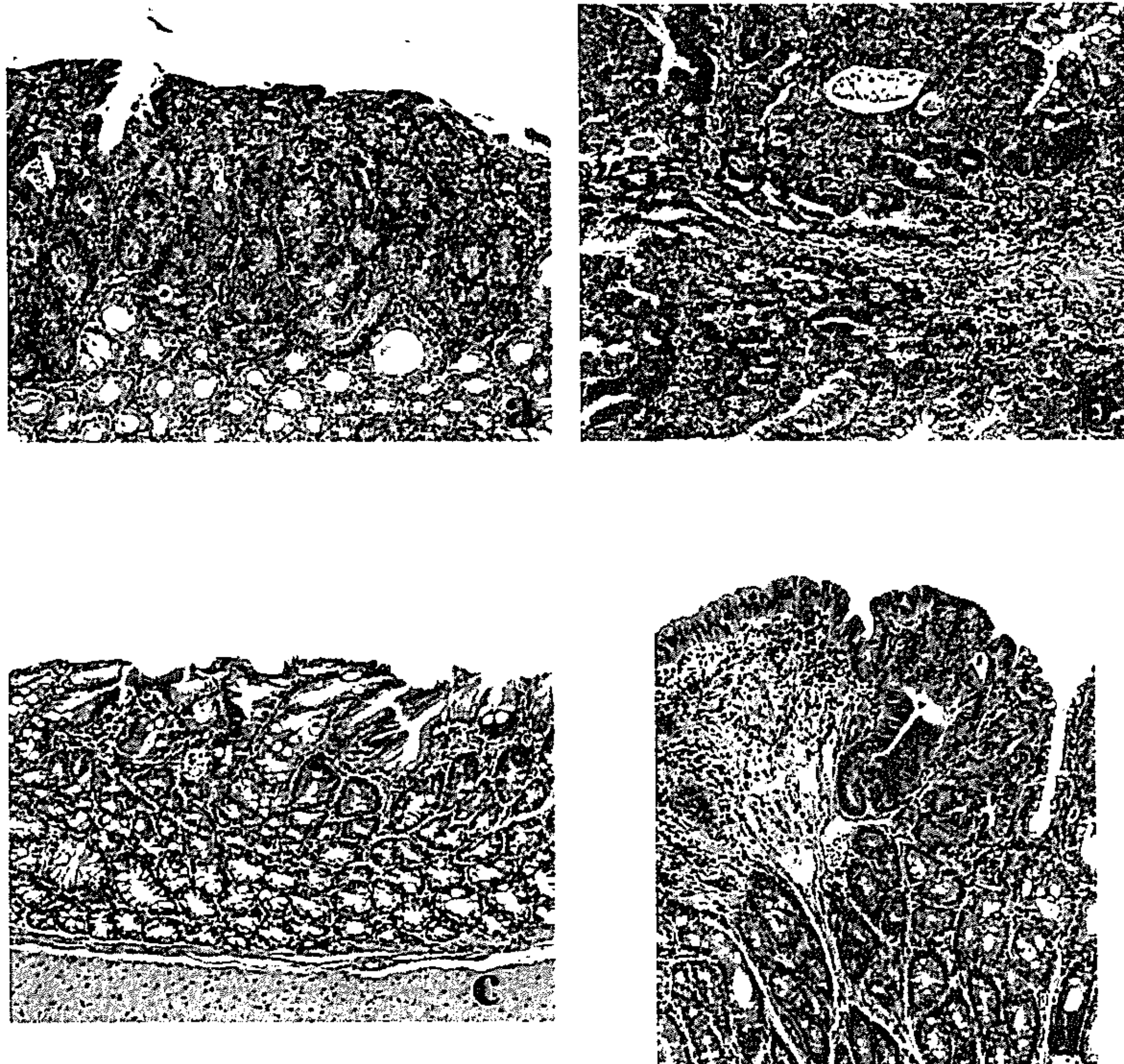

Figure 2
Histopathology of colonic lesions in mice of group 1. (a) adenoma, (b) adenocarcinoma, (c) mild dysplasia, and (d) severe dysplasia. Hematoxylin and eosin stain. Original magnifications, × 10.

Pathological findings
Macroscopically, nodular, polypoid or caterpillar-like tumors were observed in the middle and distal colon of mice in groups 1 through 4. They were histologically tubular adenoma (Figure 2a) or well-/moderately-differentiated tubular adenocarcinoma (Figure 2b). Dysplasia (Figure 2c–e) was also developed in mice of these groups. Animals of groups 5–10 did not have large bowel neoplasms and dysplasia. The incidences and multiplicity of colon neoplasma are shown in Table 2. Group 1 (AOM/

Table 4: PCNA and apoptosis indices and scores of β-catenin, COX-2, iNOS and nitrotyrosine expression in colonic adenocarcinomas.

Group no.	Treatment	PCNA-labeling index (%)	Apoptotic index (%)	Scores for:			
				β-Catenin	COX-2	iNOS	Nitrotyrosine
1	AOM/DSS	62.4 ± 13.7[a] (30)	4.1 ± 1.9 (30)	3.7 ± 0.7 (30)	3.3 ± 0.7 (30)	3.0 ± 1.0 (30)	3.1 ± 0.8 (30)
2	AOM/DSS/ 0.04% Nimesulide	38.3 ± 11.1 [b] (6)	11.8 ± 2.9 [b] (6)	2.3 ± 1.0 [c] (6)	1.3 ± 0.5 [b] (6)	1.3 ± 0.5 [b] (6)	1.7 ± 0.5 [b] (6)
3	AOM/DSS/ 0.05% Troglitazone	43.6 ± 9.0 [b] (9)	10.0 ± 2.4 [b] (9)	2.7 ± 0.7 [c] (9)	1.8 ± 0.8 [b] (9)	1.6 ± 0.5 [b] (9)	1.8 ± 0.6 [b] (9)
4	AOM/DSS/ 0.05% Bezafibrate	40.5 ± 12.7 [b] (15)	9.7 ± 2.7 [b] (15)	3.0 ± 0.8 (15)	1.8 ± 0.8 [b] (15)	1.6 ± 0.6 [b] (15)	2.2 ± 1.1 [b] (15)

Numbers in parentheses are numbers of lesions examined.
[a]: means ± SD.
[b,c] Significantly different from group 1 by Student's t-test ([b]$P < 0.001$ and [c]$P < 0.05$).

DSS) induced 100% incidence of colon adenocarcinomas with a multiplicity of 3.0 ± 1.8. The incidences of colorectal adenocarcinomas in groups 2 (AOM/DSS/0.04% nimesulide), 3 (AOM/DSS/0.05% troglitazone), and 4 (AOM/DSS/0.05% bezafibrate) were significantly smaller than that of group 1 ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). The multiplicities of colon adenocarcinomas in groups 2 and 3 were also significantly lower than that of group 1 ($P < 0.005$ and $P < 0.05$, respectively). While the multiplicity of colon adenocarcinoma of group 4 (AOM/DSS/0.05% bezafibrate) was smaller than group 1, the difference was insignificant. In this study, mucosal ulcer with or without focal dysplasia (Figure 2c–e) were also found in the distal colon of mice in groups 1 through 4. The incidences and multiplicity of colonic ulceration and dysplasia are shown in Table 3. The incidences and multiplicities of colorectal mucosal ulcer and dysplasia of groups 2, 3, and 4 were smaller than group 1, but the differences did not reach to statistical significance.

Immunohistochemistry for PCNA, ssDNA, β-catenin, COX-2, iNOS and nitrotyrosine in colonic adenocarcinoma

As summarized in Table 4, PCNA-labeling index (Figure 3a) of colonic adenocarcinomas developed in groups 2, 3, and 4 was significantly smaller than group 1 ($P < 0.001$). Apoptotic index measured by ssDNA immunohistochemistry (Figure 3b) in groups 2, 3, and 4 was significantly greater than group 1 ($P < 0.001$).

Strong β-catenin expression was seen in the nucleus and cytoplasm of adenocarcinoma cells (Figure 3c). Although the intensity was relatively weaker than carcinoma cells, adenoma cells showed positivity for β-catenin in their cytoplasm and cell membrane. β-catenin immunoreactivity was also found in the cell membrane and cytoplasm of dysplastic cells. Non-lesional cryptal cells showed weak positivity of β-catenin in their cell membrane. In the positive cases of COX-2 (Figure 3d), and iNOS (Figure 3e) expression in the dysplasia and adenocarcinoma, the staining pattern was granular and localized to cytoplasm and/or nuclei. Slight immunoreactivity for COX-2 and iNOS was observed in the superficial layers of the non-lesional colonic mucosa and in parts of basal layer in all groups. The expression pattern between COX-2 and iNOS of colorectal adenocarcinomas was well correlated. Furthermore, a positive staining for nitrotyrosine, a marker of nitrosative injury, was mainly observed in mononuclear cells infiltrated in the colonic mucosa (Figure 3f). Neoplastic cells also showed negative or very weakly positive immnoreactivity of nitrotyrosine. Scores for β-catenin, COX-2 and iNOS expression in colonic adenocarcinomas are given in Table 4. β-Catenin expression scores of colorectal adenocarcinomas in groups 2 and 3 were significantly decreased when compared with that in group 1 ($P < 0.05$ and $P < 0.05$, respectively). Scores for COX-2 and iNOS expression of colorectal adenocarcinomas in groups 2, 3, and 4, were significantly smaller than those in group 1 ($P < 0.001$). The scores of nitrotyrosine positivity in groups 2, 3, and 4 were significantly lower than group 1 ($P < 0.001$).

Discussion

The results of the present work clearly indicated that a COX-2 inhibitor nimesulide and a PPARγ ligand troglitazone effectively inhibited AOM/DSS-induced colitis-related colonic carcinogenesis in mice. Inhibitory effect of

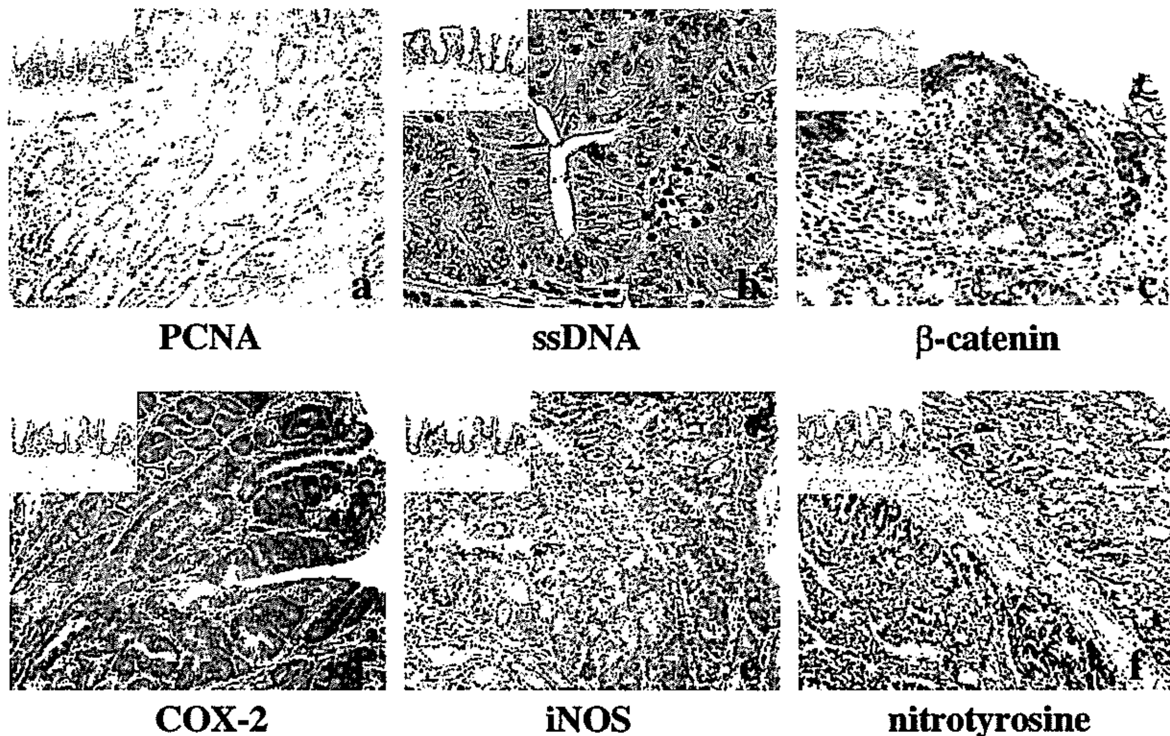

Figure 3
Immunohistochemistry of PCNA, ssDNA, β-catenin COX-2, iNOS, and nitrotyrosine in mice of group 1. (a) PCNA immunohistochemistry, (b) ssDNA immunohistochemistry, (c) β-catenin immunohistochemistry, (d) COX-2 immunohistochemistry, (e) iNOS immunohistochemistry, and (f) nitrotyrosine immunohistochemistry. Original magnifications: a, d, e, f, × 10; c, × 20; b, × 40. Insets, immunohistochemistry staining for each antibody in the mouse colon from group 10 (Original magnifications, × 10).

nimesulide was superior to that of troglitazone. Bezafibrate also reduced the occurrence of colonic adenocarcinoma, but the ability was relatively weaker than that of nimesulide and troglitazone. The suppressive effects of nimesulide, troglitazone and bezafibrate on the development of colonic adenocarcinoma was well correlated with the inhibition of cell proliferation activity, induction of apoptosis, and lowered immunoreactivity of β-catenin, COX-2, iNOS, and nitrotyrosine in the colonic malignancies. However, no differences on the frequency of dysplastic lesions could be observed among the groups. These data may suggest that the pharmacological classes tested under the present investigation slow down the time course of tumor development rather than completely preventing it.

