cells seeded on round coverslips (diameter 18mm) in 12-well plates were incubated for 1h of contact with HNE 40 μM or fecal water (dilution 10x) at 37°C. Following saturation (PBS, 0.1% Triton, 0.5% bovine serum albumin), each coverslip was incubated with primary antibodies directed against Nrf2 (Santa Cruz rabbit polyclonal IgG (33), 2.5 μg/ml in PBS, 0.1% Triton, 0.5% bovine serum albumin) for 1h at room temperature. After washing with PBS, the cells were incubated with secondary fluorophore-labeled antibodies (Invitrogen Alexa Fluor 488 (green) donkey anti-rabbit IgG, 10 μg/ml in PBS, 0.1% Triton, 0.5% bovine serum albumin) for 1h at room temperature. All cover slips were then sealed on microscope slides using anti-fading solution (Invitrogen Prolong Gold antifade reagent with DAPI). The slides were analyzed using confocal microscope Leica SP8 with lasers 488 nm (Alexa 488) and 405 nm (DAPI) under 63x oil immersion objective. The localization of Nrf2 and nuclei was observed by using fluorescence intensity graphs presenting the intensity of blue (nuclei) and green (Nrf2) fluorescence detected along determined regions of interest (ROI). Nuclear localization of Nrf2 was deduced from blue and green fluorescence pattern.

**RT-qPCR**

Cells were incubated for 4h of contact with HNE 40 μM or fecal water (dilution 10x) at 37°C. RNA was isolated using TRI Reagent (Euorgenetec) according to manufacturer’s instructions. Two-step RT-qPCR was performed using TBP (TATA binding protein) as the housekeeping gene. cDNA synthesis was done using the iScript cDNA Synthesis kit (Bio-Rad) with 1 μg RNA. Quantitative PCR was conducted using VFLA™ 7 Real-Time PCR System (Life Technologies), with final reaction volume of 5 μl (1 μl cDNA, 1.5 μl primers and 2.5 μl IQ SYBR Green Supermix (Bio-Rad). Thermocycling conditions were set as following: initial denaturation at 95°C (10 min), denaturation at 95°C (15 s), annealing at 60°C (30 s) and extension at 72°C (30 s). The sequences of the primers (5’-3’ used are listed below: H01 (F-CAA CCC CAC CAA GAT CAA ACA, R-AGG CGG TCG TAG CCT CTT CTG A), ALDH5A1 (F-AAA GTA GCC CAT GGA GGC AC, R-GTC ATT GCC TAT CAC CCC AC), XCT (F-CCT GGC ATT TGG ACA CTG CAT, R-TCA GAA TGG CTG TGA GGT TGC A), GSTA4 (F-AGA CTT CTA GCA ACC TCC TTT T, R-TGG AGC TGC TGG TAT GTC TGT), TBP (F-AGA ACA ATC ACT ACC AGG A, R-GCG AAC TCC ACA TCA CAG CTC).

**Western blot of Nrf2**

Cells were incubated for 1h with HNE 40 μM or fecal water (dilution 10x) at 37°C. Whole cell extracts were fractionated using Nuclear Extraction kit (Active Motif). Western blot of nuclear extracts (10 μg) was performed by SDS-PAGE and blots were transferred to nitrocellulose membranes. Membranes were then saturated and incubated with primary antibodies against Nrf2 (Cell Signaling rabbit polyclonal IgG (34), dilution 1:1000) and lamin A/C (Sigma Aldrich mouse monoclonal IgG (35), dilution 1:5000) at 4°C for a night under agitation. Fluorescent secondary antibodies (Thermo Scientific Pierce DyLight 800 donkey anti-rabbit IgG and Thermo Scientific Pierce DyLight 800 donkey anti-mouse IgG, 0.25 μg/ml) were incubated for 1h and membranes were revealed using Odyssey® CLX Li-Cor Infrared Imaging System at wavelengths of 700 and 800 nm.

