Evidence That Hydrogen Sulfide Is a Genotoxic Agent

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Abstract

Hydrogen sulfide (H_2S) produced by commensal sulfate-reducing bacteria, which are often members of normal colonic microbiota, represents an environmental insult to the intestinal epithelium potentially contributing to chronic intestinal disorders that are dependent on gene-environment interactions. For example, epidemiologic studies reveal either persistent sulfate-reducing bacteria colonization or H_2S in the gut or feces of patients suffering from ulcerative colitis and colorectal cancer. However, a mechanistic model that explains the connection between H_2S and ulcerative colitis or colorectal cancer development has not been completely formulated. In this study, we examined the chronic cytotoxicity of sulfide using a microplate assay and genotoxicity using the single-cell gel electrophoresis (SCGE; comet assay) in Chinese hamster ovary (CHO) and HT29-Cl.16E cells. Sulfide showed chronic cytotoxicity in CHO cells with a %C1/2 of 368.57 μmol/L. Sulfide was not genotoxic in the standard SCGE assay. However, in a modified SCGE assay in which DNA repair was inhibited, a marked genotoxic effect was observed. A sulfide concentration as low as 250 μmol/L (similar to that found in human colon) caused significant genomic DNA damage. The HT29-Cl.16E colonocyte cell line also exhibited increased genomic DNA damage as a function of Na_2S concentration when DNA repair was inhibited, although these cells were less sensitive to sulfide than CHO cells. These data indicate that given a predisposing genetic background that compromises DNA repair, H_2S may lead to genomic instability or the cumulative mutations found in adenomatous polyps leading to colorectal cancer. (Mol Cancer Res 2006;4(1):9–14)

Introduction

Colorectal cancer is the third most frequent cancer worldwide, producing 945,000 new cases and being responsible for 492,000 deaths annually (1). Genetic and environmental factors play a significant role in the development of colorectal cancer (2-5). Although etiologically divided into sporadic (~90% of the cases), hereditary (5-10%), and inflammatory bowel disease associated (2%; ref. 1), all colorectal cancers show multistep development with several mutations (3-5). Genomic instability, a characteristic feature of colorectal cancer, potentially contributes to the multistep acquisition of mutations found in these tumors (6, 7) and to cancer formation (6, 8). Chromosomal instability is found in >80% of sporadic colorectal cancer (9), whereas microsatellite instability is highly associated with hereditary nonpolyposis colorectal cancer (9, 10). Several mechanisms have been proposed in chromosomal instability formation, including aneuploidy, cell cycle checkpoint loss, break-induced replication, defects in double-strand repair-recombination machinery, mitotic nondisjunction, and oxidative stress from free radicals produced by intraluminal bacteria (11-14). However, none of these potential mechanisms have been proven satisfactory, leaving the nature of this fundamental process in colorectal tumorigenesis unclear.

Numerous clinical and epidemiologic studies have evaluated the relationship between sulfate-reducing bacteria (often members of the normal colonic microbiota) or hydrogen sulfide (H_2S) and inflammatory bowel disease (such as ulcerative colitis) or colorectal cancer (15-20). Potential mechanisms underlying these clinical observations have focused mainly on the possibility that sulfide, through its toxic properties, damages the epithelium (21-26), thereby inciting chronic inflammation. Few studies have examined the effect of H_2S on intestinal epithelial cell fate, especially in the context of its potential contribution to sporadic colorectal cancer (27). Christl et al. (23) observed a significant increase in the proliferation of cells from a colonic biopsy incubated for 4 hours with 1 mmol/L NaHS. Deplancke and Gaskins (24) showed that H_2S may perturb the balance between apoptosis, proliferation, and differentiation in intestinal epithelial cells. Leschelle et al. (28) observed that sulfide decreased HT29 cell growth without increasing apoptosis or necrosis. In contrast to cell function studies, the toxic properties of H_2S have been extensively studied (29). Nevertheless, little is known about H_2S-induced DNA damage, its function as a mutagen or carcinogen, or its involvement in chromosomal instability formation. In the present study, using the alkaline single-cell gel electrophoresis (SCGE) assay or comet assay, we observed that H_2S is capable of generating DNA damage that may be, in part, responsible for the generation of the genomic instability and the cumulative mutations observed in colorectal cancer.

