Ingestion of sugar beet fiber enhances irradiation-induced aberrant crypt foci in the rat colon under an apoptosis-suppressed condition

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The induction of aberrant crypt foci (ACF) by irradiation of γ-rays (^{60}Co), and the effect of dietary sugar beet fiber (SBF) on irradiation-induced ACF were examined. We found that abdominal irradiation of γ-rays could induce ACF in the rat colon. The irradiation was performed once a week at a dose rate of 2 or 3 Gy per irradiation. Irradiation-induced ACF were observed in the colon at 10 weeks after the first irradiation at dose of 2 Gy for six times or 3 Gy for four times. Dietary SBF had no effect on the number of ACF, aberrant crypts (AC) or AC/focus induced by abdominal γ-irradiation. However, an ingestion of SBF resulted in an increase in the number of these parameters in apoptosis-suppressed rats by cycloheximide (CHX). An injection of CHX suppressed irradiation-induced apoptosis of the colonic epithelial cells for at least 6 h after the irradiation. In CHX-injected rats, an ingestion of SBF significantly increased the number of ACF, AC and AC/focus compared with fiber-free fed rats at 9 weeks after the first irradiation. On the other hand, in saline-injected rats, no significant difference was found between SBF and fiber-free diets in the number of ACF, AC and AC/focus through the experimental period. These results suggest that dietary SBF may be involved in the elimination of abnormal cells from an irradiated colon through the apoptosis of colonic epithelial cells. In this study, we have shown a new method for inducing ACF by using γ-rays which were not influenced by luminal contents such as bacterial enzyme, at least in the initiation stage.

Introduction

Aberrant crypt foci (ACF) are used as biomarkers of colorectal cancer because they appear in the colorectum of rodents at an early period of carcinogenesis (1). Aberrant crypts (AC) are a few times larger than normal crypts, occurring as an oval shape in the thickened epithelium, and are easily detectable in whole mounts of the colonic mucosa with methylene blue staining (1). ACF look like adenoma and have high proliferative activity because the bromodeoxyuridine (2) or the proliferating cell nuclear antigen-labeling (3) index was higher in the crypt comprising ACF than in the normal crypt. In rodents exposed to chemical carcinogens, ACF and tumors develop in their colons (1,4–7). ACF appear at an early period of colorectal

Abbreviations: AC, aberrant crypts; ACF, aberrant crypt foci; AOM, azoxymethane; CHX, cycloheximide; DMH, 1,2-dimethylhydrazine; SBF, sugar beet fiber; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

carcinogenesis and in the tumor-bearing state in rats after treatment with carcinogen (8), also in patients with sporadic and hereditary colon cancer (9). They can therefore be considered as putative early preneoplastic lesions. On the other hand, there are complicated results that ACF formation is inhibited by oral administration of cholic acid, which promotes the formation of colonic tumors (10–12). It is interesting to investigate the effect of dietary factors on the growth characteristics of ACF to evaluate the biological significance of ACF or to elucidate the mechanism of tumor formation thereafter.

We have studied the effect of dietary fibers on the apoptosis of the epithelial cells, ACF and tumors, induced by a carcinogen, 1,2-dimethylhydrazine (DMH) (13–15). DMH, or azoxymethane (AOM), is frequently used as colonic carcinogen to investigate influence of dietary factors on carcinogenesis (16). DMH or AOM require conversion by intestinal bacteria into a carcinogen, methylazoxymethanol (17). Ionizing radiation is known to induce tumors in the colon (18,19) and to increase the risk of colorectal cancer (20). In this study, we investigated whether low dose and frequent irradiation could induce ACF in the rat colon by using γ-rays as an ionizing radiation. We also studied the effects on the irradiation induced ACF of sugar beet fiber (SBF) as a dietary fiber source, and the involvement of apoptosis in ACF formation after exposure to radiation, using an apoptosis inhibitor, cycloheximide.