**Detection of HNE adducts**

Cells were incubated for 6h of contact with HNE 40 μM at 37°C. Whole cell extracts were fractionated using Nuclear Extraction kit (Active Motif). HNE-histidine adducts in cytoplasmic fraction were measured as previously described [36] using anti-HNE-his mouse monoclonal primary antibody generously given by Drs G. Weg and N. Zarkovic (Laboratory for Oxidative Stress, Zagreb, Croatia) [37,38].
Figure 1. Fecal water of Hb- and Bf-fed rats (Hb and Bf) exerted higher toxicity towards normal cells compared with preneoplastic cells. Cells were treated with HNE 40 µM and fecal water. Control Hb and Control Bf (respective controls for Hb and Bf) were fecal water issued from standard diet-fed rats. Apoptosis was observed by detecting caspase-3/7 activity and membrane permeability using time-lapse microscopy and by APC Annexin V/propidium iodide (PI) flow cytometry. (A) Measures of the percentage of cells exhibiting caspase-3/7 activity upon exposure to fecal water recorded over time. (B) Percentage of cells with positive caspase-3/7 activity and (C) permeable membrane upon treatments with HNE 40 µM and fecal water (t = 6h). (D) Repartition of cell population exhibiting positive and negative staining of APC Annexin V and PI upon treatments with HNE 40 µM and fecal water (t = 6 and 24h). *Significant difference between normal and preneoplastic cells (P < 0.05). #Significant difference between a treatment (HNE, Hb, Bf) and its respective control (Control, Control Hb, Control Bf) (P < 0.05). †Significant difference between unstained (Annexin V(-) PI(-)) normal and preneoplastic cells (P < 0.05). Data are expressed as mean ± SD (n = 3).
Cystine uptake assay

L-[¹⁴C]-Cystine (PerkinElmer, 100 mCi/mmol) uptake was measured according to Giraudi et al. with some modifications (39). Cells were seeded in a 12-well plate and incubated for 6h of contact with fecal water (dilution 10×) at 37°C. The cells were then rinsed with warmed uptake buffer (NaCl 140 mM, HEPES 25 mM, KCl 1.8 mM, MgSO₄ 0.8 mM, glucose 5 mM, pH = 7.5). Cystine uptake was started by incubating the cells in uptake buffer containing L-[¹⁴C]-Cystine 0.1 µCi at room temperature for 10 min, and stopped by rinsing with ice-cold unlabelled uptake buffer. The cells were then lysed by adding NaOH 0.1 N. Lysate was mixed with scintillation cocktail (UltimaGold, PerkinElmer), protein quantification was done (Pierce bicinchoninic acid enzymatic kit) and the radioactivity was determined using a scintillation counter (Hewlett Packard). The results were expressed as nmol of incorporated cystine reported to protein content.

Statistical analysis

The results (n ≥ 3) were analyzed using software GraphPad Prism 4 for Windows. Different responses of treatments and genotype effect were analyzed by two-way ANOVA and student’s t-test, respectively. When ANOVA showed a statistically significant effect (P < 0.05), comparison among data was done using Tukey’s HSD Post-hoc test. Pearson correlation test was done to analyze the correlation between the percentage of apoptotic nuclei and TBARS or free HNE concentration in fecal water.

Results

Fecal water of Hb- and Bf-fed rats induced cell death by apoptosis and preneoplastic cells exhibited higher resistance compared with normal cells

To characterize cell death, we analyzed caspase-3/7 activation, membrane phosphatidylserine translocation to the outer leaflet and cell membrane permeabilization (Figure 1A–D; Supplementary Movies 1–4, available at Carcinogenesis Online) in normal and preneoplastic cells following exposure to fecal water, according to the guidelines of apoptosis identification (40). Fecal water induced apoptosis in both cell lines (Figure 1A) but at a much higher extend when diets were supplemented with Hb or Bf. The differential of apoptosis in normal and preneoplastic cells was observed only upon exposure to fecal water of Hb- and Bf-fed rats, but not to the fecal water control issued from rats fed with standard diet. Compared to preneoplastic cells, normal cells exhibited earlier and higher apoptosis upon exposure to fecal water of Hb- and Bf-fed rats.