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Results and Discussion

The adverse chronic cellular effect of H_2S was studied using a microplate method that measured the reduction in cell density as a function of the Na_2S concentration over a 72-hour period. In this assay, a decreased cell density could be due to a disruption in the cell cycle, to growth retardation, or to cell toxicity (30, 31). The data from repeated cytotoxicity experiments were averaged and plotted (Fig. 1), and regression analysis was used to calculate the %C1/2 value for Na_2S (which is the Na_2S concentration that causes a 50% reduction of the cell density compared with the negative control; ref. 31). Sodium sulfide had a %C1/2 value of 368.6 μmol/L, indicating that Na_2S is a moderate cytotoxic agent when compared with a previously published cytotoxicity library (31, 32). Nevertheless, Na_2S was more cytotoxic than the positive controls ethylmethane sulfonate (%C1/2 = 4.19 mmol/L) and potassium bromate (%C1/2 = 964 μmol/L; ref. 32). Because sulfide was cytotoxic at a concentration range similar to those found in the mouse intestine (0.2-1 mmol/L; ref. 33) and human feces (0.3-3.4 mmol/L H_2S; refs. 34-36), it is not clear how cells can survive long periods of exposure to H_2S. Different cell-specific detoxification mechanisms (37) or constant cell removal may be the strategy that the intestinal epithelium follows to maintain its integrity. It is also noticeable that the %C1/2 value is much higher than the 50 μmol/L previously reported to inhibit mitachondrial oxidative phosphorylation (38). In fact, in our study, at 50 μmol/L, no growth difference was observed compared with control cells.

The acute cytotoxic and the genotoxic characteristics of Na_2S were evaluated with Chinese hamster ovary (CHO) cells using the SCGE microplate assay. Significant changes in the average median tail moment were not observed in the concentration range of 25 to 5,000 μmol/L compared with the negative control (Fig. 2). Acute cytotoxicity after 4 hours of treatment was minimal for concentrations similar to those reported for the human colon and feces but increased drastically at ≥7,500 μmol/L concentrations, where only a few cells remained attached to the well after 4 hours of treatment.

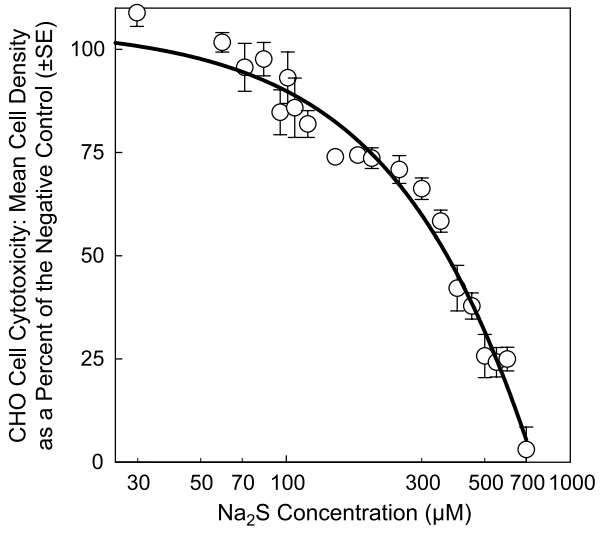

FIGURE 1. Sulfide cytotoxicity in CHO cells. Each concentration corresponds to at least eight observations. Calculated %C1/2 = 368.57 μmol/L.

A modified SCGE assay, where DNA repair was inhibited using hydroxyurea and 1-β-D-arabinofuranosylcytosine (AraC), was used to evaluate whether the previous negative observation was due to the lack of sulfide genotoxicity or was a consequence of active DNA repair. Hydroxyurea depletes the pools of deoxynucleotide triphosphate (39) by inhibiting ribonucleotide reductase, the enzyme responsible for deoxyribonucleotide biosynthesis (40). AraCTP, which is the major intracellular form of AraC, is incorporated into cellular DNA blocking DNA elongation by inhibiting DNA polymerase (39, 41). Figure 3 illustrates a concentration response increase of the average median tail moment values as a function of Na_2S concentration in the hydroxyurea and AraC pretreated cells. A significant difference ($F_{5,34} = 91.71$; $P < 0.001$) in the average median SCGE tail moments was observed compared with the negative control. Sulfide concentrations as low as 250 μmol/L, which are commonly found in human feces (34-36), were genotoxic in mammalian cells ($P < 0.001$), indicating its possible role as an environmental insult in the development of mutations and eventually colorectal cancer. Examples of ethidium bromide–stained nuclei showing the concentration-dependent genotoxic effect of sulfide are presented in Fig. 4.