Materials and methods

Animals

Four-week-old male Wistar/ST rats (Japan SLC, Shizuoka, Japan), were used throughout the experiments. All rats were housed in individual cages with free access to drinking water and experimental diets, under controlled conditions of temperature (23 ± 2°C) and lighting (12 h light–dark cycle). A fiber-free diet contained casein, corn oil, a mineral mixture and a modified AIN-76 vitamin mixture as described previously (21). For SBF ingestion study, rats were divided into two dietary groups, which were given either fiber-free diet or the diet supplemented with SBF (100 g/kg diet) after acclimatization with a fiber-free diet. The SBF was donated by Nippon Beet Sugar MFG (Obihiro, Japan). These experiments were approved by the Hokkaido University Animal Use Committee, and animals were maintained according to the guidelines of Hokkaido University for the care and use of laboratory animals.

Study design

In the experiment for the induction of ACF by γ-irradiation, 20 Wistar/ST rats were divided into two groups. They were γ-irradiated on their abdomens once a week at doses of 2 Gy for six times or 3 Gy for four times. The total absorbed dose was identical between both groups (12 Gy). Rats were killed to collect their colons for ACF determination at 5 or 9 weeks after the first irradiation. In the experiment for the effects of ingestion of SBF on the irradiation-induced ACF, 32 Wistar/ST rats had been fed a fiber-free or an SBF diet from 5 days before irradiation. Three rats in every group were not irradiated and were kept as a control. The dose of irradiation was six times at 3 Gy, and the colonic ACF measurement was performed at 15 weeks after the first irradiation. In the experiment for suppression of apoptosis of colonic epithelial cells by CHX injection, rats were irradiated once and killed at 3, 6 or 9 h after the irradiation. For CHX-injection groups, CHX (1 mg/kg body wt; Sigma, St Louis, MO) was injected intraperitoneally into rats at 10–15 min before and 3 h after the irradiation. Control rats were injected with saline at the same time point. In the experiment for the influence of CHX injection and SBF ingestion on ACF induced by γ-rays, 47 Wistar/ST rats were

purchased. Three rats were not irradiated and were kept with fiber-free diet throughout the experimental period as a control. Forty-four rats were divided into two dietary groups (fiber-free or SBF) for 7 days after acclimatization, then irradiated three times at a dose of 3 Gy once a week (total 9 Gy). In each group, rats were injected with CHX or saline as described above. ACF determination was performed at 5 (CHX), 7 (CHX) or 9 weeks (CHX and saline) after the first irradiation. In the experiment for the effect of CHX injection and SBF ingestion on the irradiation-induced apoptosis of colonic epithelial cells, 36 Wistar/ST rats were divided into two dietary groups (fiber-free or SBF) for 3 days after acclimatization and irradiated once, twice or three times. The dose of irradiation was 3 Gy, once (after the first irradiation), twice (after the second irradiation) or three times (after the third irradiation). All rats were injected with CHX before and after irradiation as described above. CHX was not injected after the last irradiation in this experiment, because irradiation-induced apoptosis cells using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method were evaluated 1 h after the irradiation in these studies. These colons were removed 1 h after every irradiation and used for morphological evaluation of apoptotic epithelial cells to confirm whether CHX injection inhibits the irradiation-induced apoptosis of the colonic epithelial cells.

γ-Irradiation

The abdomens of rats were γ-irradiated using a ^{60}Co-γ-irradiator (Cobalt-60 Teletherapy Apparatus RCR-120-C3; Toshiba, Kanagawa, Japan) under anesthesia with 30 mg/kg body wt sodium pentobarbital (Abbott Laboratories, North Chicago, IL). Irradiations were performed between 1100 and 1400. γ-Rays from a ^{60}Co source were given once a week at dose rates of 0.66–0.77 Gy/min.

Determination of ACF in colon

Colonic samples for ACF determination were performed at various times (5, 7, 9, 10 or 15 weeks) after the first irradiation, and the rats were killed by exsanguination under anesthesia with sodium pentobarbital. The colons were removed, flushed with saline, then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h on flat plates. These samples were stained with 0.2% methylene blue for 15 min. ACF and AC were counted with a light microscope.