Following 6h of exposure to fecal water of Hb- and Bf-fed rats, approximately 50% of normal cells population exhibited caspase-3/7 activation and membrane permeabilization while only approximately 30% of preneoplastic cells population exhibited the same phenomena (Figure 1B and C). The number of unstained living cells was higher in preneoplastic cells than in normal cells even at a longer incubation time of 24h (Figure 1D). HNE was used in our experiments as positive control for apoptosis induction by lipid peroxidation byproducts. Like HNE, fecal water of Hb- and Bf-fed rats induced significantly higher apoptosis in normal cells than in preneoplastic cells (P < 0.05). This difference was not observed upon exposure to fecal water control.

Depletion of carbonyl compounds in fecal water of Hb- and Bf-fed rats abolished differential apoptosis in normal and preneoplastic cells

To support the fact that lipid peroxidation byproducts are involved in fecal water toxicity towards normal and preneoplastic cells, we used CTR to deplete carbonyl compounds (including

Figure 2. Carbonyl-trapping resin (CTR) was shown to be effective in depleting carbonyl compounds (including aldehydes) in fecal water of Hb- and Bf-fed rats (Hb and Bf). (A) HNE-histidine adducts in protein extract of cells treated with HNE 40 µM treated or not with CTR (t = 6h). # Concentration of lipid peroxidation byproducts TBARS and (C) free HNE in fecal water (Hb and Bf) and the respective controls (Control Hb and Control Bf) treated or not by CTR. # Significant difference between a treatment (HNE, Hb, Bf) and its respective control (Control, Control Hb, Control Bf) (P < 0.05). • Significant difference between a treatment using untreated and CTR-treated HNE or fecal water (P < 0.05). Data are expressed as mean ± SD (n = 3).
aldehydes) in fecal water. Figure 2A shows the efficacy of CTR in scavenging HNE, leading to omitted HNE-histidine adducts in the cells exposed to CTR-treated HNE. In Figure 2B and C, diet enriched in n-6 fatty acid and heme iron (under the form of Hb and Bf) led to a significant increase in fecal lipid peroxidation compared with standard diet (P < 0.05). Treatment by CTR was effective in reducing fecal TBARS and free HNE significantly to a concentration observed in fecal water control (P < 0.05).

We incubated normal and preneoplastic cells in untreated and CTR-treated fecal water to assess apoptosis by analyzing the percentage of nuclei with apoptotic appearance (fragmented or condensed) (Figure 3A), cells with positive caspase-3/7 activity (Figure 3B) and cells with permeable membrane (Figure 3C). The depletion of carbonyl compounds was shown to decrease significantly fecal water-induced apoptosis in both cell lines and abolish the differential apoptosis in normal and preneoplastic cells.

**Exposure to fecal water of Hb- and Bf-fed rats induced Nrf2 activation in normal and preneoplastic cells**

To analyze Nrf2 activation in normal and preneoplastic cells induced by exposure to fecal water, we investigated the nuclear localization of Nrf2. Figure 4A displays the fluorescence intensity graphs representing the localization of nuclei (blue channel) and Nrf2 (green channel) in normal and preneoplastic cells under basal conditions and upon exposure to fecal water of Hb- and Bf-fed rats. Figure 4A shows that Nrf2 was active in preneoplastic cells under basal conditions, indicated by its nuclear localization. In contrast, Nrf2 in normal cells tended to display less nuclear accumulation than preneoplastic cells. Following exposure to HNE and to fecal water of Hb- and Bf-fed rats, both normal and preneoplastic cells exhibited Nrf2 activation.