To evaluate the effect of Na_2S on cultured human colonocytes, we used the Cl.16E subclone of the human colonic cancer cell line HT29 (42) in the modified SCGE assay. The preliminary results are illustrated in Fig. 5. Compared with the transgenic CHO cells that were specifically generated for genotoxicity assays, the HT29-Cl.16E cells were less sensitive to Na_2S. Treatment with Na_2S alone or treatment with hydroxyurea and AraC without Na_2S did not induce a significant increase in genotoxic damage. However, Na_2S in a range from 500 to 2,000 μmol/L induced a positive concentration response with 2,000 μmol/L Na_2S significantly higher than the hydroxyurea + AraC negative control ($F_{5,14} = 13.63$; $P < 0.001$). Under all treatment groups, the viability of the HT29-Cl.16E cells was >95%. Additional studies with a range of colonocyte cell lines or, ideally, primary colonocytes are needed to determine the extent to which colonocytes are resistant to genotoxic properties of sulfide.

To our knowledge, only one previous study investigated the mutagenic potential of H_2S (43). Using the *Salmonella typhimurium* mutagenicity assay with and without Aroclor-induced hamster and rat liver S9 fractions, Hughes et al. found that H_2S vapor (17-1750 g/plate) was not mutagenic in *S. typhimurium* strains TA97, TA98, or TA100 with and without metabolic activation (43). Despite that, Berglin and Carlsson showed that H_2S gas potentiated the mutagenicity of hydrogen peroxide in *S. typhimurium* strain TA102 (44), a strain specifically sensitive to DNA damage mediated by oxidative stress. Sulfide facilitated the formation of iron sulfide, which converts hydrogen peroxide to hydroxyl radicals more efficiently than ferrous iron. Consistently, Moore et al. (45) described the formation of sulfur-centered radicals as a consequence of superoxide production by *Enterococcus faecalis* that may be responsible in part for the action of sulfide *in vivo*.

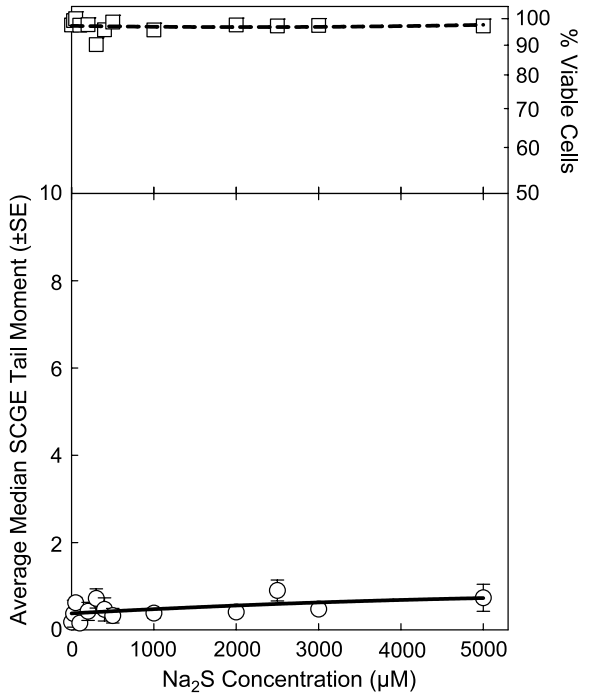

FIGURE 2. SCGE analysis of Na_2S in CHO cells. Top, acute toxicity of CHO cells from treatment groups used in the SCGE assay; bottom, linear-linear plot of the genotoxic concentration-response curve. No significant difference in the average median SCGE tail moment was observed as a function of Na_2S concentration.

c-Jun NH_2-terminal kinase is one of the mitogen-activated protein kinases that phosphorylate and activate the transcriptional activities of c-Jun and ATF2 in response to DNA damage (46, 47). Activation of c-Jun and ATF2 leads to a large-scale coordinated gene expression of several genes involved in DNA repair (48). Deplancke and Gaskins (24) observed an increase in steady-state c-Jun mRNA expression after exposing IEC-18 cells to 0.05 to 5 mmol/L NaHS. This observation was accompanied by a 3-fold increase in the DNA damage–inducible protein (Gadd45), consistent with the present observation that sulfide is capable of generating DNA damage.