Determination of apoptotic epithelial cells in colon

Colonic samples were embedded in OCT compound, rapidly frozen with liquid nitrogen, and stored at –80°C. Frozen sections from these samples were stained with hematoxylin for the effect of CHX on irradiation-induced apoptosis of the colonic epithelial cells or for the TUNEL method to evaluate the effect of CHX injection and SBF ingestion on irradiation-induced apoptosis of the colonic epithelial cells. These sections were fixed with 4% paraform-aldehyde in PBS for 10 min. For TUNEL staining, after treatment with a microwave for 5 min, these sections were immersed in hydrogen peroxide in methanol to reduce endogenous peroxidase activity, then washed with PBS. *In situ* labeling was performed using a solution containing 0.01 nmol/μl biotin-11-dUTP and 0.3 U/μl TdT in TdT buffer (30 mM Tris–HCl, 140 mM sodium cacodylate and 1 mM $CaCl_2$, pH 7.2) at 37°C for 60 min. The sections were placed in 4% bovine serum albumin in PBS to block non-specific biotin binding. They were then incubated with peroxidase-conjugated streptavidin (Cosmo Bio, Tokyo, Japan) at 37°C for 30 min. 3,3′-Diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) was used as the chromogen. These sections were counterstained with hematoxylin.

Morphological evaluation of apoptotic epithelial cells and crypt cells

For an evaluation of apoptotic cells stained with hematoxylin, the number of apoptotic epithelial cells in a well-shaped 10-crypt section were counted. Then, the total number of apoptotic cells per 10 crypts was expressed as the index. For an evaluation of the number of apoptotic cells stained with the TUNEL method and crypt cells, TUNEL-positive epithelial cells in a well-shaped crypt section and total epithelial cells were counted in the same crypt. The labeling index was the percentage calculated by counting the number of TUNEL-positive cells against the total number of epithelial cells in the same crypt and expressing the result as APO-LI. This assessment was carried out without knowledge of diet or treatments. Ten crypts were counted per segment of colon in each histological parameter.

Statistics

The differences in mean values among groups were tested by analysis of variance. When the probability was <0.05, the significant differences between means were tested by Student's *t*-test or Tukey's honestly significant difference test for growth parameters, the number of apoptotic epithelial cells and crypt cells of the same section. A *P*-value <0.05 was considered significant. All statistical calculations were carried out by using JMP computer software (SAS, Cary, NC). Values in the text are means ± SEM.

Table I. Growth parameters for 5 or 9 weeks after the first γ-irradiation

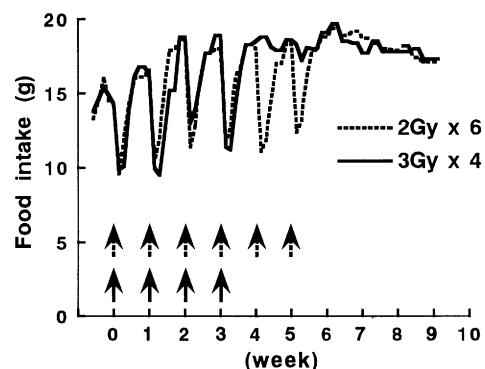|	Irradiation	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
5 weeks	2 Gy×6	187.9 ± 2.4	328.8 ± 7.9	140.9 ± 7.5
	3 Gy×4	184.0 ± 2.8	340.1 ± 8.6	156.2 ± 8.0
9 weeks	2 Gy×6	182.7 ± 2.4	415.6 ± 8.6	232.9 ± 8.0
	3 Gy×4	187.2 ± 1.9	410.9 ± 14.5	223.7 ± 13.3

Fig. 1. Changes in mean food intake for 9 weeks after the first γ-irradiation. Rats were irradiated on their abdomens once a week six times at 2 Gy (broken arrows) or four times at 3 Gy (solid arrows).

Table II. ACF, AC and AC/focus of the rat colon 5 and 9 weeks after the first γ-irradiation

	Irradiation	Incidence	No. of ACF/ colon	No. of AC/ colon	No. of AC/ focus
5 weeks	2 Gy×6	3/5	0.80 ± 0.37	1.40 ± 0.75	1.67 ± 0.33
	3 Gy×4	3/5	1.40 ± 0.75	1.80 ± 1.11	1.17 ± 0.17
9 weeks	2 Gy×6	5/5	3.60 ± 1.36	7.00 ± 2.02	2.11 ± 0.29
	3 Gy×4	5/5	4.20 ± 1.56	11.80 ± 4.45*	2.52 ± 0.50

Values are means ± SEM ($n = 5$).
*Significantly different from the same irradiation group (3 Gy×4) at 5 weeks.