The pathogenesis of IBD-associated colorectal carcinogenesis is widely believed to involve a step-wise progression from inflamed and hyperplastic epithelia through flat dysplasia to finally adenocarcinoma [30]. IBD-associated colorectal carcinogenesis is probably promoted by chronic inflammation, but the mechanism is still unclear. However, mucosal inflammation may result in colonic carcinogenesis through several proposed mechanisms such as induction of genetic mutations, increased-cryptal cell proliferation, changes in crypt cell metabolism, changes in bile acid enterohepatic circulation, and alterations in bacteria flora [4,34]. These events are considered to promote IBD-associated CRC development. Given the correlation between increased COX-2 expression and colonic carcinoma and/or inflammation, the chemopreventive effects of NSAIDs seem to be mediated, at least in part, by COX inhibition [35]. We [36] and others [29,37] demonstrated that NSAIDs including nimesulide inhibited both colon tumorigenesis and colitis. In the current study, powerful chemopreventive ability of nimesulide

was observed in our colitis-related mouse colon carcinogenesis model, suggesting that nimesulide can be applied as an effective chemopreventor of both sporadic and IBD-associated cololectral carcinogenesis.

We previously demonstrated that dietary administration of PPARα and PPARγ ligands inhibits AOM and/or DSS-induced ACF in rodents [38]. In the present study, cancer chemopreventive ability of the PPARγ ligand, troglitazone, or the PPARα ligand, bezafibrate, was found in AOM/DSS-induced mouse colon carcinogenesis model, although their ability was lower than nimesulide. Inhibition of colonic inflammation and decrease in cell proliferation activity by these PPARs ligands might be responsible for their chemopreventive effects on colitis-associated colon carcinogenesis [38]. DNA damage caused by reactive oxygen and nitrogen species may contribute to colitis-related colon tumorigenesis [39]. Several NSAIDs can bind to PPARα and PPARγ [40]. Their anti-inflammatory activities might be mediated through inhibition of COX-1 and/or COX-2. PPARα could suppress COX-2 induction [41]. In addition, immunomodulation by the PPARs ligands might contribute to inhibition of colitis and colon carcinogenesis [42].

Expression and activity of iNOS is increased in colonic mucosa in patients with IBD [43] and colonic adenomas [44]. Several studies using experimental colon carcinogenesis models indicate that chemically induced colon tumors have higher expression and/or activity of iNOS compared with those in their adjacent non-tumorous tissues [12,25]. Numerous iNOS-positive and nitrotyrosine-positive inflammatory cells are observed in non-cancerous colonic mucosa of mice treated with DSS [25]. PPARα [45] and PPARγ [46] involve in inflammation control, and can inhibit iNOS expression [47]. In addition, Rao *et al.* [48] showed that an iNOS-selective inhibitor suppresses AOM-induced colonic ACF development and iNOS activity. Furthermore, nitrotyrosine may originate from the reaction of iNOS generated NO with reactive oxygen species [49] or the myeloperoxidase-dependent pathway [50]. In the present study, we found a positive immunoreactivity for iNOS and nitrotyrosine in the inflamed colon, suggesting the formation of peroxynitrite and other NO-derived oxidants. These results may suggest that one of the mechanisms by which tested agents exert chemopreventive ability might be related to suppression of iNOS activity and/or expression.

Cell proliferation plays an important role in multi-step carcinogenesis [51]. In the colon, the number of cryptal cells is strictly regulated by a balance between cell proliferation and cell death that maintains homeostasis [52]. Changes in cell proliferation and apoptosis are regarded as a common denominator in the pathogenesis of tumor formation [53]. Reduced tumor incidence is generally associated with decreases in cellular proliferation and/or increases in apoptosis [54]. An increased COX-2 expression in CRC [55,56] may confer a survival advantage on cells by inhibition of apoptosis and a change in cellular adhesion to the extracellular matrix [57]. Cancer cells treated with PPARγ ligands induce cell differentiation and apoptosis [14,17]. Recently, Tardieu *et al.* [58] demonstrated that nimesulide increases apoptosis in colonic mucosa of DSS-treated rats. Our findings that nimesulide and troglitazone inhibited cell proliferation activity and induced apoptosis in colorectal mucosa are in accordance with these findings. Thus, cellular responses like cell growth and/or apoptosis to nimesulide and troglitazone may contribute to chemopreventive effects against colon carcinogenesis processes.

β-Catenin is a key regulator of the cadherin-mediated cell-cell adhesion system and an important element in the Wnt signal transduction pathway [59]. Accumulated β-catenin interacts with T-cell factor (Tcf) or lymphoid-enhancer factor (Lef) and translocates to the nucleus, in which it transactivates target genes including *c-myc* and *cyclin D1* that are the potentially oncogenic [47,60] in the cytoplasm or nucleus as a consequence of mutant *Apc* or *β-catenin* genesis frequently observed in early stages of colorectal carcinogenesis [61,62]. Recently, Girnun *et al.* [21] indicated that a ligand of PPARγ suppresses β-catenin levels and colon carcinogenesis in *Pparγ*$^{+/-}$ mice treated with AOM. Furthermore, COX-2 is regulated by nuclear β-catenin accumulation [63]. In the current study, treatment with nimesulide, troglitazone, and bezafibrate significantly suppressed β-catenin expression in colorectal adenocarcinomas. Thus, it seems likely that the preventive efficacies of the COX-2 inhibitor (nimesulide) or the PPAR ligands (troglitazone and bezafibrate) against AOM/DSS induced mouse colon carcinogenesis might be mediated, at least in part, by β-catenin down-regulation.

Chemoprevention of cancer might be defined as the deliberate introduction of these selected non-toxic substances into the diet for the purpose of reducing cancer development. In the present study, the mean liver weights of mice in dietary feeding of troglitazone and bezafibrate were significantly increased. However, there were no pathological alterations suggesting toxicity of the drugs examined. It is known that administration of PPARs ligands to rodent exhibit hepatomegaly due to both cellular hypertrophy and hyperplasia [64]. Increase in liver weight by exposure of troglitazone and bezafibrate in this study may be caused by these pathological changes. The estimated daily intakes of nimesulide, troglitazone, and bezafibrate in mice given the diets containing 400 ppm and 500 ppm were approximately 160 mg/kg and 200 mg/kg in the present study. In a direct extrapolation to a 60 kg person,

these doses are slightly lower than those of clinical trial [65-67]. These findings may suggest that the efficacy of these agents at dietary dose-levels has a direct practical and translational relevance to human.

Conclusion

In conclusion, dietary administration of nimesulide, troglitazone, and bezafibrate could effectively suppress colon carcinogenesis induced by AOM and DSS in female ICR mice. Our on-going study on molecular profiles in colonic samples from the current experiment will provide precise molecular mechanisms involved in their inhibitory action in AOM/DSS-induced mouse colon carcinogenesis.

Abbreviations

AOM, azoxymethane; ACF, aberrant crypt foci; CRC, Colorectal cancer; COX-2, cyclooxygenase-2; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; NSAID, non-steroidal anti-inflammatory drug; PPAR, peroxisome proliferator-activated receptor; PCNA, proliferating cell nuclear antigen; ssDNA, single stranded DNA; UC, ulcerative colitis.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

HK participated in study design, performed the animal studies, and drafted the manuscript. RS carried out tissue collection and data analysis. SS participated in the histopathological and immunohistological analysis. TT participated in study design, coordination, and manuscript preparation. All authors read and approved the final manuscript.

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References

1. Eaden JA, Abrams KR, Mayberry JF: **The risk of colorectal cancer in ulcerative colitis: a meta-analysis.** *Gut* 2001, **48:**526-535.
2. Ekbom A, Helmick C, Zack M, Adami HO: **Ulcerative colitis and colorectal cancer. A population-based study.** *N Engl J Med* 1990, **323:**1228-1233.
3. Weitzman SA, Gordon LI: **Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis.** *Blood* 1990, **76:**655-663.
4. Seril DN, Liao J, Yang GY, Yang CS: **Oxidative stress and ulcerative colitis-associated carcinogenesis: stuides in human and animal models.** *Carcinogenesis* 2003, **24:**353-362.
5. Thun MJ, Namboodiri MM, Heath CWJ: **Aspirin use and reduced risk of fatal colon cancer.** *N Engl J Med* 1991, **325:**1593-1596.
6. Reddy BS, Tokumo K, Kulkarni N, Aligia C, Kelloff G: **Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds.** *Carcinogenesis* 1992, **13:**1019-1023.
7. Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kelloff GJ, Verma A, Moser AR, Dove WF: **Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam.** *Cancer Res* 1996, **56:**710-714.
8. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ: **Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis.** *N Engl J Med* 1993, **328:**1313-1316.
9. Taniguchi Y, Ikesue A, Yokoyama K, Noda K, Debuchi H, Nakamura T, Toda A: **Selective inhibition by nimesulide, a novel nonsteroidal anti-inflammatory drug, with prostaglandin endoperoxide synthase-2 activity in-vitro.** *Pharm Sci* 1995, **1:**173-175.
10. Cipollini F, Mecozzi V, Altilia F: **Endoscopic assessment of the effects on nimesulide on the gastric mucosa: comparison with indomethacin.** *Curr Ther Res* 1989, **45:**1042-1048.
11. Takahashi M, Fukutake M, Yokota S, Ishida K, Wakabayashi K, Sugimura T: **Suppression of azoxymethane-induced aberrant crypt foci in rat colon by nimesulide, a selective inhibitor of cyclooxygenase 2.** *J Cancer Res Clin Oncol* 1996, **122:**219-222.
12. Nakatsugi S, Fukutake M, Takahashi M, Fukuda K, Isoi T, Taniguchi Y, Sugimura T, Wakabayashi K: **Suppression of intestinal polyp development by nimesulide, a selective cyclooxygenase-2 inhibitor, in Min mice.** *Jpn J Cancer Res* 1997, **88:**1117-1120.
13. Rosen ED, Spiegelman BM: **PPARg : a nuclear regulator of metabolism, differentiation, and cell growth.** *J Biol Chem* 2001, **276:**37731-37734.
14. Tontonoz P, Singer S, Forman BM, Sarraf P, Fletcher JA, Fletcher CD, Brun RP, Mueller E, Altiok S, Oppenheim H, Evans RM, Spiegelman BM: **Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor.** *Proc Natl Acad Sci USA* 1997, **94:**237-241.
15. Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM: **Differentiation and reversal of malignant changes in colon cancer through PPARg.** *Nat Med* 1998, **4:**1046-1052.
16. Suh N, Wang Y, Williams CR, Risingsong R, Gilmer T, Willson TM, Sporn MB: **A new ligand for the peroxisome proliferator-activated receptor-g (PPAR-g), GW7845, inhibits rat mammary carcinogenesis.** *Cancer Res* 1999, **59:**5671-5673.
17. Takahashi N, Okumura T, Motomura W, Fujimoto Y, Kawabata I, Kohgo Y: **Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells.** *FEBS Lett* 1999, **455:**135-139.
18. Tanaka T, Kohno H, Yoshitani S, Takashima S, Okumura A, Murakami A, Hosokawa M: **Ligands for peroxisome proliferator-activated receptors a and g inhibit chemically induced colitis and formation of aberrant crypt foci in rats.** *Cancer Res* 2001, **61:**2424-2428.
19. Osawa E, Nakajima A, Wada K, Ishimine S, Fujisawa N, Kawamori T, Matsuhashi N, Kadowaki T, Ochiai M, Sekihara H, Nakagama H: **Peroxisome proliferator-activated receptor g ligands suppress colon carcinogenesis induced by azoxymethane in mice.** *Gastroenterology* 2003, **124:**361-367.
20. Niho N, Takahashi M, Kitamura T, Shoji Y, Itoh M, Noda T, Sugimura T, Wakabayashi K: **Concomitant suppression of hyperlipidemia and intestinal polyp formation in Apc-deficient mice by peroxisome proliferator-activated receptor ligands.** *Cancer Res* 2003, **63:**6090-6095.
21. Girnun GD, Smith WM, Drori S, Sarraf P, Mueller E, Eng C, Nambiar P, Rosenberg DW, Bronson RT, Edelmann W, Kucherlapati R, Gonzalez FJ, Spiegelman BM: **APC-dependent suppression of colon carcinogenesis by PPARg.** *Proc Natl Acad Sci USA* 2002, **99:**13771-13776.
22. Lefebvre AM, Chen I, Desreumaux P, Najib J, Fruchart JC, Geboes K, Briggs M, Heyman R, Auwerx J: **Activation of the peroxisome