Our blot analysis of nuclear Nrf2 shows Nrf2 activation in normal and preneoplastic cells following exposure to HNE and fecal water of Hb- and Bf-fed rats, but not to fecal water control (Figure 4B and Supplementary Figure 5, available at Carcinogenesis Online). Interestingly, CTR-treated HNE or fecal water did not trigger Nrf2 nuclear translocation, showing the importance of carbonyl compounds in such an activation.

We analyzed the expression of heme oxygenase 1 (HO1), an Nrf2 target gene following exposure to HNE and fecal water for 4h. Figure 4C shows higher HO1 induction in normal cells than in preneoplastic cells upon exposure to HNE and fecal water of Hb- and Bf-fed rats (P < 0.05). Depletion of carbonyl compounds was shown to block the upregulation of HO1 expression carried out by untreated fecal water in both cell lines.

**Nrf2 was involved in the resistance of preneoplastic cells towards fecal water toxicity**

To analyze whether Nrf2 is involved in the resistance of preneoplastic cells towards fecal water toxicity, we downregulated Nrf2 expression by transfecting control Nrf2-specific siRNA (siNrf2).
Figure 4. *Nrf2* was activated in normal and preneoplastic cells upon exposure to fecal water of Hb- and Bf-fed rats (Hb and Bf). Following treatments with fecal water (t = 1 h), *Nrf2* detection in cells was done by immunofluorescence and western blot. (A) Images of immunofluorescence analysis of fecal water-treated normal and preneoplastic cells. Blue channels indicate nuclei and green channels indicate *Nrf2*. The fluorescence intensity graphs represent the colocalization of blue signal (nuclei) and green signal (*Nrf2*) along regions of interest (ROI). The presented images are representative of three independent experiments. (B) Western blotting analysis of nuclear *Nrf2* following exposure to HNE 40 μM and fecal water treated or not with carbonyl-trapping resin (CTR). Lamin A/C was used as loading control. The presented blots are representative of three independent experiments. (C) Relative mRNA expression of HO1 following exposure to HNE 40 μM and fecal water treated or not with CTR. Significant difference between normal and preneoplastic cells (P < 0.05). Significant difference between a treatment in a cell line (HNE, Hb, Bf) and its respective control (Control, Control Hb, Control Bf) (P < 0.05). Significant difference between a treatment using untreated and CTR-treated HNE or fecal water (P < 0.05). Data are expressed as mean ± SD (n = 3).
This down-regulation diminished the level of nuclear Nrf2 in preneoplastic cells both under basal conditions and upon exposure to HNE (Figure 5A). In preneoplastic cells, siNrf2 transfection reduced cell viability and increased apoptosis following 6h of exposure to HNE and fecal water of Hb- and Bf-fed rats (Figure 5B), highlighting the importance of Nrf2 in their resistance towards fecal water toxicity. In normal cells, probably because of their high sensitivity to HNE and fecal water, no enhancement of apoptosis induction following Nrf2 invalidation was observed (Figure S6).

Exposure to fecal water of Hb- and Bf-fed rats induced higher expression of HNE detoxification enzymes in preneoplastic cells

To further investigate the role of Nrf2 in cellular response to fecal water toxicity, we analyzed the expression of three Nrf2 target genes involved in HNE detoxification: aldehyde dehydrogenase 3A1 (ALDH3A1), glutathione S-transferase A4 (GSTA4) and x-C-type cystine/glutamate antiporter (xCT). Figure 6A (left panel) shows that these genes were more expressed in preneoplastic cells than in normal cells, both under basal conditions and following exposure to HNE and fecal water of Hb- and Bf-fed rats (P < 0.05). Treatment with CTR set down and normalized the effects of HNE and fecal water on the expression of these genes, resulting in gene expression profiles which resemble basal conditions. Despite higher final genes expression in preneoplastic cells, we found that these genes were more upregulated in normal cells than in preneoplastic cells upon exposure to HNE and fecal water of Hb- and Bf-fed rats (Figure 6A right panel).