The present data show that sulfide is genotoxic at concentrations as low as 250 μmol/L similar to that found in rat colon and human feces. DNA strand breaks have been directly associated with the induction of mutation promotion of induced carcinogenesis (49-51); therefore, H_2S may be responsible for some of the mutations needed in the development of colorectal cancer. Furthermore, H_2S may also be at least in part responsible for the genomic instability observed in colorectal cancer that potentially contributes to the multistep acquisition of mutations found in these tumors.

Colorectal cancer is a disease with both genetic and environmental determinants (3-5, 52). Polymorphisms in DNA repair genes have been associated with an increase in cancer risk (53, 54). Clinical studies have investigated the association in polymorphisms in *xeroderma pigmentosum* [*XPD* (or *ERCC2*), *XPF, XPG*, and *ERCC1*] and *X-ray repair cross-complementing groups 1 and 3* (*XRCC1* and *XRCC3*) genes and colorectal cancer risk (55-58). These studies show that polymorphisms in *XRCC1* and *XRCC3* genes are linked with an increase in colorectal cancer risk, although some results are ambiguous and depend on environmental and racial differences (55, 56, 58). In this study, the DNA damage generated by sulfide was only observed when DNA repair was inhibited, indicating that CHO cells actively repaired the damaged DNA. Individuals with a reduced ability to repair damaged DNA due to allelic polymorphisms in genes involved in DNA repair or harboring germ-line mutations in mismatch repair genes (Lynch syndrome; refs. 4, 59) or in the *MYH* (*MUTYH*) gene (59, 60) would be more sensitive to the environmental insult that H_2S represents. Even more, if alleles that favor colonic colonization by sulfate-reducing bacteria are also present, these subjects would be more susceptible to acquire different mutations and eventually develop colorectal cancer.

In summary, we observed that sulfide, which is produced by commensal sulfate-reducing bacteria in the large intestine, is capable of generating genomic DNA damage. Given a predisposing genetic background, this may lead to genomic instability or the cumulative mutations found in adenomatous polyps and colorectal cancer.

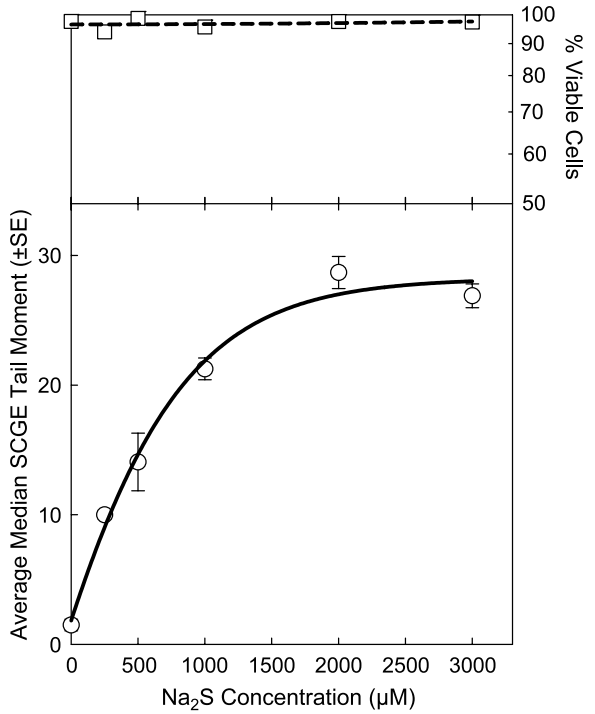

FIGURE 3. Modified SCGE analysis of Na_2S in CHO cells using hydroxyurea and AraC. CHO cells (4×10^4) were incubated with hydroxyurea for 24 hours and then treated with the Na_2S in F12 with hydroxyurea and AraC without FBS for 2 hours. Top, acute toxicity of CHO cells from treatment groups used in the SCGE assay; bottom, linear-linear plot of the genotoxic concentration-response curve. A significant difference ($F_{5,34}$ = 91.71; $P < 0.001$) in the average median SCGE tail moment values was observed compared with the negative control. The genotoxic response of cells treated with hydroxyurea and AraC alone without Na_2S was an average ± SE median tail moment value of 1.49 ± 0.45.

Materials and Methods

Reagents, Cell Culture Medium, and Biologicals

General laboratory reagents were purchased from Fisher (Itasca, IL) or Sigma (St. Louis, MO). $Na_2S{\cdot}9H_2O$ was obtained from Sigma. Media supplies and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT). Clone 11-4-8 of CHO cell line AS52 was maintained in Ham's F12 medium containing 5% FBS, 1% antibiotics (100 units/mL sodium penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2 (61). HT29-Cl.16E cells (ref. 42; kind gift from C.L. Laboisse, Université de Nantes, France) were maintained in DMEM supplemented with 10% heat-inactivated FBS and 0.5% antibiotics (50 units/mL penicillin G, 50 mg/mL streptomycin sulfate, 0.25 mg/mL amphotericin B, 0.85% saline) at 37°C in a humidified atmosphere of 5% CO_2.