proliferator-activated receptor g promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. *Nat Med* 1998, **4**:1053-1057.
23. Saez E, Tontonoz P, Nelson MC, Alvarez JGA, Ming UT, Baird SM, Thomazy VA, Evans RM: **Activators of the nuclear receptor PPARg enhance colon polyp formation.** *Nat Med* 1998, **4**:1058-1061.
24. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R: **Novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice.** *Gastroenterology* 1990, **98**:694-702.
25. Seril DN, Liao J, Ho KLK, Warsi A, Yang CY, Yang GY: **Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice.** *Dig Dis Sci* 2002, **47**:1266-1278.
26. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD: **A novel therapy for colitis utilizing PPAR-g ligands to inhibit the epithelial inflammation response.** *J Clin Invest* 1999, **104**:383-389.
27. Nakajima A, Wada K, Miki H, Kubota N, Nakajima N, Terauchi Y, Ohnishi S, Saubermann LJ, Kadowaki T, Blumberg RS, Nagai R, Matsuhashi N: **Endogenous PPAR g mediates anti-inflammatory activity in murine ischemia-reperfusion injury.** *Gastroenterology* 2001, **120**:460-469.
28. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H: **A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate.** *Cancer Sci* 2003, **94**:965-973.
29. Fukutake M, Nakatsugi S, Isoi T, Takahashi M, Ohta T, Mamiya S, Taniguchi Y, Sato H, Fukuda K, Sugimura T, Wakabayashi K: **Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice.** *Carcinogenesis* 1998, **19**:1939-1942.
30. Riddell RH, Goldman H, Ransohof DF, Appleman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC, Sommers SC, Yardley JH: **Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical application.** *Human Pathol* 1983, **14**:931-968.
31. Pascal RR: **Dysplasia and early carcinoma in inflammatory bowel disease and colorectal carcinomas.** *Human Pathol* 1994, **25**:1160-1171.
32. Ward JM: **Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats.** *Lab Invest* 1974, **30**:505-513.
33. Watanabe I, Toyoda M, Okuda J, Tenjo T, Tanaka K, Yamamoto T, Kawasaki H, Sugiyama T, Kawarada Y, Tanigawa N: **Detection of apoptotic cells in human colorectal cancer by two different in situ methods; antibody against single-stranded DNA and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and end-labeling (TUNEL) methods.** *Jpn J Cancer Res* 1999, **90**:188-193.
34. Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S: **Colitis-related rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate (Review).** *Oncol Rep* 2000, **7**:501-508.
35. Wakabayashi K: **NSAIDs as cancer preventive agents.** *Asian Pacific J Cancer Prev* 2000, **1**:97-113.
36. Tanaka T, Kojima T, Yoshimi N, Sugie S, Mori H: **Inhibitory effect of the non-steroidal anti-inflammatory drug, indomethacin on the naturally occurring carcinogen, 1-hydroxyanthraquinone in male ACI/N rats.** *Carcinogenesis* 1991, **12**:1949-1952.
37. Kawamori T, Rao CV, Seibert K, Reddy BS: **Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis.** *Cancer Res* 1998, **58**:409-412.
38. Kohno H, Yoshitani S, Takashima S, Okumura A, Hosokawa M, Yamaguchi N, Tanaka T: **Troglitazone, a ligands for peroxisome proliferator-activated receptor g inhibits chemically-induced aberrant crypt foci in rats.** *Jpn J Cancer Res* 2001, **92**:396-403.
39. Maeda H, Akaike T: **Review: Nitric oxide and oxygen radicals in infection, inflammation, and cancer.** *Biochemistry (Mosc)* 1998, **63**:854-865.
40. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA: **Peroxisome proliferator-activated receptors a and g are activated by indomethacin and other non-steroidal anti-inflammatory drugs.** *J Biol Chem* 1997, **272**:3406-3410.
41. Vamecq J, Latruffe N: **Medical significance of peroxisome proliferator-activated receptor.** *Lancet* 1999, **354**:141-148.
42. Gelman L, Fruchart JC, Auwerx J: **An update on the mechanisms of action of the peroxisome proliferator-activated receptors (PPARs) and their roles in inflammation and cancer.** *Cell Mol Life Sci* 1999, **55**:932-943.
43. Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, Stenson WF: **Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease.** *Gastroenterology* 1996, **111**:871-885.
44. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, Klein S, Shields PG, Billiar TR, Harris CC: **Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression.** *Cancer Res* 1998, **58**:334-341.
45. Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W: **The PPARa-leukotriene B4 pathway to inflammatory control.** *Nature* 1996, **384**:39-43.
46. Jiang C, Ting AT, Seed B: **PPAR-g agonists inhibit production of monocyte inflammatory cytokines.** *Nature* 1998, **391**:82-86.
47. Colville-Nash PR, Qureshi SS, Willis D, Willoughby DA: **Inhibition of inducible nitric oxide synthase by peroxisome proliferator-activated receptor agonists: correlation with induction of heme oxygenase 1.** *J Immunol* 1998, **161**:978-984.
48. Rao CV, Kawamori T, Hamid R, Reddy BS: **Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor.** *Carcinogenesis* 1999, **20**:641-644.
49. Zingarelli B, Szabo C, Salzman AL: **Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase.** *Gut* 1999, **45**:199-209.
50. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A: **Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils.** *Nature* 1998, **391**:393-397.
51. Cohen SM, Ellwein LB: **Cell proliferation in carcinogenesis.** *Science* 1990, **249**:1007-1011.
52. Kellett M, Potten CS, Rew DA: **A comparison of in vivo cell proliferation measurements in the intestine of mouse and man.** *Epithelial Cell Biol* 1992, **1**:147-155.
53. McGarrity TJ, Peiffer LP, Colony PC: **Cellular proliferation in proximal and distal rat colon during 1,2-dimethylhydrazine-induced carcinogenesis.** *Gastroenterology* 1988, **95**:343-348.
54. Barnes CJ, Cameron IL, Hardman WE, Lee M: **Non-steroidol anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis.** *Br J Cancer* 1998, **77**:573-580.
55. DuBois RN, Radhika A, Reddy BS, Entingh AJ: **Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors.** *Gastroenterology* 1996, **110**:1259-1262.
56. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN: **Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas.** *Gastroenterology* 1994, **107**:1183-1188.
57. Tsujii M, DuBois RN: **Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2.** *Cell* 1995, **83**:493-501.
58. Tardieu D, Jaeg JP, Deloly A, Corpet DE, Cadet J, Petit CR: **The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxy-deoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats.** *Carcinogenesis* 2000, **21**:973-976.
59. Miller JR, Moon RT: **Signal transduction through b-catenin and specification of cell fate during embryogenesis.** *Genes Dev* 1996, **10**:2527-2539.
60. Tetsu O, McCormick F: **b-Catenin regulates expression of cyclin D1 in colon carcinoma cells.** *Nature* 1999, **398**:422-426.
61. Iwao K, Nakamori S, Kameyama M, Imaoka S, Kinoshita M, Fukui T, Ishiguro S, Nakamura Y, Miyoshi Y: **Activation of the b-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations.** *Cancer Res* 1998, **58**:1021-1026.
62. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: **Identification of c-MYC as a target of the APC pathway.** *Science* 1998, **281**:1509-1512.

63. Araki Y, Okamura S, Hussain SP, Nagashima M, He P, Shiseki M, Miura K, Harris CC: **Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways.** *Cancer Res* 2003, **63**:728-734.
64. Fahimi HD, Maumgart E, Beier K, Pill J, Hartig F, Volkl A: **Ultrastructural and biochemical aspects of peroxisome proliferation and biogenesis in different mammalian species.** In *Peroxisomes: biology and importance in toxicology and medicine* Edited by: Gibson G and Lake B. London, Taylor and Francis Ltd.; 1993:395-424.
65. Reiner M, Massera E, Magni E: **Nimesulide in the treatment of fever: a double-blind, crossover clinical trial.** *J Int Med Res* 1984, **12**:102-107.
66. Kumar S, Prange A, Schulze J, Lettis S, Barnett AH: **Troglitazone, an insulin action enhancer, improves glycaemic control and insulin sensitivity in elderly type 2 diabetic patients.** *Diabet Med* 1998, **15**:772-779.
67. Wheeler KA, West RJ, Lloyd JK, Barley J: **Double blind trial of bezafibrate in familial hypercholesterolaemia.** *Arch Dis Child* 1985, **60**:34-37.

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Indole-3-carbinol inhibits the growth of human colon carcinoma cells but enhances the tumor multiplicity and volume of azoxymethane-induced rat colon carcinogenesis

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Abstract. Indole-3-carbinol (I3C) is a naturally occurring phytochemical which exerts a broad range of biological activities. The purpose of this study was to examine the effects of I3C on colon carcinogenesis, cell proliferation, cell-cycle progression and apoptosis, and on the levels of expression of several cell-cycle control molecules. We used a long-term rat model by using azoxymethane (AOM) to induce tumors (adenomas and adenocarcinomas) in the colon. In the present study, we found that after AOM injection, the treatment of male F344 rats with 0.01 and 0.05% I3C caused a significant increase in the tumor multiplicity of adenocarcinomas by 2.2- ($P<0.05$ for 0.01% I3C) and 2.1-fold ($P<0.0002$ for 0.05% I3C) respectively, when compared to the control rats. In addition, the tumor multiplicity of adenoma plus adenocarcinoma and the volume of adenocarcinoma were also increased by 2.0- ($P<0.00001$) and 2.1-fold ($P<0.05$) respectively, compared to the control. I3C significantly increased the proliferating cell nuclear antigen labeling index (PCNA LI) ($P<0.008$) and decreased the apoptotic index ($P<0.05$) of the colon adenocarcinoma. In contrast, in HCT 116 and HT29 human colon carcinoma cells, I3C inhibited growth and induced G1-phase cell-cycle arrest and apoptosis. Furthermore, I3C caused approximately a 2- to 4-fold increase in the cellular levels of $p27^{KIP1}$ and $p21^{CIP1}$ mRNA. These results suggest that I3C inhibits the growth of human colon carcinoma cells, at least in part, by inducing $p27^{KIP1}$ and $p21^{CIP1}$-mediated G1 cell-cycle arrest but dietary I3C promotes AOM-induced rat colon carcinogenesis by inhibiting the apoptosis of colon tumors. Therefore, the present study may provide further evidence for the ambivalent modulatory activity of I3C and this information may be useful when including I3C in cancer chemoprevention and/or extensive clinical therapy trials.