In accordance with the upregulation of xCT expression, we analyzed the level of cystine uptake in normal and preneoplastic cells following exposure to fecal water. Exposure to fecal water of Hb- and Bf-fed rats resulted in higher final uptake in preneoplastic cells than in normal cells (Figure 6B). Depletion of carbonyl compounds abolished the differential cystine uptake due to fecal water exposure.

Discussion

The aim of this study was to understand the mechanisms involved in the differential of fecal water toxicity prepared from Hb- or Bf-fed rats towards normal and preneoplastic mouse colonocytes. Here, we show that Nrf2 activity, via a higher expression of HNE-detoxification enzymes in preneoplastic cells, explains at least in part this difference. We also demonstrate that carbonyl compounds (including aldehydes) are the main agents of fecal water toxicity determining differential susceptibility of normal and preneoplastic cells.

Fecal water from Hb- and Bf-fed rats were associated to high lipid peroxidation and colorectal cancer promotion in vivo

Fecal water of Hb- and Bf-fed rats is a complex matrix derived from real luminal contents which is relevant for studying the effects of heme on luminal neoformation of aldehydes. In comparison with Hb- and Bf-fecal water, HNE was used as pure model molecule representative of lipid peroxidation-derived aldehydes since fecal waters were obtained from feces of rats fed with diets rich in n−6 fatty acids. Indeed, HNE is an important lipoperoxidation endproduct of n−6 fatty acid by heme iron, both in terms of presence and toxicity (10). The HNE concentration used in this study (40 µM) was deduced from TBARS concentration in the feces of Hb-fed rats (Figure 2B) to mimic the global level of lipoperoxidation end-products in fecal water.

Our hypothesis on the role of lipid peroxidation in the promotion of colorectal carcinogenesis by red meat emphasizes the importance of lipid peroxidation-derived aldehydes in generating a selection of colon cells. During our in vivo experiments, rats were fed with standard diet enriched in safflower oil and heme under the form of Hb or raw Bf. Heme contained in Hb and raw Bf is a potent catalyst of lipid peroxidation (41,42).

As observed in Figure 2B and C, supplementation of heme under the form of Hb and Bf in rats increased fecal lipid peroxidation...
and HNE level correlating positively with increasing fecal toxicity towards colon cells (Figure 1). Indeed, Hb (10) and Bf supplementation (not published) increased significantly number or size of preneoplastic lesions in the colon of azoxymethane-induced rats. These lesions are mucin-depleted foci (MDF) with absent or scant mucus production (43). After 100 days, we found that the Hb-fed...
rats developed significantly higher amount of MDF compared with standard diet-fed rats meat (3.4±1.2 versus 2.2±1.5 MDF per colon, respectively; P < 0.01) (10). Rats consuming Bf-based diet had significantly larger MDF in their colon compared with those not consuming red meat (2.6±0.6 versus 2.2±0.3 crypts by MDF, respectively; P = 0.03). Taken together, these results indicate that Hb or Bf consumption promoted colorectal carcinogenesis.

Higher Nrf2 activity in preneoplastic cells led to resistance to apoptosis towards exposure to fecal water of Hb- and Bf-fed rats

We showed that fecal water induced apoptosis in normal and preneoplastic cells through caspase-3/7 activation, membrane phosphatidylserine translocation, membrane permeabilization and finally DNA fragmentation (Figures 1 and 3). These endpoints prompted us to classify the cell death as apoptosis (40). Exposure to fecal water of Hb- and Bf-fed rats activated Nrf2 in both normal and preneoplastic cells (Figure 4). Normal cells appeared to be more responsive in upregulating HNE detoxification genes expression compared with preneoplastic cells (Figure 6A right panel). However, final expression of cytoprotective enzymes in preneoplastic cells remained higher than in normal cells, asserting that preneoplastic cells had better cellular defense towards fecal water toxicity (Figure 6A left panel). These enzymes (ALDH3A1, xCT and GSTA4) are implicated in HNE detoxification (44) and regulated by Nrf2 (45–48). Thus, preneoplastic cells were more resistant to pro-apoptotic effects of HNE and fecal water than normal cells (Figure 1). Down-regulation of Nrf2 in preneoplastic cells reduced their resistance to fecal water toxicity, confirming its protective role (Figure 5).