Microplate Cytotoxicity Assay

Chronic cytotoxicity to mammalian cells was measured as described previously (30-32). Flat-bottomed, tissue culture 96-well microplates were used; eight replicate wells were prepared for each concentration of $Na_2S{\cdot}9H_2O$. Eight wells were reserved for the blank control consisting of 200 μL F12 + 5% FBS. The negative control consisted of eight wells containing 100 μL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 μL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS, and a known concentration of Na_2S for a total of 200 μL. To prevent crossover contamination between wells due to volatilization of the test agent, a sheet of sterile AlumnaSeal (RPI Corp., Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The plate was placed on a rocking platform for 10 minutes to uniformly distribute the cells, and the microplate was placed in a tissue culture incubator for 72 hours. Each well was gently aspirated, fixed in 100% methanol for 10 minutes, and stained for 30 minutes with a 1% crystal violet solution in 50% methanol. The plate was gently washed, and 50 μL DMSO was added to each well for 30 minutes. The plate was analyzed in a Bio-Rad (Hercules, CA) microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet in a microcomputer connected to the microplate reader.

SCGE Assay

The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 μL F12 + 5% FBS and incubated. The next day, the cells were washed with HBSS and treated with the $Na_2S{\cdot}9H_2O$ in F12 without FBS in a total volume of 50 μL for 4 hours at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal. After incubation, the cells were washed twice with HBSS and harvested with 50 μL of 0.005% trypsin + 53 μmol/L EDTA. The trypsin was inactivated with 70 μL F12 + FBS. A 10 μL aliquot was removed to measure acute cytotoxicity using 10 μL of 0.05% trypan blue vital dye in PBS. SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low-melting point agarose prepared with PBS on clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. Detailed methods for preparing and electrophoresing the SCGE slides were published previously (31). Cellular membranes were removed by an overnight immersion in lysing solution at 4°C. The slides were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 minutes. The slides were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 minutes at 4°C. The slides were removed, neutralized with Tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis, the slides were hydrated in cold water for 20 minutes and stained with 65 μL of 20 μg/mL ethidium bromide for 3 minutes. The slides were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, two slides were prepared per treatment group. The slides were coded, and 25 randomly chosen nuclei were

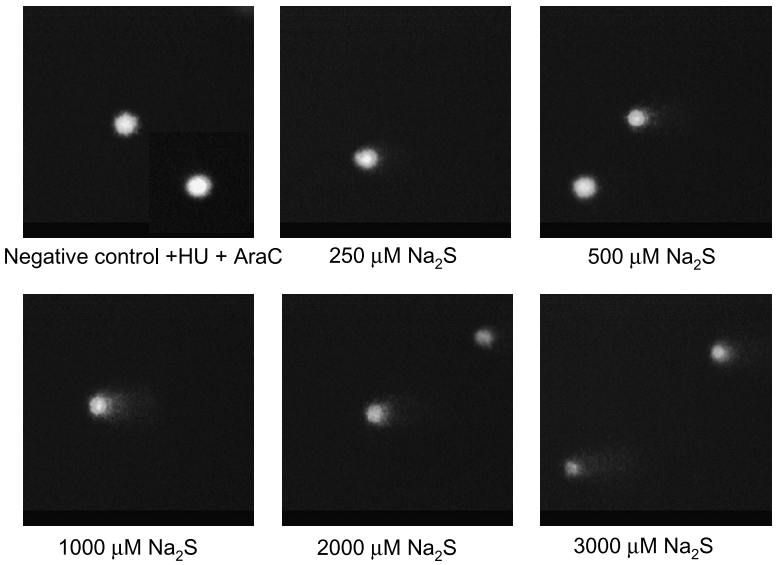

FIGURE 4. Genomic DNA damage induced by Na_2S. Each picture represents nuclei exposed to different concentrations of Na_2S.