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Key words: indole-3-carbinol, colon carcinogenesis, cancer prevention, apoptosis, cell cycle

Introduction

Phytochemicals are non-nutritive components in the plant-based diet that possess substantial anticarcinogenic and antimutagenic properties (1). There has been considerable current interest in dietary phytochemicals in the prevention or treatment of human carcinomas. Indole-3-carbinol (I3C) is a naturally occurring phytochemical and can be found in cruciferous vegetables including cabbage, broccoli, Brussels sprouts, and cauliflower (2) and these vegetables are commonly consumed in ordinary life as a dietary supplement. I3C has been shown to have growth-inhibitory activity in animal models of carcinogenesis and in several human colon carcinoma cell lines. Dietary I3C inhibited rat colonic aberrant crypt foci (ACF) formation induced by heterocyclic amines (HCA) or azoxymethane (AOM) during initiation or post-initiation stages (3-5). I3C also inhibited the growth of SW480 and HT29 human colon carcinoma cell lines in a dose-dependent manner (6,7). In addition, I3C increases the levels of a drug-metabolizing protein, cytochrome P450 1A1 (CYPA1), and induces apoptosis in the CaCo-2 human colon carcinoma cell line (8). As described above, most *in vivo* and *in vitro* studies demonstrate I3C's inhibitory or preventive activities for colon carcinogenesis but some provide obvious evidence for promotion or enhancement of colon carcinogenesis. For instance, I3C caused a clear enhancement of 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis, when animals were treated with I3C during the post-initiation phase (9). Also, cabbage-supplemented Swiss mice had a higher incidence and multiplicity of colon tumors induced by DMH (10). Thus, the effect of I3C in response to cancer prevention appears controversial.

In the present study, we used a rat colon carcinogenesis bioassay system and human colon carcinoma cell lines to further investigate the range of activity of I3C. To obtain insights into its mechanism of action, we examined the effects of I3C on colon carcinogenesis, cell proliferation, cell-cycle progression and apoptosis, and on the levels of expression of several cell-cycle control molecules.

Table I. Body, liver, and relative liver weight.

Group no.	Treatment (no. of rats)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM (22)	360.8±18.0[a,b]	11.5±1.2	3.18±0.26[b]
2	AOM → 0.01% I3C (17)	367.6±36.0	12.4±1.0[b]	3.39±0.32[b]
3	AOM → 0.05% I3C (18)	377.4±12.7[b]	12.7±0.8[b]	3.36±0.22[b]
4	AOM → 0.01% DIM (18)	369.8±21.1	12.2±1.4	3.28±0.27
5	AOM → 0.05% DIM (17)	386.7±18.5[b]	13.2±1.2[b]	3.41±0.21[b]
6	0.05% I3C (8)	380.3±14.0	13.2±0.6	3.48±0.10[b]
7	0.05% DIM (8)	390.7±12.6	12.0±1.1	3.08±0.26
8	No treatment (8)	389.4±21.3	11.4±1.2	2.93±0.18

[a]Mean ± SD. [b]Statistically significant.

Table II. Incidence, multiplicity, and volume of colon tumors.

Group no.	Treatment	No. of rats examined	Incidence (no. of rats bearing tumors)			Mutiplicity (no. of tumors/rat, mean ± SD)			Volume of tumor (mm³, mean ± SD)
			Total (%)	Ad (%)	Adc (%)	Total	Ad	Adc	Adc
1	AOM	22	19 (86)	8 (36)	15 (68)	1.36±0.90	0.45±0.67	0.91±0.75	100.9±131.9
2	AOM → 0.01% I3C	17	14 (82)	2 (12)	13 (76)	1.58±1.17	0.11±0.33	2.09±2.59[a]	211.5±187.4[a]
3	AOM → 0.05% I3C	18	17 (94)	11 (61)	16 (89)	2.72±1.01[b]	0.72±0.73	2.00±1.03[c]	212.5±268.6
4	AOM → 0.01% DIM	18	15 (83)	3 (17)	12 (67)	1.33±0.90	0.27±0.75	1.06±0.99	88.9±68.3
5	AOM → 0.05% DIM	17	13 (76)	5 (29)	10 (59)	1.35±1.05	0.50±0.68	1.00±1.00	187.3±225.2
6	0.05% I3C	8	0	0	0	0	0	0	0
7	0.05% DIM	8	0	0	0	0	0	0	0
8	No treatment	8	0	0	0	0	0	0	0

[a]Significantly different from group 1 by Welch's t-test (P<0.05). [b]Significantly different from group 1 by Student's t-test (P<0.00001). [c]Significantly different from group 1 by Student's t-test (P<0.0002). Ad, adenoma; Adc, adenocarcinoma.

Materials and methods

Animals. Four-week-old male F344 rats were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). All rats were housed 3-4/wire cage with free access to tap water and basal diet (CE2, Clea Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2˚C). The rats were maintained in the Animal Facility of the Kanazawa Medical University according to the Institutional Animal Care Guidelines.

Treatment. I3C (>97% pure) and its dimer 3,3'-diindolylmethane (DIM) (>97% pure) were purchased from Sigma Chemical Company (St. Louis, MO) and LKT Labs. (St. Paul, MN), respectively. Experimental diets were made on a weekly basis by mixing I3C or DIM powder at doses of 0.01 and 0.05% (w/w) with a powdered CE-2 basal diet and stored in a cold room (<4˚C) until used. One hundred and sixteen rats (5 weeks old) were randomly assigned to eight experimental groups as shown in Tables I and II. Rats in groups 1-5 were given subcutaneous (s.c.) injections of AOM (20 mg/kg body weight) (Sigma) once a week for 2 weeks. One week after the last injection of AOM, rats in groups 2 and 3 were fed a diet containing 0.01 and 0.05% I3C, respectively, for 35 weeks, and rats in groups 4 and 5 were fed a diet containing 0.01 and 0.05% DIM, respectively, for 35 weeks. Rats in groups 6 and 7 were fed a diet containing 0.05% I3C and DIM, respectively, throughout the experiment. Rats in group 8 were given the basal diet alone and served as untreated controls. The rats

were carefully observed and weighed weekly during the experiment. Consumption of the experimental diets was also recorded to estimate the intake of the test compounds. The experiment was terminated at 38 weeks after the start of the experiment. All animals were then euthanized under CO_2 anesthesia and their colons were carefully removed, washed with saline, opened longitudinally and then fixed with 10% buffered formalin. Colon tumors were noted grossly for their location, number, and size. The mean volume per tumor was calculated using the formula: V (mm^3) = a x $b^2/2$, where V is the volume, and a is the longest and b is the shortest diameter of the tumor (11). All of the identified colon tumors were carefully removed and processed for histopathological examination and immunohistochemical analysis. Colon tumors were histopathologically diagnosed based on the criteria described elsewhere (11,12). Liver was weighed and processed for histopathological examination.

Immunohistochemical staining and measurement of PCNA labeling index. These assays were performed using an established method as described elsewhere (13). In brief, 4-μm-thick paraffin sections were prepared to include the colon tumor or adjacent normal mucosa. These sections were treated in 3% H_2O_2 for 20 min to block the endogenous peroxidase activity and then incubated with a primary antibody of the proliferating cell nuclear antigen (PCNA) (1:50 dilution) (Dako Co. Ltd. Japan, Kyoto, Japan) at room temperature for 60 min. Sections were then stained using a Simple Stain kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. PCNA was measured in cells consisting of the colon tumor or normal mucosa. The PCNA labeling index (PCNA LI) was determined by calculating the ratio of PCNA-positive nuclei/total number of nuclei counted as described in a recent report (13). More than 4 adenocarcinomas were examined in each treatment group and >300 cells were counted in each lesion.

Detection of apoptosis. Because the standard for determination of apoptosis has been set through observation of characteristic morphological changes by either electron microscopy or light microscopy (14), we evaluated the apoptotic cells in sections stained with hematoxylin and eosin (HE) using light microscopy, as performed in a previous study (15). Apoptotic cells are identified by cell shrinkage, nuclear condensation, and apoptotic body formation. Some apoptotic cells contain pyknotic chromatin and lack a nuclear component (16). Apoptotic index, which represents the percentage of cells exhibiting apoptosis, was determined by counting at least 300 cells in randomly chosen fields of the colon adenocarcinoma (14,17). All sections were scored by one investigator who was blind to the experimental listing. More than 3 adenocarcinomas were examined in each treatment group. In cell cultures, apoptosis was detected by observing DNA fragmentation on agarose gel electrophoresis. In brief, after treatment of cells with the indicated concentrations of I3C for 48 h, both adherent and floating cells were harvested, centrifuged, and washed twice with phosphate-buffered saline (PBS). The cell pellet was then homogenized in 400 μl of 50 mM SEDTA (0.1 M NaCl and 50 mM EDTA). After supplementation of 1% sodium dodecyl sulfate (SDS), the homogenate was digested with proteinase K and then extracted twice with phenol and chloroform, and DNA was precipitated with ethanol. After RNase treatment, DNA fragmentation was visualized by agarose gel electrophoresis and ethidium bromide staining. In addition, apoptosis was also detected by flow cytometry analysis as described in this section.

Cell lines and cell culture. The HCT116 and HT29 human colon carcinoma cell lines were generously provided by Dr I. Bernard Weinstein (Columbia University College of Physicians and Surgeons, NY) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen) in an incubator with humidified air at 37°C with 5% CO_2.

Cell proliferation assays. Cell proliferation assays were performed as described previously (18). To determine cell viability, exponentially dividing $2x10^4$ cells were plated into 6-well/35-mm diameter culture dishes and treated with the indicated concentrations of I3C in DMEM plus 10% FBS for 48 h. Each concentration of I3C was tested in triplicate. After trypsinization, the number of attached viable cells was counted using a Coulter Counter (Beckman Coulter Co., Fullerton, CA). As an untreated solvent control, cells were treated with dimethyl sulfoxide (DMSO) (Sigma), at a final concentration of <0.1%. Results were expressed as a percentage of growth, with 100% representing control cells treated with DMSO alone.

Flow cytometry analysis. Flow cytometry analysis was performed as described in a recent report (19). HCT116 and HT29 cells were plated onto 10-cm-diameter culture dishes ($0.5x10^5$ cells/dish) in DMEM plus 10% FBS and cultured to yield 50-60% confluence. Cells were then treated with DMSO (<0.1%) or increasing concentrations (0.1-1,000 μM) of I3C. After treatment of cells for 48 h, both adherent and floating cells were collected, fixed with 70% ethanol, centrifuged, resuspended in 400 μl of PBS containing 2 mg/ml RNase (Sigma), and stained with 400 μl of 0.1 mg/ml propidium iodide (PI) (Sigma). The cell suspension was filtered through a 60 μm nylon filter (Ikemoto Scientific Technology Co. Ltd., Tokyo). Samples of 10,000-20,000 cells were then analyzed for DNA histograms and cell-cycle phase distributions by flow cytometry using a FACScalibur instrument (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed using the CellQuest computer program (Becton Dickinson), as previously described (20). The percentage of cells that had undergone apoptosis was also determined as the sub-G1 fraction of at least 10,000 cells using the above-described PI staining method. All experiments were conducted in duplicate and yielded similar results.

Reverse transcription-PCR (RT-PCR) analysis. These assays were performed by established procedures (19). Total RNA was isolated from frozen cells using a TRIzol reagent (Invitrogen Life Technologies) as recommended by the manufacturer. cDNA was amplified from total RNA (1.0 μg) using a SuperScript One-Step RT-PCR system (Invitrogen Life Technologies). PCR was conducted for 35-40 cycles in a GeneAmp PCR system 9700 (Applied

Table III. PCNA labeling and apoptotic indices of adenocarcinoma.