Results

Induction of ACF by γ-irradiation

Table I shows the growth parameters of γ-irradiated rats. No significant differences in final body weight and body weight gain were observed between these two irradiated groups. Food intake temporarily decreased immediately after the irradiation (Figure 1). It was, however, recovered within 1 week until the next irradiation. No rats died in these experimental periods. ACF were induced at 5 or 9 weeks after the first irradiation (Table II). At 9 weeks after first irradiation, all rats had ACF in their colon. The numbers of ACF, AC and AC/focus tended to be greater in 3-Gy- than in 2-Gy-irradiated groups and greater at 9 weeks than at 5 weeks after the first irradiation.

Effects of dietary SBF on ACF induced by γ-rays

Final body weight and body weight gain were significantly suppressed by γ-irradiation in fiber-free rats (Table III). A similar trend was observed in SBF-fed rats, but no significant difference was observed. Colonic ACF were detected in irradiated rats. No ACF were observed in irradiation-untreated rats. The number of irradiation-induced ACF was not significantly influenced by the ingestion of SBF (Table IV). Moreover,

Table III. Effect of γ-irradiation and dietary sugar beet fiber on the growth parameters for 15 weeks

Treatment	Diet	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Irradiated	Fiber-free	173.5 ± 1.8	451.6 ± 7.8[a]	278.1 ± 7.8[a]
	SBF	174.0 ± 1.7	439.6 ± 7.9[a]	265.6 ± 8.0[a]
Unirradiated	Fiber-free	174.7 ± 2.8	518.4 ± 13.3[b]	343.7 ± 15.9[b]
	SBF	172.3 ± 4.9	476.8 ± 22.0[ab]	304.5 ± 26.8[ab]

Values are means ± SEM (Irradiated, $n = 13$; unirradiated, $n = 3$).
[a,b]Within a column, values not sharing a common superscript differ significantly ($P < 0.05$).

Table IV. Effect of dietary SBF on ACF induced by γ-rays (3 Gy×6) 15 weeks after the first irradiation

	No. of ACF/colon	No. of AC/colon	AC/focus
Fiber-free	3.85 ± 0.73	11.00 ± 2.18	2.89 ± 0.28
SBF	5.00 ± 0.52	15.92 ± 1.92	3.19 ± 0.21

Values are expressed mean±SEM ($n = 13$). No significant difference was observed in parameters.

Table V. Effect of ingestion of sugar beet fiber on cell numbers and TUNEL positive cells of the crypt of rat distal colon 1 h after every irradiation under CHX-treated conditions

	Diet	No. of cells/crypt	APO-LI (%)
After the first irradiation	Fiber-free	64.7 ± 1.1[a]	3.0 ± 0.2
	SBF	66.7 ± 1.4[a,b]	3.2 ± 0.2
After the second irradiation	Fiber-free	68.3 ± 1.1[a,b,c]	2.8 ± 0.2
	SBF	71.2 ± 1.3[b,c,d]	3.0 ± 0.3
After the third irradiation	Fiber-free	73.2 ± 1.3[c,d]	2.7 ± 0.3
	SBF	74.4 ± 1.1[d]	3.0 ± 0.2

Values are means ± SEM ($n = 6$).
[a–d]Within a column, values not sharing a common superscript differ significantly ($P < 0.05$).

no significant difference in AC or AC/focus between both dietary groups was observed.

Suppression of apoptosis of colonic epithelial cells by CHX injection

γ-Irradiation induced apoptosis of the colonic epithelial cells, and the apoptosis was observed until 9 h after the irradiation (Figure 2). CHX injection clearly suppressed apoptosis of the colonic epithelial cells induced by irradiation at 3 h. Then, the apoptosis was gradually reduced, but the apoptosis-suppressing effect of CHX was maintained until at least 6 h after irradiation. The i.p. injections of CHX (1 mg/kg body wt) markedly inhibited apoptosis of the colonic epithelial cells.