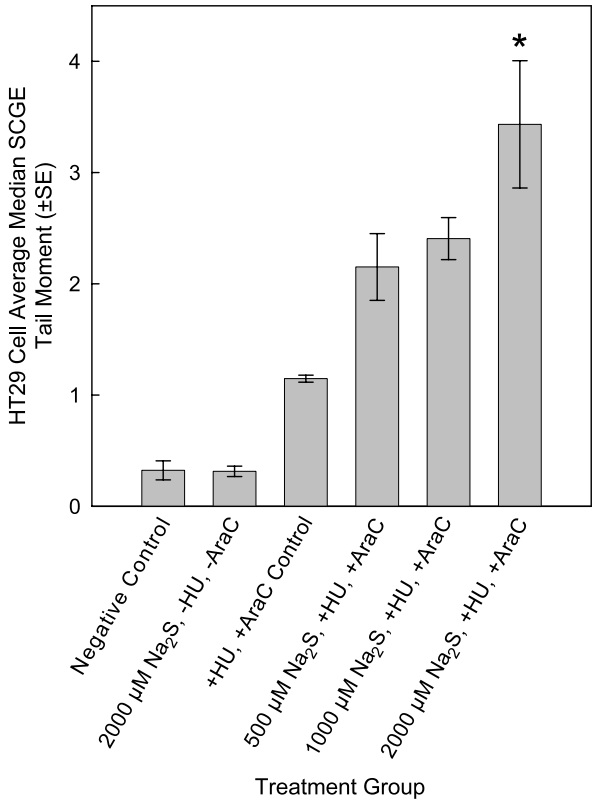

FIGURE 5. Preliminary results of a modified SCGE analysis of Na_2S using HT29-Cl.16E colonocyte cells treated with hydroxyurea and AraC. Induction of genomic DNA damage as measured by SCGE tail moment values. The three control groups (negative control, Na_2S without hydroxyurea or AraC, and hydroxyurea + AraC control) were not significantly different. HT29-Cl.16E cells treated with hydroxyurea + AraC expressed increased genomic DNA damage as a function of Na_2S concentration. *, $P < 0.001$, compared with the hydroxyurea + AraC control ($F_{5,14} = 13.63$). Cell viability for all treatment groups was >95%.

analyzed in each slide using a charge coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, United Kingdom) was employed to determine the tail moment (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as an index of DNA damage. The digitalized data were automatically transferred to a computer-based spreadsheet for subsequent statistical analysis.

SCGE Assay with Modifications to Decrease DNA Repair

This method was done as developed by Wagner and Plewa.[6] Briefly, the day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 μL F12 + 5% FBS and 100 μmol/L hydroxyurea and incubated. The next day, the cells were washed with HBSS and treated with the Na_2S in F12 with 100 μmol/L hydroxyurea and 100 μmol/L AraC without FBS in a total volume of 50 μL for 2 hours at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal. After incubation, the protocol was the same for the SCGE assay described above.

[6] E.D. Wagner and M.J. Plewa, in preparation.

Safety and Data Handling

Manipulations of toxic and mutagenic chemicals were conducted in certified biological/chemical safety hoods. For chronic cytotoxicity assays, the experiments were repeated at least twice with a minimum of eight independent replicates for each chemical concentration per experiment, and for the SCGE assay, three experiments were conducted with eight slides analyzed per treatment group. The median tail moment value for each slide was determined, and the data from all of the slides representing each Na_2S concentration were averaged. Averaged median values express a normal distribution according to the central limit theorem and were used with a one-way ANOVA test (62). A Holm-Sidak method for multiple comparisons versus the control group analysis was conducted.

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References

1. Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW. Colorectal cancer. Lancet 2005;365:153–65.

2. Potter JD. Colorectal cancer: molecules and populations. J Natl Cancer Inst 1999;91:916–32.

3. Rhodes JM, Campbell BJ. Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. Trends Mol Med 2002;8:10–6.

4. de la Chapelle A. Genetic predisposition to colorectal cancer. Nat Rev Cancer 2004;4:769–80.

5. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.

6. Stoler DL, Chen N, Basik M, et al. The onset and extent of genomic instability in sporadic colorectal tumor progression. Proc Natl Acad Sci U S A 1999;96:15121–6.

7. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

8. Shih IM, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. Cancer Res 2001;61:818–22.

9. Lindblom A. Different mechanisms in the tumorigenesis of proximal and distal colon cancers. Curr Opin Oncol 2001;13:63–9.

10. Breivik J, Gaudernack G. Genomic instability, DNA methylation, and natural selection in colorectal carcinogenesis. Semin Cancer Biol 1999;9:245–54.