Group no.	Treatment	PCNA labeling index (no. of adenocarcinomas examined)	Apoptotic index (no. of adenocarcinomas examined)
1	AOM	35.4±4.9% (6)	6.8±2.8% (3)
2	AOM → 0.01% I3C	42.9±8.6% (4)	4.8±3.2% (5)
3	AOM → 0.05% I3C	45.5±6.1%[a] (7)	3.8±1.4%[b] (7)
4	AOM → 0.01% DIM	48.2±9.1%[c] (5)	4.3±1.8% (6)
5	AOM → 0.05% DIM	39.7±2.7% (4)	3.8±0.8% (5)

Significantly different from group 1 by Student's t-test ([a]P<0.008, [b]P<0.04, and [c]P<0.02).

Biosystems, Foster City, CA). The primers (19) used for amplification were as follows: cyclin D1-specific primer set, CD1F (5'-CTG GCC ATG AAC TAC CTG GA-3') and CD1R (5'-GTC ACA CTT GAT CAC TCT GG-3'); p21[CIP1]-specific primer set, C1F (5'-CTC AGA GGA GGC GCC ATG TCA-3') and C2R (5'-GCC GTT TTC GAC CCT GAG AGT-3'); and p27[KIP1]-specific primer set, K2F (5'-GCT CAC GGC TCT GCG ACT CC-3') and K1R (5'-GGG CTC CCG TTA GAC ACT CG-3'). ß-actin-specific PCR products for the same RNA samples were simultaneously amplified and served as internal controls. Primers, FBA (5'-CCA GGC ACC AGG GCG TGA TG-3') and RBA (5'-CGG CCA GCC AGG TCC AGA CG-3'), were used for amplification of ß-actin. Each amplification cycle consisted of 0.5 min at 94˚C for denaturing, 0.5 min at 55˚C for primer annealing, and 1 min at 72˚C for extension. In all of the amplification procedures we included RT-free control assays consisting of the amplification cocktail, the RNA sample and distilled water in place of RT, to check for possible contamination of the RNA samples with DNA. After PCR amplification, the fragments were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The results were confirmed by repeating experiments.

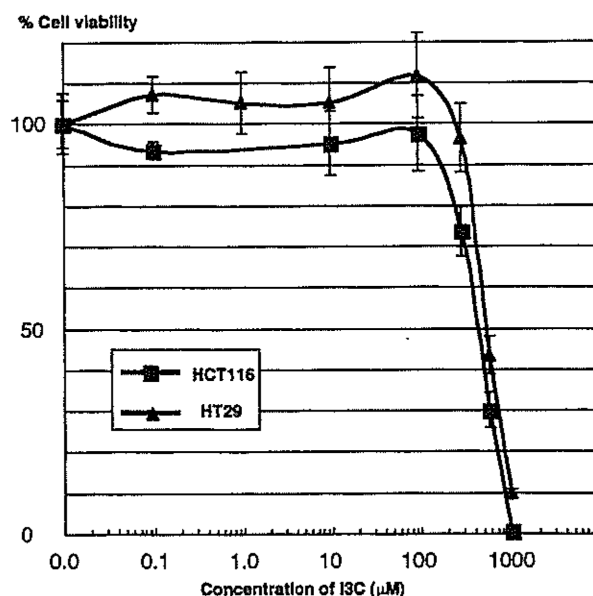

Figure 1. Inhibition of cell growth by I3C in HCT116 and HT29 human colon carcinoma cell lines. Cells were treated with the indicated concentrations of I3C for 48 h in DMEM containing 10% FBS. Results were expressed as a percentage of growth, with 100% representing control cells treated with DMSO alone. Each concentration of I3C was examined in triplicate and gave similar results.

Statistical analysis. Tumor incidence, multiplicity, and volume were compared between the animals of group 1 and those of groups 2-5. Tumor incidence was analyzed by Fisher's exact probability test, and tumor multiplicity was analyzed by Student's or Welch's t-test. Differences in tumor volume, body weight, relative liver weight, PCNA LI, and apoptotic index between groups were also analyzed by Student's or Welch's t-test. All statements of significance are P<0.05.

Results

General observation. A total of 116 rats survived the experiment. No macroscopic metastases were observed in any of these rats. To examine the effect of I3C or DIM on body weight gain, the rats were monitored on a routine basis. The body, liver, and relative liver weights are shown in Table I. The body weights of groups 1, 3, and 5 were significantly lower than those of group 8 (untreated control) (P<0.005). The liver weights of groups 2, 3, and 5 were significantly higher than those of group 8 (P<0.05). Also, the relative liver weights of groups 1-3, 5, and 6 were significantly higher than those of group 8 (P<0.05). These data indicate a toxic effect of AOM on the carcinogen-treated animals. Also, an increase in relative liver weight of AOM-treated animals is presumably due to decreased body weight. After AOM injection, treatment of rats with either I3C (groups 2 and 3) or DIM (groups 4 and 5) did not cause significant body weight loss or liver weight loss, when compared to the rats treated with AOM alone (group 1). This result indicates that I3C or DIM did not affect body weight. No symptomatic side effects were found in any of the rats.

Histology. The tumors were macroscopically sessile or pedunculated in shape. They were histologically well-differentiated tubular adenocarcinomas. Adenocarcinomas mainly showed tubular or papillary growth with respect to the structure of glandular formation as described elsewhere (11). A small number of mucinous or signet-ring cell carcinoma were found. In liver tissues, centrilobular hypertrophy of hepatocytes (21) was observed in the rats treated with I3C or DIM.

I3C causes an increase in colon tumor multiplicity and volume. In the present study, we used the well-established

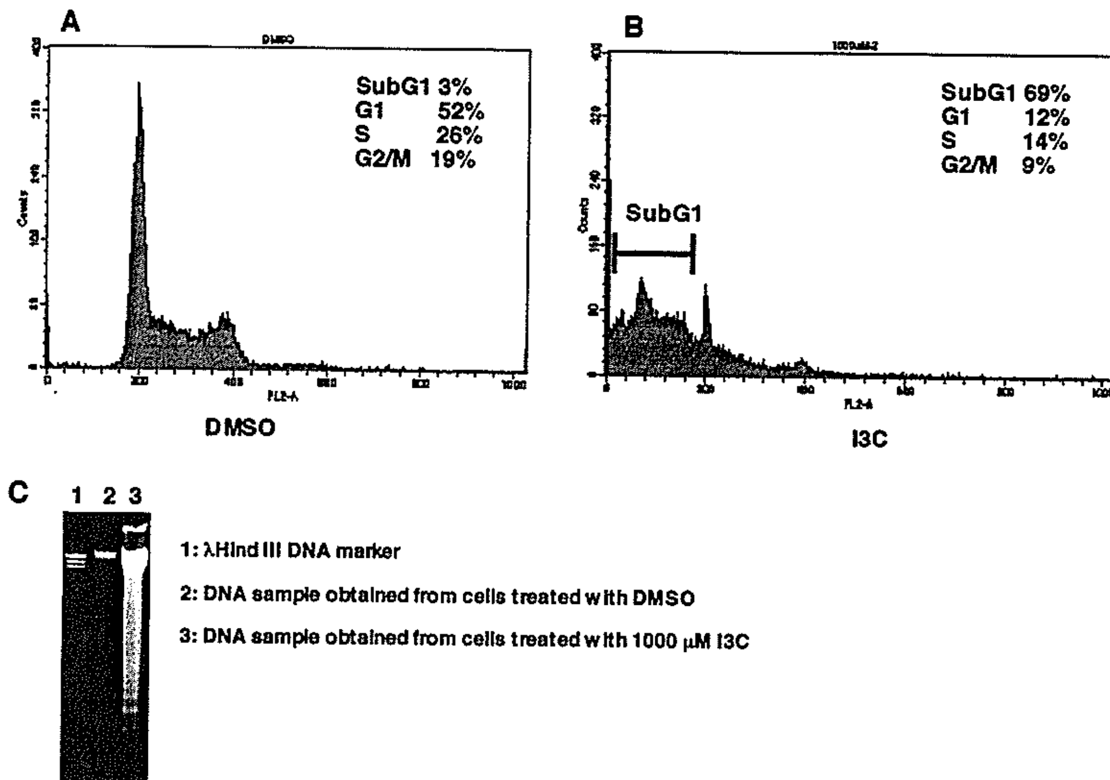

Figure 2. Apoptosis induction by I3C. HCT116 cells were treated with DMSO (A) or 1,000 μM I3C (B) for 48 h in DMEM plus 10% FBS. The data indicate the percentage of cells in the indicated phase of the cell cycle. Note evidence of apoptosis by an increase in the sub-G1 population of cells. DNA fragmentation was seen in agarose gel electrophoresis (C). DNA was extracted from cells treated with DMSO or 1,000 μM I3C.

and long-term protocol of the rat colon carcinogenesis model system to examine the effect of I3C. We also examined the effects of DIM because this compound is a biologically active dimmer of I3C (22). In this system, we treated rats with AOM to induce colon tumors and fed them with I3C or DIM during the post-initiation phase. We also used 0.01 and 0.05% dose levels of I3C in their diets because, at this dose range, we found obvious effects of dietary I3C on uterine adenocarcinoma (21). This compound can be easily converted to a biologically active dimer DIM under acidic conditions in gastrointestinal tracts (23).

Tumor incidence (number of rats with tumors), multiplicity (number of tumors per rat), and volume are shown in Table II. After AOM injection, treatment of rats with both dose levels [0.01% (group 2) and 0.05% (group 3)] of I3C caused a significant increase in tumor multiplicity of adenocarcinomas by 2.2- ($P<0.05$ for 0.01% I3C) and 2.1-fold ($P<0.0002$ for 0.05% I3C) respectively, when compared to the control rats (group 1), although both I3C and DIM did not affect the incidence of colon tumors. In group 3, tumor multiplicity of adenoma plus adenocarcinoma and volume of adenocarcinoma were also increased by 2.0- ($P<0.00001$) and 2.1-fold ($P<0.05$) compared to the control (group 1). In DIM-treated groups (groups 2 and 3), however, there was not a significant difference in the incidence, multiplicity, and volume of colon tumors compared to the control (group 1). These results indicate that I3C increases tumor multiplicity and volume, thereby enhancing the colon carcinogenesis induced by AOM in F344 rats.

I3C increases PCNA LI and decreases the apoptotic index of colon adenocarcinoma. In view of the above-mentioned tumor-enhancing effect, we were interested in determining whether I3C affects cell proliferation and apoptosis by measuring PCNA and apoptotic indices. PCNA LI of adenocarcinoma in groups 3 and 4 was significantly higher than that in group 1 ($P<0.008$ for group 3, $P<0.02$ for group 4) (Table III). PCNA LI of normal mucosa in rats of groups 6-8 was 21.7±5.48, 26.3±9.33, and 19.2±5.9, respectively (these values are not shown in Table III). Whereas, the apoptotic index of adenocarcinoma in group 3 was significantly lower by about 1.8-fold than that in group 1 ($P<0.05$) (Table III). Similar results were also obtained with ssDNA immunohistochemical staining (data not shown). These results indicate that, after injection of AOM, treatment of rats with I3C increased cell proliferation and decreased apoptosis in the colon adenocarcinoma.

I3C inhibits the growth of human colon carcinoma cells and induces G1 cell-cycle arrest and apoptosis. Because we found that I3C enhances cell proliferation and inhibits apoptosis *in vivo*, it was of interest to examine the effects of I3C on cell growth, cell-cycle progression, and apoptosis in exponentially dividing cell cultures of human colon carcinoma cell lines. HCT 116 and HT29 cells were treated with DMSO (control) or increasing dose levels (0.1-1,000 μM) of I3C. In these cell lines, I3C caused a marked growth inhibition in a dose-dependent fashion, with IC_{50} values of about 400 and 500 μM, respectively, when cells were grown in DMEM plus 10%

Table IV. Changes in cell-cycle progression in HCT116 cells.