Influence of CHX injection and SBF ingestion on ACF induced by γ-rays

Figure 3 shows the effects of the ingestion of SBF on irradiation-induced ACF in CHX-treated rats. No significant differences in body weight and body weight gain were observed at every time point regardless of irradiation, diet and CHX injection. In CHX-injected groups, the ingestion of SBF promoted the numbers of ACF, AC and AC/focus as the duration after the irradiation increased. There was no significant effect of dietary SBF on ACF in saline-injected groups. At 9
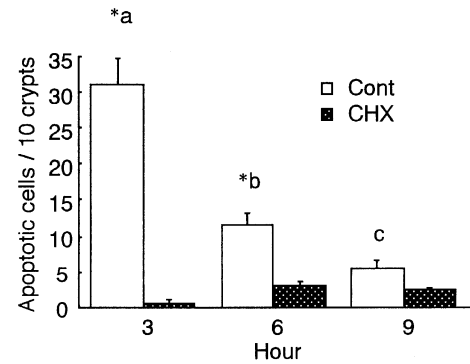

Fig. 2. Effect of CHX injection on irradiation-induced apoptosis of epithelial cells in the distal colon. Injection of CHX was performed 10–15 min before and 3 h after γ-irradiation. Control rats were injected with saline. Values are means ± SEM (3 h after irradiation, $n = 3$; 6 h after irradiation, $n = 7$; 9 h after irradiation, $n = 13$). *, Significantly different from control group ($P < 0.05$). Bars not sharing a letter are significantly different ($P < 0.05$).
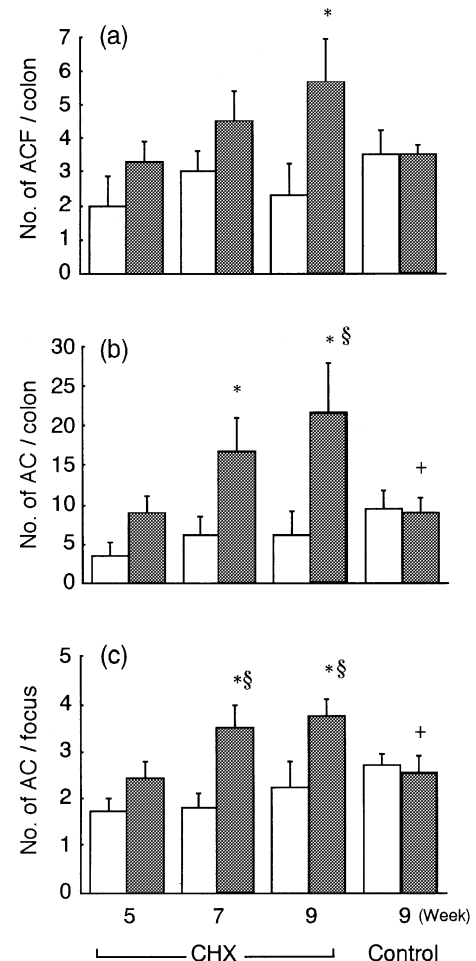

Fig. 3. Effect of ingestion of SBF (filled bars) or fiber-free (open bars) on ACF induced by γ-irradiation in CHX-treated or untreated rats. Control rats were injected with saline. Values are means ± SEM ($n = 6$, except for fiber-free and control group at 9 weeks, $n = 4$; fiber-free group at 5 and 7 weeks, $n = 5$). *, Significantly different from fiber-free and CHX-injected group at the same time point; §, significantly different from SBF group at 5 weeks; +, significantly different from SBF and CHX-injected group at the same time point.

weeks after the first irradiation, a significantly large number of ACF and AC/focus was observed by CHX injection in comparison with the saline-injected control group in SBF-fed rats. In fiber-free rats, there was no significant change in the numbers of ACF, AC and AC/focus through the experimental period, regardless of CHX injection.

Effect of CHX injection and SBF ingestion on the irradiation-induced apoptosis of colonic epithelial cells

APO-LI was at a constantly low level (2.7–3.2%) at 1 h after each irradiation, and no significant change of APO-LI was observed in this experiment (Table V). In our preliminary results, APO-LI after the second irradiation in the SBF group (15.9 ± 0.3) was significantly larger than in the fiber-free group (14.5 ± 0.3) ($P < 0.05$) in CHX-untreated condition. CHX injection therefore has an inhibitory effect on the apoptosis of colonic epithelial cells. The crypt cell number tended to be larger as the number of irradiations increases but it did not differ between the dietary groups.