11. Babbs CF. Free radicals and the etiology of colon cancer. Free Radic Biol Med 1990;8:191–200.

12. Duesberg P, Rausch C, Rasnick D, Hehlmann R. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A 1998;95:13692–7.

13. Huycke MM, Abrams V, Moore DR. *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis 2002;23:529–36.

14. Thiagalingam S, Laken S, Willson JK, et al. Mechanisms underlying losses of heterozygosity in human colorectal cancers. Proc Natl Acad Sci U S A 2001;98:2698–702.

15. Gibson GR, Cummings JH, Macfarlane GT. Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. FEMS Microbiol Lett 1991;86:103–11.

16. Pitcher MCL, Beatty ER, Gibson GR, Cummings JH. Incidence and activities of sulphate-reducing bacteria in patients with ulcerative colitis. Gut 1995;36:A63.

17. Kanazawa K, Konishi F, Mitsuoka T, et al. Factors influencing the development of sigmoid colon cancer. Bacteriologic and biochemical studies. Cancer 1996;77:1701–6.

18. Roediger WE, Moore J, Babidge W. Colonic sulfide in pathogenesis and treatment of ulcerative colitis. Dig Dis Sci 1997;42:1571–9.

19. Levitt MD, Furne J, Springfield J, Suarez F, DeMaster E. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. J Clin Invest 1999;104:1107–14.

20. Moore J, Babidge W, Millard S, Roediger W. Colonic luminal hydrogen sulfide is not elevated in ulcerative colitis. Dig Dis Sci 1998;43:162–5.

21. Babidge W, Millard S, Roediger W. Sulfides impair short chain fatty acid β-oxidation at acyl-CoA dehydrogenase level in colonocytes: implications for ulcerative colitis. Mol Cell Biochem 1998;181:117–24.

22. Roediger WE, Duncan A, Kapaniris O, Millard S. Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. Gastroenterology 1993;104:802–9.

23. Christl SU, Eisner HD, Dusel G, Kasper H, Scheppach W. Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: a potential role for these agents in the pathogenesis of ulcerative colitis. Dig Dis Sci 1996;41: 2477–81.

24. Deplancke B, Gaskins HR. Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. FASEB J 2003;17: 1310–2.

25. Roediger WE, Duncan A, Kapaniris O, Millard S. Sulphide impairment of substrate oxidation in rat colonocytes: a biochemical basis for ulcerative colitis? Clin Sci (Lond) 1993;85:623–7.

26. Moore JW, Millard S, Babidge W, Rowland R, Roediger WE. Hydrogen sulphide produces diminished fatty acid oxidation in the rat colon *in vivo*: implications for ulcerative colitis. Aust N Z J Surg 1997;67:245–9.

27. Huycke MM, Gaskins HR. Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. Exp Biol Med 2004;229:586–97.

28. Leschelle X, Goubern M, Andriamihaja M, et al. Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. Biochim Biophys Acta 2005;1725:201–12.

29. Chou S, Bitter P, Longstreth J. Toxicological profile for hydrogen sulfide. Atlanta (GA): U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry; 1999.

30. Plewa MJ, Kargalioglu Y, Vankerk D, Minear RA, Wagner ED. Development of a quantitative comparative cytotoxicity and genotoxicity assays for environmental hazardous chemicals. Water Sci Technol 2000;42:109–16.

31. Plewa MJ, Kargalioglu Y, Vankerk D, Minear RA, Wagner ED. Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. Environ Mol Mutagen 2002;40:134–42.

32. Plewa MJ, Wagner ED, Jazwierska P, Richardson SD, Chen PH, McKague AB. Halonitromethane drinking water disinfection byproducts: chemical characterization and mammalian cell cytotoxicity and genotoxicity. Environ Sci Technol 2004;38:62–8.

33. Deplancke B, Finster K, Graham WV, Collier CT, Thurmond JE, Gaskins HR. Gastrointestinal and microbial responses to sulfate-supplemented drinking water in mice. Exp Biol Med 2003;228:424–33.

34. Florin T, Neale G, Gibson GR, Christl SU, Cummings JH. Metabolism of dietary sulphate: absorption and excretion in humans. Gut 1991;32:766–73.

35. Pochart P, Dore J, Lemann F, Goderel I, Rambaud JC. Interrelations between populations of methanogenic archaea and sulfate-reducing bacteria in the human colon. FEMS Microbiol Lett 1992;77:225–8.