	Concentration of I3C (μM)					
	0	1	10	100	500	1,000
Sub-G1	3	3	3	3	4	69
G1	52	50	53	53	61	12
S	26	24	21	23	13	14
G2/M	19	23	23	21	22	9

The data indicate the percentage of cells in each phase of the cell cycle. HCT116 cells were treated with the indicated concentrations of I3C in DMEM/10% FBS for 48 h and flow cytometry analysis was performed. All experiments were conducted in duplicate and gave similar results.

FBS for 48 h (Fig. 1). Because of the effects on cell growth seen in Fig. 1, we further examined the effects of I3C on cell-cycle progression in the HCT116 cell line. A representative histogram for the HCT116 cells treated with DMSO alone is shown in Fig. 2A, and the cell-cycle distribution data obtained in the same cell line are summarized in Table IV. When HCT116 cells were treated with 500 μM I3C, there was a marked increase (about 10% increase) of cells in the G1 phase of the cell cycle after 48 h of treatment, with a concomitant decrease of cells in the S and G2-M phases (Table IV). When these cells were treated with a higher concentration (1,000 μM) of I3C for 48 h, they began to detach from the bottom of the culture plate and display evidence of apoptosis as detected in a prominent sub-G1 fraction (Table IV, Fig. 2B). The appearance of a 'DNA ladder' was seen in a sample treated with 1,000 μM I3C on agarose gel electrophoresis (Fig. 2C). Similar results were also obtained with the HT29 cell line (data not shown). These results suggest that I3C inhibits the growth of human colon carcinoma cells in a dose-dependent manner and induces G1 cell-cycle arrest and apoptosis.

I3C causes a 2- to 4-fold increase in the cellular levels of $p27^{KIP1}$ and $p21^{CIP1}$ mRNA. In the above studies, we found that I3C arrests colon carcinoma cells in the G1 phase of the cell cycle after 48 h of treatment. Thus, we performed RT-PCR analysis to determine whether treatment of these cells with I3C affects the level of expression of cyclin D1, $p21^{CIP1}$, and $p27^{KIP1}$ mRNA. As shown in Fig. 3, we treated HT29 cells with increasing doses (1-500 μM) of I3C in DMEM plus 10% FBS. We then isolated RNA at 48 h after the addition of the compound. To quantify the expression levels of mRNA, PCR products were generated during both plateau and log-phase reactions by conducting 35-40 cycles of PCR as described in our recent work (19). With this approach, these products have been shown to reflect corresponding levels of mRNA by northern blot assays (19,20). There was about a 4-fold increase in the $p27^{KIP1}$ band intensity in samples treated with 10, 100, and 500 μM I3C, and also about a 4-fold increase in the $p21^{CIP1}$ band intensity in samples treated with 100 and 500 μM I3C, when compared to that of samples treated with DMSO alone (Fig. 3). I3C also caused about a 2-fold increase in the $p27^{KIP1}$ and $p21^{CIP1}$ band intensities in samples

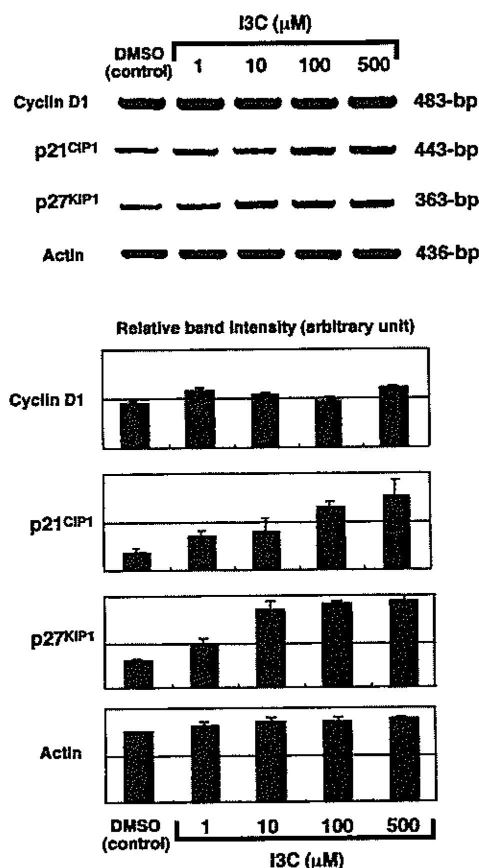

Figure 3. Effects of I3C on mRNA expression of cell-cycle control molecules. Upper panel, representative results of RT-PCR assays. HT29 cells were treated with DMSO (control) or the indicated concentrations of I3C for 48 h in DMEM containing 10% FBS. RNA samples were then analyzed by RT-PCR using the pairs of primers shown in Materials and methods. Actin was used as an internal control. The results were confirmed by repeating experiments. Lower panel, densitometric analysis representing relative band intensity in each RNA sample. The results are the means ± SD from repeated experiments.

treated with 1 μM I3C and 1 and 10 μM I3C, respectively. However, RT-PCR assays for cyclin D1 mRNA indicated no appropriate change in cellular levels of mRNA in samples treated with 1-500 μM I3C (Fig. 3). These results suggest that, at doses ranging from 10-100 μM, I3C appears to increase the level of expression of $p27^{KIP1}$ and $p21^{CIP1}$ mRNA. Taken together with the results of the flow cytometry analysis, at doses around its IC_{50} value, I3C causes a G1 cell-cycle arrest by inducing $p27^{KIP1}$ and $p21^{CIP1}$ mRNA expression in human colon carcinoma cells.

Discussion

In recent decades, there has been considerable interest in naturally occurring phytochemicals in the treatment of human diseases. Despite the reported benefits of these compounds in individual patients, it still remains unclear whether these agents have anti-cancer properties. Also, the precise mechanisms by which phytochemical I3C promotes or inhibits carcinogenesis are not known. Therefore, it was of interest to examine the effects of I3C on rat colon carcinogenesis and human colon carcinoma cells. In our initial studies, we found that, in an AOM-induced rat colon carcinogenesis bioassay system, I3C

significantly increased colon tumor multiplicity and volume when animals were fed with I3C during the post-initiation phase. Similar tumor-promoting effects of post-initiation I3C treatment have been seen by Pence *et al* (9) and Temple and El-Khatib (10) with male F344 rats and Swiss mice respectively. Bailey *et al* (24) also demonstrated that I3C feeding, before and during (initiation phase) aflatoxin B1 (AFB_1) exposure in rainbow trout, inhibited hepatocarcinogenesis but, after (post-initiation phase) the carcinogen AFB1 treatment, I3C strongly promoted hepatocarcinogenesis. In addition, the extent of promotion increased linearly with time of dietary I3C post-initiation exposure (25,26). As mentioned above, although treatment of animals with I3C led to a promotion in tumor multiplicity and volume, dietary I3C initiation exposure caused an inhibition of the development of colon, liver, breast, tongue, and forestomach tumors in rodents or trout (3-5,27-30). In some of these studies, I3C significantly inhibited the occurrence of HCA or AOM-induced ACF in the rat colon, using initiation or post-initiation protocols (3-5). These results suggest that the exposure time-point (initiation or post-initiation) affects the ambivalent modulatory effects of I3C on colon carcinogenesis. Also, in the present study, F344 rats received s.c. injections of AOM (20 mg/kg body weight) once a week for 2 weeks and 0.05% dietary I3C was administered for a total of 35 weeks. Using this protocol, I3C enhanced colon carcinogenesis. Whereas, in a previous study by Xu *et al* (4), F344 rats were given HCA by oral gavage (50 mg/kg body weight) and animals had continuous exposure of 0.1% I3C through their diets for 8 weeks, resulting in a decrease of ACF formation. Therefore, the characteristics of rat colon carcinogenesis may depend on the cumulative dose and duration of I3C treatment, and/or the carcinogen used for cancer development and the method of administrating the carcinogens.

While analyzing the effects of I3C on the cell proliferation activity in the PCNA LI assays, we found that in AOM-induced colon adenocarcinomas the PCNA LI significantly increased by about 10% ($P<0.008$) after 0.05% I3C treatment, demonstrating that I3C enhances cell proliferation in these lesions. In addition, dietary I3C caused a significant decrease in apoptotic index ($P<0.05$) in randomly chosen colon adenocarcinomas. These findings, together with our evidence that treatment of rats with I3C significantly increases the multiplicity ($P<0.05$) and volume ($P<0.05$) of colon adenocarcinoma induced by AOM, suggest that dietary I3C promotes AOM-induced colon carcinogenesis by inhibiting the apoptosis of adenocarcinoma. Furthermore, the indole compounds including I3C can form mutagenic *N*-nitroso products by treatment with nitrite under acidic conditions (31,32), suggesting potential genotoxic and tumor-promoting activity *in vivo*. DIM has recently been shown to inhibit growth and induce apoptosis in MCF10CA1a human breast and C33A human cervical carcinoma cells (33,34). Its derivatives also inhibit the growth of HT29 and HCT15 human colon carcinoma cells (35). Therefore, these DIM's apoptosis-inducing and growth-inhibitory activities may contribute to the present results that dietary DIM did not affect either tumor multiplicity or volume despite this compound increased PCNA LI (Tables II and III). However, these aspects warrant further study.

In view of the above-mentioned growth-enhancing and apoptosis-inhibiting effects of I3C, we were interested in investigating the effects of I3C on cell growth, cell cycle progression, apoptosis, and the levels of expression of several cell-cycle control molecules after treating human colon carcinoma cells with I3C. In the present study, we found that I3C exerted a marked and dose-dependent inhibition on the growth of the HCT116 and HT29 human colon carcinoma cell lines, with IC_{50} values of about 400 and 500 μM, respectively, when the cells were grown in DMEM containing 10% FBS. These values are consistent with those reported to exert biological activity in a range of other human colon carcinoma cell lines (600 μM for LS174 and CaCo-2 cell lines) and immortalized human colon epithelial cells (>1,000 μM for HCEC cell line) (8) when they were grown in conditions similar to that of our study. In addition, I3C has recently been shown to inhibit cell growth after treatment with 30-300 μM in MCF7 [estrogen receptor (ER)-positive] and MDA-MB-435 (ER-negative) human breast (36-38), CaSki and C33A cervical (33), and PC3 prostate (39) carcinoma cell lines. Therefore, our present findings together with the above-mentioned recent data demonstrate the broad range of anti-cancer properties of I3C in human carcinoma cell lines.

To further characterize the effects of I3C on cell progression, we examined whether I3C arrests cells in specific phases of the cell cycle using flow cytometry analysis. Treatment of HCT116 cells with 500 μM I3C for 48 h resulted in 61% of cells remaining in G1 phase, in comparison to DMSO-treated control cells in which 51% of cells remained in G1. This result is in accordance with a body of evidence that I3C induces G1 cell-cycle arrest in MCF7 human breast carcinoma cells (37) when cells are treated with 100 μM I3C for 96 h. In contrast, combination treatment of I3C and polyamine putrescine in the SW480 human colon carcinoma cell line caused a slight increase in the proportion of cells in the G2-M phase of the cell cycle (7). Thus, the effects of I3C on cell-cycle progression may vary in different experimental systems. We have further investigated whether, in human colon carcinoma cells, I3C induces apoptosis by detecting a sub-G1 fraction of cells using flow cytometry analysis and by observing DNA fragmentation using agarose gel electrophoresis. We found that I3C induces apoptosis in the HCT116 cell line, after treatment of cells with 1,000 μM I3C for 48 h (Fig. 2B and C). Our results are consistent with a previous study demonstrating the apoptotic cell death-inducing activity of I3C in MDA-MB-435 human breast and PC3 prostate carcinoma cells, when these cells were treated with 30-60 μM and 100 μM I3C, respectively (38,39).