Discussion

We have shown that low doses (2 or 3 Gy) and frequent irradiations of γ-rays induce ACF in the rat colon. In many studies, the carcinogen is injected at least a few times every week (16). In this study, ACF parameters tended to increase as the dose per week increased and as sampling time was delayed after the first irradiation if total doses were the same (Table II). The initiation of this irradiation-induced ACF may hardly be influenced by intestinal environmental factor as compared with carcinogen-induced ACF. Chemical carcinogens, for example DMH or AOM, require conversion by intestinal bacteria into a real carcinogen, methylazoxymethanol (17), though ionizing radiation such as γ-rays directly affects DNA in the intestinal epithelial cells.

The ingestion of SBF as a dietary fiber had no effect on the irradiation-induced ACF in a CHX-untreated condition. Our previous result showed that dietary SBF significantly reduced DMH-induced ACF in the rat colon (15). The difference between these results is caused by a difference in the method for inducing ACF. One possible explanation is that a reduction of carcinogen-induced ACF by SBF was due to the enhancement of defecation and the excretion of carcinogen. To explain this difference, we must examine how dietary SBF influences ACF generation or development. In this connection, it is also interesting to investigate the effects of dietary cholic acid against irradiation-induced ACF. Comparing responses with dietary factors between the irradiation- and carcinogen-induced ACF will lead to useful information on the effect of dietary factors on the formation of ACF.

Another possible reason for dietary SBF having no effect on irradiation-induced ACF in a CHX-untreated condition is a disruption of the immune system by γ-irradiation. Intraepithelial lymphocytes, which have chemotactic activity and mediate various forms of cytotoxicity (22–25), may be involved in elimination of abnormal epithelial cells. We have shown that the administration of anti-asialo GM1 increased the number of DMH-induced ACF in the rat colon (15,21). So, the reduction of intraepithelial lymphocytes or other peripheral lymphocytes in the initiating period probably results in increasing abnormal cell through escape from immune surveillance. The relationship between the immune system and ACF formation remains to be clarified.

Apoptosis of the colonic epithelial cells appears a few hours

after γ-irradiation or the injection of carcinogen (26). The elimination of abnormal cells by apoptosis may lower the frequency of ACF and tumors thereafter. CHX was used as an inhibitor of apoptosis (27,28). Our preliminary experiment showed that this dose of CHX (1 mg/kg body wt) almost completely suppressed the irradiation-induced apoptosis of the colonic epithelial cells a few hours after the irradiation. We then investigated the effect of CHX injection and/or SBF ingestion on irradiation-induced ACF. Interestingly, dietary SBF clearly increased irradiation-induced ACF in the apoptosis-inhibiting condition (Figure 3). We confirmed that apoptosis of the colonic epithelial cells was inhibited (2–3%) by the CHX-injection, even in SBF-fed rats. We also showed that SBF ingestion increased irradiation-induced apoptosis. These results demonstrated that apoptosis after irradiation is involved in the generation of ACF by ionizing irradiation. Mechanisms associated with increasing ACF by SBF were unknown.

Many organic acids have appeared as products of the fermentation after the ingestion of dietary fibers (29,30). In the previous report, the ingestion of SBF significantly increases the concentration of short chain fatty acids, especially butyrate in cecal contents (31). This is easily used as an energy source (32,33), it induces mucosal proliferation (34) and is a potent apoptosis-inducer against some cell lines originated from colorectal cancers (35,36). It was uncertain if butyrate acted as an apoptosis-inducing reagent in this study.

In SBF-fed and CHX-injected rats, the number of remaining abnormal cells that escape from irradiation-induced apoptosis may be greater than in fiber-free rats, which resulted in a significant increase in the number of ACF and their multiplicity (Figure 3). The apoptosis inhibitory period in this experimental condition is thought to be at least 6 h after the irradiation, as shown in the results. The administration of the apoptosis inhibiting reagent with ionizing irradiation is considered to raise the risk of colorectal carcinogenesis.

In conclusion, we found a novel method to induce ACF by γ-irradiation as well as by colonic carcinogen, and we suggest that apoptosis is involved in irradiation-induced ACF generation. Comparing the responses of the irradiation- and carcinogen-induced ACF with dietary factors will give us useful information.

Acknowledgement

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