36. Magee EA, Richardson CJ, Hughes R, Cummings JH. Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans. Am J Clin Nutr 2000;72:1488–94.

37. Picton R, Eggo MC, Merrill GA, Langman MJ, Singh S. Mucosal protection against sulphide: importance of the enzyme rhodanese. Gut 2002;50:201–5.

38. Powell MA, Somero GN. Hydrogen-sulfide oxidation is coupled to oxidative-phosphorylation in mitochondria of *Solemya reidi*. Science 1986; 233:563–6.

39. Skoog L, Nordenskjold B. Effects of hydroxyurea and 1-β-D-arabinofuranosyl-cytosine on deoxyribonucleotide pools in mouse embryo cells. Eur J Biochem 1971;19:81–9.

40. Thelander L, Reichard P. Reduction of ribonucleotides. Annu Rev Biochem 1979;48:133–58.

41. Severin I, Padieu M, Lhuguenot JC, Chagnon MC. Toxic interaction between hydroxyurea and 1-β-D-arabino-furanosylcytosine on the DNA of a human hepatoma cell line (HEPG2). Toxicol Lett 2003;145:303–11.

42. Augeron C, Laboisse CL. Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Res 1984;44:3961–9.

43. Hughes TJ, Sparacino C, Frazier S. Validation of chemical and biological techniques for evaluation of vapors in ambient air testing of twelve (12) vapor-phase compounds (project summary). Research Triangle Park (NC): Environmental Protection Agency; 1984.

44. Berglin EH, Carlsson J. Effect of hydrogen sulfide on the mutagenicity of hydrogen peroxide in *Salmonella typhimurium* strain TA102. Mutat Res 1986; 175:5–9.

45. Moore DM, Kotake Y, Huycke MM. *Enterococcus faecalis* produces thiyl radicals while colonizing the colon. Free Radic Biol Med 2003;35:S175.

46. Derijard B, Hibi M, Wu IH, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994;76:1025–37.

47. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene 2001; 20:2390–400.

48. Hayakawa J, Mittal S, Wang Y, et al. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. Mol Cell 2004;16:521–35.

49. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature 2001;411:366–74.

50. Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis 2002;23:687–96.

51. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2001;27:247–54.

52. Ierardi E, Principi M, Francavilla R, et al. Epithelial proliferation and ras p21 oncoprotein expression in rectal mucosa of patients with ulcerative colitis. Dig Dis Sci 2001;46:1083–7.

53. Shen MR, Jones IM, Mohrenweiser H. Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans. Cancer Res 1998;58:604–8.

54. Goode EL, Ulrich CM, Potter JD. Polymorphisms in DNA repair genes and associations with cancer risk. Cancer Epidemiol Biomarkers Prev 2002;11: 1513–30.

55. Abdel-Rahman SZ, Soliman AS, Bondy ML, et al. Inheritance of the ^{194}Trp and the ^{399}Gln variant alleles of the DNA repair gene XRCC1 are associated with increased risk of early-onset colorectal carcinoma in Egypt. Cancer Lett 2000; 159:79–86.

56. Yeh CC, Hsieh LL, Tang R, Chang-Chieh CR, Sung FC. MS-920: DNA repair gene polymorphisms, diet and colorectal cancer risk in Taiwan. Cancer Lett 2005;224:279–88.

57. Yeh CC, Sung FC, Tang R, Chang-Chieh CR, Hsieh LL. Polymorphisms of the XRCC1, XRCC3, & XPD genes, and colorectal cancer risk: a case-control study in Taiwan. BMC Cancer 2005;5:12.

58. Mort R, Mo L, McEwan C, Melton DW. Lack of involvement of nucleotide excision repair gene polymorphisms in colorectal cancer. Br J Cancer 2003;89: 333–7.

59. Baglioni S, Genuardi M. Simple and complex genetics of colorectal cancer susceptibility. Am J Med Genet C Semin Med Genet 2004;129:35–43.

60. Al-Tassan N, Chmiel NH, Maynard J, et al. Inherited variants of MYH associated with somatic G:C->T:A mutations in colorectal tumors. Nat Genet 2002;30:227–32.

61. Wagner ED, Rayburn AL, Anderson D, Plewa MJ. Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. Environ Mol Mutagen 1998; 32:360–8.

62. Lovell DP, Thomas G, Dubow R. Issues related to the experimental design and subsequent statistical analysis of *in vivo* and *in vitro* comet studies. Teratog Carcinog Mutagen 1999;19:109–19.