Because we found that I3C arrests human colon carcinoma cells in the G1 phase of the cell cycle, with a concomitant decrease of cells in the S and G2-M phases, we have further examined I3C's molecular mechanism of action in the G1 progression of the cell cycle. Cyclin D1 complexes with cyclin-dependent kinase (cdk)-4 and cdk-6 and thereby regulates transition from G1 to S phase (40). The activities of these cyclin D1/cdk complexes are negatively regulated by the cdk inhibitors (CDKIs), including $p21^{CIP1}$ and $p27^{KIP1}$ (40). Therefore, we examined the effects of I3C on the level of mRNA expression of cyclin D1, $p21^{CIP1}$, and $p27^{KIP1}$ by performing RT-PCR assays. RT-PCR analysis indicated that

the I3C-treated HT29 cells displayed a 2- to 4-fold increase in their levels of expression of $p27^{KIP1}$ and $p21^{CIP1}$ mRNA at 48 h after the addition of the compound (Fig. 3). However, the I3C-treated cells did not display changes in the level of mRNA expression of cyclin D1. Similar findings have been previously demonstrated in MCF7 human breast and PC3 prostate carcinoma cells (22,36,37). These findings suggest that $p27^{KIP1}$ and $p21^{CIP1}$ play a role in G1 cell-cycle arrest and thereby further contribute to I3C-induced growth inhibition of human colon carcinoma cells. In the present study, we found that I3C inhibits the growth of human colon carcinoma cells but enhances AOM-induced rat colon carcinogenesis. Hence, the present study may provide advisory information for the use of I3C in cancer chemoprevention and/or clinical therapy trials. Nevertheless, the precise reasons why I3C possesses an ambivalent modulatory response to colon carcinogenesis remains to be solved. The CYP1 family, which has recently been shown to play critical roles in I3C-mediated apoptosis induction (8) or carcinogenesis promotion (21), may be affected. This aspect warrants further investigation.

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References

1. Surh YJ: Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3: 768-780, 2003.
2. Jongen WM: Glucosinolates in Brassica: occurrence and significance as cancer-modulating agents. Proc Nutr Soc 55: 433-446, 1996.
3. Wargovich MJ, Chen CD, Jimenez A, Steele VE, Velasco M, Stephens LC, Price R, Gray K and Kelloff GJ: Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. Cancer Epidemiol Biomarkers Prev 5: 355-360, 1996.
4. Xu M, Bailey AC, Hernaez JF, Taoka CR, Schut HA and Dashwood RH: Protection by green tea, black tea, and indole-3-carbinol against 2-amino-3-methylimidazo[4,5-f]quinoline-induced DNA adducts and colonic aberrant crypts in the F344 rat. Carcinogenesis 17: 1429-1434, 1996.
5. Guo D, Schut HA, Davis CD, Snyderwine EG, Bailey GS and Dashwood RH: Protection by chlorophyllin and indole-3-carbinol against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced DNA adducts and colonic aberrant crypts in the F344 rat. Carcinogenesis 16: 2931-2937, 1995.
6. Frydoonfar HR, McGrath DR and Spigelman AD: Inhibition of proliferation of a colon cancer cell line by indole-3-carbinol. Colorectal Dis 4: 205-207, 2002.
7. Hudson EA, Howells LM, Gallacher-Horley B, Fox LH, Gescher A and Manson MM: Growth-inhibitory effects of the chemopreventive agent indole-3-carbinol are increased in combination with the polyamine putrescine in the SW480 colon tumour cell line. BMC Cancer 3: 2, 2003.
8. Bonnesen C, Eggleston IM and Hayes JD: Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res 61: 6120-6130, 2001.
9. Pence BC, Buddingh F and Yang SP: Multiple dietary factors in the enhancement of dimethylhydrazine carcinogenesis: main effect of indole-3-carbinol. J Natl Cancer Inst 77: 269-276, 1986.
10. Temple NJ and El-Khatib SM: Cabbage and vitamin E: their effect on colon tumor formation in mice. Cancer Lett 35: 71-77, 1987.
11. Suzui M, Okuno M, Tanaka T, Nakagama H and Moriwaki H: Enhanced colon carcinogenesis induced by azoxymethane in min mice occurs via a mechanism independent of ß-catenin mutation. Cancer Lett 183: 31-41, 2002.
12. Ward JM: Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. Lab Invest 30: 505-513, 1974.
13. Morioka T, Suzui M, Nabandith V, Inamine M, Aniya Y, Nakayama T, Ichiba T and Yoshimi N: Modifying effects of Terminalia catappa on azoxymethane-induced colon carcinogenesis in male F344 rats. Eur J Cancer Prev 14: 101-105, 2005.
14. Samaha HS, Kelloff GJ, Steele V, Rao CV and Reddy BS: Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: apoptotic index as a biomarker in colon cancer chemoprevention and promotion. Cancer Res 57: 1301-1305, 1997.
15. Tanaka T, Kohno H, Murakami M, Kagami S and El-Bayoumy K: Suppressing effects of dietary supplementation of the organoselenium 1,4-phenylenebis(methylene)selenocyanate and the Citrus antioxidant auraptene on lung metastasis of melanoma cells in mice. Cancer Res 60: 3713-3716, 2000.
16. Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV and Reddy BS: Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res 59: 597-601, 1999.
17. Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S, Sumida T, Azuma Y and Ogawa H: Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices rich in beta-cryptoxanthin and hesperidin. Int J Cancer 88: 146-150, 2000.
18. Shimizu M, Suzui M, Deguchi A, Lim JT and Weinstein IB: Effects of acyclic retinoid on growth, cell cycle control, epidermal growth factor receptor signaling, and gene expression in human squamous cell carcinoma cells. Clin Cancer Res 10: 1130-1140, 2004.
19. Suzui M, Shimizu M, Masuda M, Lim JT, Yoshimi N and Weinstein IB: Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells. Mol Cancer Ther 3: 309-316, 2004.
20. Suzui M, Masuda M, Lim JT, Albanese C, Pestell RG and Weinstein IB: Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. Cancer Res 62: 3997-4006, 2002.
21. Yoshida M, Katashima S, Ando J, Tanaka T, Uematsu F, Nakae D and Maekawa A: Dietary indole-3-carbinol promotes endometrial adenocarcinoma development in rats initiated with N-ethyl-N'-nitro-N-nitrosoguanidine, with induction of cytochrome P450s in the liver and consequent modulation of estrogen metabolism. Carcinogenesis 25: 2257-2264, 2004.
22. Kim YS and Milner JA: Targets for indole-3-carbinol in cancer prevention. J Nutr Biochem 16: 65-73, 2005.
23. Grose KR and Bjeldanes LF: Oligomerization of indole-3-carbinol in aqueous acid. Chem Res Toxicol 5: 188-193, 1992.
24. Bailey GS, Hendricks JD, Shelton DW, Nixon JE and Pawlowski NE: Enhancement of carcinogenesis by the natural anticarcinogen indole-3-carbinol. J Natl Cancer Inst 78: 931-934, 1987.
25. Dashwood RH, Fong AT, Williams DE, Hendricks JD and Bailey GS: Promotion of aflatoxin B1 carcinogenesis by the natural tumor modulator indole-3-carbinol: influence of dose, duration, and intermittent exposure on indole-3-carbinol promotional potency. Cancer Res 51: 2362-2365, 1991.
26. Dashwood RH: Indole-3-carbinol: anticarcinogen or tumor promoter in brassica vegetables? Chem Biol Interact 110: 1-5, 1998.
27. Wattenberg LW and Loub WD: Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. Cancer Res 38: 1410-1413, 1978.
28. Bresnick E, Birt DF, Wolterman K, Wheeler M and Markin RS: Reduction in mammary tumorigenesis in the rat by cabbage and cabbage residue. Carcinogenesis 11: 1159-1163, 1990.
29. Tanaka T, Kojima T, Morishita Y and Mori H: Inhibitory effects of the natural products indole-3-carbinol and sinigrin during initiation and promotion phases of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Jpn J Cancer Res 83: 835-842, 1992.

30. Tanaka T, Mori Y, Morishita Y, Hara A, Ohno T, Kojima T and Mori H: Inhibitory effect of sinigrin and indole-3-carbinol on diethylnitrosamine-induced hepatocarcinogenesis in male ACI/N rats. Carcinogenesis 11: 1403-1406, 1990.
31. Sasagawa C and Matsushima T: Mutagen formation on nitrite treatment of indole compounds derived from indole-glucosinolate. Mutat Res 250: 169-174, 1991.
32. Tiedink HG, Davies JA, Visser NA, Jongen WM and van Broekhoven LW: The stability of the nitrosated products of indole, indole-3-acetonitrile, indole-3-carbinol and 4-chloroindole. Food Chem Toxicol 27: 723-730, 1989.
33. Chen DZ, Qi M, Auborn KJ and Carter TH: Indole-3-carbinol and diindolylmethane induce apoptosis of human cervical cancer cells and in murine HPV16-transgenic preneoplastic cervical epithelium. J Nutr 131: 3294-3302, 2001.
34. Rahman KW and Sarkar FH: Inhibition of nuclear translocation of nuclear factor-{kappa}B contributes to 3,3'-diindolylmethane-induced apoptosis in breast cancer cells. Cancer Res 65: 364-371, 2005.
35. Chintharlapalli S, Smith R III, Samudio I, Zhang W and Safe S: 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes induce peroxisome proliferator-activated receptor gamma-mediated growth inhibition, transactivation, and differentiation markers in colon cancer cells. Cancer Res 64: 5994-6001, 2004.
36. Cover CM, Hsieh SJ, Tran SH, Hallden G, Kim GS, Bjeldanes LF and Firestone GL: Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. J Biol Chem 273: 3838-3847, 1998.
37. Cover CM, Hsieh SJ, Cram EJ, Hong C, Riby JE, Bjeldanes LF and Firestone GL: Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells. Cancer Res 59: 1244-1251, 1999.
38. Rahman KM, Aranha O, Glazyrin A, Chinni SR and Sarkar FH: Translocation of Bax to mitochondria induces apoptotic cell death in indole-3-carbinol (I3C) treated breast cancer cells. Oncogene 19: 5764-5771, 2000.
39. Chinni SR, Li Y, Upadhyay S, Koppolu PK and Sarkar FH: Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 20: 2927-2936, 2001.
40. Weinstein IB: Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. Carcinogenesis 21: 857-864, 2000.

Dietary Supplementation with Silymarin Inhibits 3,2′-Dimethyl-4-Aminobiphenyl–Induced Prostate Carcinogenesis in Male F344 Rats

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Abstract **Purpose:** Silymarin has been shown to be a potent anticarcinogenic agent. Here, we investigated the modifying effects of dietary feeding with a naturally occurring polyphenolic antioxidant flavonoid silymarin on 3,2′-dimethyl-4-aminobiphenyl (DMAB)–induced prostatic carcinogenesis in male F344 rats.

Experimental Design: Male F344 rats were given s.c. injections of DMAB (25 mg/kg body weight) every other week for 20 weeks. They also received the experimental diet containing 100 or 500 ppm silymarin for 40 weeks, starting 1 week after the last dosing of DMAB. All of the rats were sacrificed 60 weeks after the start of the experiment. Histopathology and immunohistochemistry for proliferative cell nuclear antigen, cyclin D1, and apoptotic indices were done in the prostatic lesions, including invasive adenocarcinomas, intraepithelial neoplasms, and nonlesional glands.

Results: Dietary feeding with 500 ppm silymarin significantly inhibited the incidence of prostatic adenocarcinoma when compared with the DMAB-alone group (17.6% versus 50.0%, $P < 0.05$). The proliferative cell nuclear antigen– and cyclin D1–positive indices in adenocarcinomas, prostatic intraepithelial neoplasm, and nonlesional glands in rats treated with DMAB and silymarin were slightly lower than that of the DMAB-alone group. Also, dietary administration of silymarin increased apoptotic index in prostatic adenocarcinoma by measuring immunohistochemically positive nuclei for ssDNA.

Conclusions: Our results indicate that silymarin exerts chemopreventive ability against chemically induced prostatic carcinogenesis through apoptosis induction and modification of cell proliferation.

Prostate cancer is the most common type of cancer found in older men and the leading cause of cancer mortality in men (1). In Japan, the incidence and mortality rates of this malignancy are lower compared with Western populations (2), but they have gradually increased (3). Furthermore, migrant studies have shown that the incidence of prostate cancer increases generation by generation after immigration in Japanese-Americans (4). These observations strongly suggest that the wide disparity in prostate cancer incidence worldwide is attributable to dietary habits, among which are a regimen rich in several flavonoids and isoflavones that inhibits the progression of prostate cancer by modulating epigenetic events (5). It is, therefore, necessary to intensify our efforts to better understand this disease and develop novel approaches for its prevention and treatment.