The mechanisms by which preneoplastic cells exert higher resistance towards fecal water of Hb- and Bf-fed rats than normal cells implicate conjugation pathway with glutathione. The expression of xCT and GSTA4, two enzymes involved in glutathione conjugation pathway and cystine uptake, was higher in preneoplastic cells than in normal cells towards exposure to fecal water of Hb- and Bf-fed rats (Figure 6).

Fecal carbonyl compounds were responsible for Nrf2 activation in colon cells

We proposed that the promotion of colorectal carcinogenesis was linked to the increase of fecal lipid peroxidation level and the toxicity of fecal water. In this study, the importance of carbonyl compounds, including aldehydes derived from lipid peroxidation in fecal toxicity was clearly shown in all our results using CTR. Under activation, this latter contains reactive hydrazine derivatives which are able to scavenge carbonyl groups. Regarding its chemical specificity towards carbonyl groups under the present reaction conditions, activated CTR is very unlikely to bind other potentially cytotoxic molecules which are possibly present in fecal water such as bile acid, bilirubin and lysophospholipids.

CTR was shown to reduce the content of fecal lipid peroxidation byproducts (TBARS) and free HNE (Figure 2A–C). The percentage of apoptotic nuclei (Figure 3) was significantly correlated with the concentration of fecal TBARS (r = 0.95, P < 0.01 for normal cells; r = 0.90, P < 0.01 for preneoplastic cells) and the concentration of fecal free HNE (r = 0.96, P < 0.01 for normal cells; r = 0.93, P < 0.01 for preneoplastic cells). In our previous in vivo studies, we demonstrated that the limitation of lipid peroxidation was correlated to the promoting effect of heme on colorectal carcinogenesis (29,49). This protection was associated to a decrease of fecal water toxicity without causative link between lipid peroxidation and toxicity. Here, we proposed that heme-induced lipid peroxidation was at least in part responsible for the toxicity of fecal water of heme-fed rats. Indeed, as indicated above, CTR traps carbonyl compounds, including aldehydes derived from lipid peroxidation. However, other carbonyl compounds such as ketones and endogenous aldehydes can also be trapped by CTR. Therefore, our study with CTR does not demonstrate that lipid peroxidation-derived aldehydes are solely responsible for the effects of fecal water towards cells. Nevertheless, the results of free HNE trapping (Figure 2C) correlating positively with cytotoxic activity and the kinetically slower reaction of ketones towards hydrazine compounds compared to aldehydes (50) encouraged us to propose that lipid peroxidation-derived aldehydes are the main agents of fecal water toxicity.

In this study, we showed that the presence of carbonyl compounds in fecal water was potentially fundamental in determining Nrf2 activation in normal and preneoplastic cells following exposure to fecal water of Hb- and Bf-fed rats. Figure 4B indicates that Nrf2 activation stimulated by fecal water of Hb- and Bf-fed rats was nullified by depletion of carbonyl compounds. These phenomena were also observed in the expression of Nrf2 downstream genes reported in Figure 6A. In addition, we also reported that all profiles induced by treatments with HNE showed similar tendency with those induced by treatments with fecal water of Hb- and Bf-fed rats. Based on these findings, we highlight the central role of carbonyl compounds (including aldehydes) in Nrf2 activation in colon cells and in the toxicity of fecal water of heme-fed rats.

The different resistance between normal and preneoplastic cells towards exposure to fecal water of Hb- and Bf-fed rats led us to propose a mechanism of colorectal carcinogenesis through positive selection of preneoplastic cells. This phenomenon would favor the survival of preneoplastic cells and thus promote colorectal carcinogenesis.

Supplementary material

Supplementary Movies S1–S4 and Supplementary Figures 5 and 6 can be found at http://carcin.oxfordjournals.org/

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