Silymarin, the collective name for an extract from the milk thistle [*Silybum marianum* (L.) Gaertneri], is a naturally occurring polyphenolic flavonoid antioxidant. It is composed mainly of silibinin (~80%, w/w; also called silybin, silibin, or sibilinin) with smaller amounts of other stereoisomers (isosilybin, dihydrosibilyn, silydianin, and silychristin, etc.; ref. 6). Silymarin has strong antioxidative properties and is able to scavenge both free radicals and reactive oxygen species (7, 8). In Europe, for over 20 years, silymarin, as an antihepatotoxic, is used clinically for the treatment of alcoholic liver disease (9, 10). In recent years, silymarin has also been used in Asia as a therapeutic agent for liver diseases (6). Silymarin is well tolerated and largely free of adverse effects (6, 11). Silymarin acts as a potent anticarcinogenic agent against *in vitro* and *in vivo* carcinogenesis experiments (12–14). Silymarin and silibinin, which is the major active constituent of silymarin, can inhibit the growth of human prostate carcinoma LNCaP, PC-3, and DU145 cells in culture (15–17). Moreover, silymarin and

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silibinin inhibit cell growth and induce apoptosis in rat prostate cancer cell lines (18). However, chemoprevention studies using silymarin in rodent were limited to skin (13, 19). Silymarin inhibits tumor promoter-caused induction of ornithine decarboxylase activity and mRNA expression in mouse epidermis (20). Silymarin inhibits mRNA expression of endogenous tumor promoter tumor necrosis factor-α (21). More recently, silymarin has been reported to inhibit activation of erbB1 signaling, induce cyclin-dependent kinase inhibitors, G_1 arrest, and cause complete inhibition of growth of human prostate carcinoma DU145 cells (17). Also, silymarin, at lower nontoxic concentrations, can inhibit transformation in cultured rat tracheal epithelial cells treated with benzo(*a*)pyrene (22). These findings led us to evaluate the possible suppressing effects of dietary silymarin on the occurrence of chemically induced neoplasms in organs other than skin of rodents. We recently have found the inhibitory effects of dietary administration of silymarin against rat tongue (23), mouse urinary bladder (24), and rat colon (25) carcinogenesis.

In the current study, we investigated the effects of silymarin on 3,2'-dimethyl-4-aminobiphenyl (DMAB)-initiated prostate carcinogenesis in male F344 rats. Also, the modulatory effects of the silymarin on the proliferating cell nuclear antigen (PCNA), cyclin D1, and apoptotic indices were immunohitochemically investigated in the prostatic lesions induced by DMAB.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male F344 rats (Charles River Japan, Inc., Kanagawa, Japan) were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in polycarbonate cages (three or four rats per cage) under controlled conditions of humidity (50 ± 10%), lighting (12-hour light/dark cycle), and temperature (23 ± 2°C). They have free access to drinking water (ion exchange water) and a basal diet, CRF-1 (Oriental Yeast, Co., Ltd., Tokyo, Japan) from which soy constituents were eliminated throughout the study. Animals were quarantined for 7 days and randomized by body weight into experimental and control groups. DMAB and silymarin were obtained from Sigma-Aldrich Japan, K.K. (Tokyo, Japan). The experimental diet containing silymarin were prepared by Oriental Yeast by adding test chemicals to soy protein-free CRF-1.

Experimental procedure. A total of 68 male F344 rats were divided into nine experimental and control groups. The animals in groups 1 through 3 were given DMAB dissolved in DMSO, s.c., at a dose of 25 mg/kg body weight every other week for 20 weeks. DMAB injection was done between 10:00 a.m. and 11:00 a.m. From 1 week after the last injection of DMAB, group 1 was given the basal diet without silymarin, groups 2 and 3 received silymarin-containing diets (100 ppm for group 2 and 500 ppm for group 3), and group 4 was fed the diet containing 500 ppm silymarin for 40 weeks. Group 5 served as an untreated control. The doses of the test compounds were selected based on previous studies (23). All rats were sacrificed at week 60 by ether overdose to assess the pathologic lesions in all organs, including prostate. At autopsy, all organs were carefully inspected and all macroscopic pathologic findings were recorded. All grossly abnormal lesions in any tissue and the organs, such as accessory sex organs including prostate, liver, kidney, lung, and heart, were fixed in 10% phosphate-buffered formalin for 2 weeks. As for the accessory sex organs, two sagittal slices of the ventral prostate, two sagittal slices of the dorsolateral prostate, which included the urethra, and three transverse slices from each side of the seminal vesicles, which included the anterior prostate, were made and embedded in paraffin. They were then sectioned and stained with H&E for histopathologic diagnosis. The prostatic lesions, including prostatic intraepithelial neoplasm (PIN; ref. 26), were histopathologically diagnosed. The diagnosis of PIN was based on the criteria described by Bostwick and Brawer (27): PIN shows the morphologic continuum of cellular proliferations with nuclear atypia that occur within prostatic ducts, ductules, or acini, and is enclosed by a basement membrane. Several architectural patterns, such as flat, tufting, micro-capillary, or cribriform, could be seen.

Immunohistochemistry. For the determination of cell proliferation and cell cycle activity of the epithelial cells, PCNA and cyclin D1 immunohistochemistry was done according to the method described previously with some modifications (28, 29). Apoptotic index was also evaluated by immunohistochemistry for ssDNA (28). Immunohistochemistry was done using a stain system kit (DAKO LSAB 2 kit/HRP, DAKO Japan Co., Ltd., Kyoto, Japan). The sections (3 μm in thickness) made from paraffin-embedded tissues were deparaffinized; they were treated sequentially with 0.3% H_2O_2, normal goat serum or horse serum, and first antibodies. A mouse anti-PCNA antibody (1:100 dilution; DAKO Japan), a rabbit polyclonal anti-cyclin D1 antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit polyclonal antibody against ssDNA (1:300; DAKO Japan) were applied to the sections according to the manufacturer's protocol (DAKO LSAB 2 kit/HRP, DAKO Japan). All incubation steps ware carried out for 15 minutes at 37°C. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. The tissues were lightly counterstained with hematoxylin to facilitate orientation. Negative controls were stained without the first antibodies. The numbers of cells with positive reactivity for PCNA, cyclin D1, and ssDNA antibody were counted in a total of 3 × 100 cells in three different areas of the tumors, PIN, and nonlesional areas, and expressed as percentage (mean ± SD).

Statistical evaluation. Where applicable, data were analyzed using Fisher's exact probability test, Student's *t* test, or Welch *t* test with $P < 0.05$ as the criterion of significance.

Table 1. Intakes of food and test chemical

Group no.	Treatment	No. rats examined	Daily intake		Total intake of test chemical (mg)
			Food (g/d/rat)	Test chemical (mg/d/rat)	
1	DMAB	18	16.1 ± 2.5*	—	—
2	DMAB → 100 ppm silymarin	17	16.0 ± 2.6	1.60	44.8
3	DMAB → 500 ppm silymarin	17	16.4 ± 2.3	8.20	2,296
4	500 ppm silymarin	8	16.3 ± 2.2	8.15	2,282
5	None	8	15.8 ± 1.7	—	—

*Mean ± SD.

Table 2. Body, liver, prostate, and testicular weights at the end of the study

Group no.	Treatment	No. rats examined	Body weight (g)	Liver weight (g)	Prostate weight (g)	Testes weight (g)
1	DMAB	18	384.8 ± 24.4*	11.82 ± 1.25	3.19 ± 0.61	2.88 ± 0.66
2	DMAB → 100 ppm silymarin	17	386.0 ± 21.3	12.30 ± 0.93	3.18 ± 0.59	2.80 ± 0.55
3	DMAB → 500 ppm silymarin	17	388.7 ± 16.1	12.42 ± 1.00	3.13 ± 0.67	2.79 ± 0.48
4	500 ppm silymarin	8	401.1 ± 11.8	11.76 ± 1.16	3.13 ± 0.45	2.98 ± 0.64
5	None	8	383.9 ± 20.9	12.42 ± 1.19	3.20 ± 0.62	3.03 ± 0.37

*Means ± SD.

Results

General observation. All animals remained healthy throughout the experimental period. During the study, no clinical signs of toxicity were present in any groups. Histologically, there were no pathologic alteration suggesting toxicity of silymarin in the liver, kidneys, lung, and heart. Food consumption (g/d/rat) did not significantly differ among the groups, as shown in Table 1. Estimated intakes of test chemicals were well correlated with doses applied (Table 1). Body, liver, prostate, and testicular weights in all groups at the end of the study are shown in Table 2. The mean body weights, liver, prostate, and bilateral testicular weights did not significantly differ among the groups.

Incidence of neoplasms of prostate and other organs. Table 3 summarizes the data on the incidence of neoplasms of prostate and other tissues. DMAB exposure could induce PIN and adenocarcinomas (Fig. 1A and B) in the ventral lobe of the prostate. Such lesions were not found in other lobes of the prostate and seminal vesicle. Treatment of DMAB alone (group 1) produced 50.0% incidence of well-differentiated prostatic adenocarcinoma (Fig. 1A). The incidence of prostatic adenocarcinoma (Fig. 1B) in group 3 that received DMAB and 500 ppm silymarin (17.6%) was significantly lower than in group 1 (50.0%, $P < 0.05$). Also, feeding with 100 ppm silymarin after DMAB administration (group 2) caused a reduction of incidence of prostatic adenocarcinoma (29.4%), but there was no statistical significance different from group 1. The incidences of prostatic PIN were 50.0% in group 1, 23.5% in group 2, and 64.7% in group 3. These values also did not show statistically significance among the groups 1 through 3. In other organs, a few neoplasms, such as colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma, were noted in a few rats of groups 1 to 3. The incidences of these tumors were not statistically significant among the groups. No prostatic neoplasms were found in groups 4 (500 ppm silymarin alone) and 5 (no treatment).

Immunohistochemical findings. The data on PCNA- (Fig. 1C and D), cyclin D1- (Fig. 1E and F), and apoptosis-positive cells (Fig. 1G and H) in the prostatic lesions are indicated in Table 4. The mean PCNA labeling indices of adenocarcinoma found in group 3 receiving DMAB and 500 ppm silymarin (6.3 ± 1.5) were significantly lower than in group 1 (10.0 ± 2.4, $P < 0.05$). The PCNA labeling indices of PIN in groups 2 (6.8 ± 1.7) and 3 (6.8 ± 2.2) were lower than that of group 1 (8.8 ± 2.9), but the differences were not statistically significant. As for the histologically normal prostatic glands, the PCNA-labeling indices of all groups were comparable. The mean cyclin D1 labeling indices of adenocarcinoma found in groups 2 and 3 were significantly lower than in group 1 ($P < 0.05$ or $P < 0.01$). The cyclin D1 labeling indices of PIN in groups 2 and 3 were slightly lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the cyclin D1 labeling indices of all groups were comparable. The apoptotic index of PIN and adenocarcinoma in group 3 was statistically greater than that of group 1 ($P < 0.05$ and $P < 0.02$, respectively). On the other hand, the apoptotic index of PIN and adenocarcinoma

Table 3. Incidence of pathologic lesions

Group no.	Treatment	No. rats examined	No. rats with incidence		
			Prostate		Others*
			PIN	Adenocarcinoma	
1	DMAB	18	9 (50.0%)	9 (50%)	4 (22.2%)
2	DMAB → 100 ppm silymarin	17	4 (23.5%)	5 (29.4%)	4 (23.5%)
3	DMAB → 500 ppm silymarin	17	11 (64.7%)	3† (17.6%)	2 (11.8%)
4	500 ppm silymarin	8	0 (0%)	0 (0%)	0 (0%)
5	None	8	0 (0%)	0 (0%)	0 (0%)

*Colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma.
†Significantly different from group 1 by Fisher's exact probability test ($P < 0.05$).

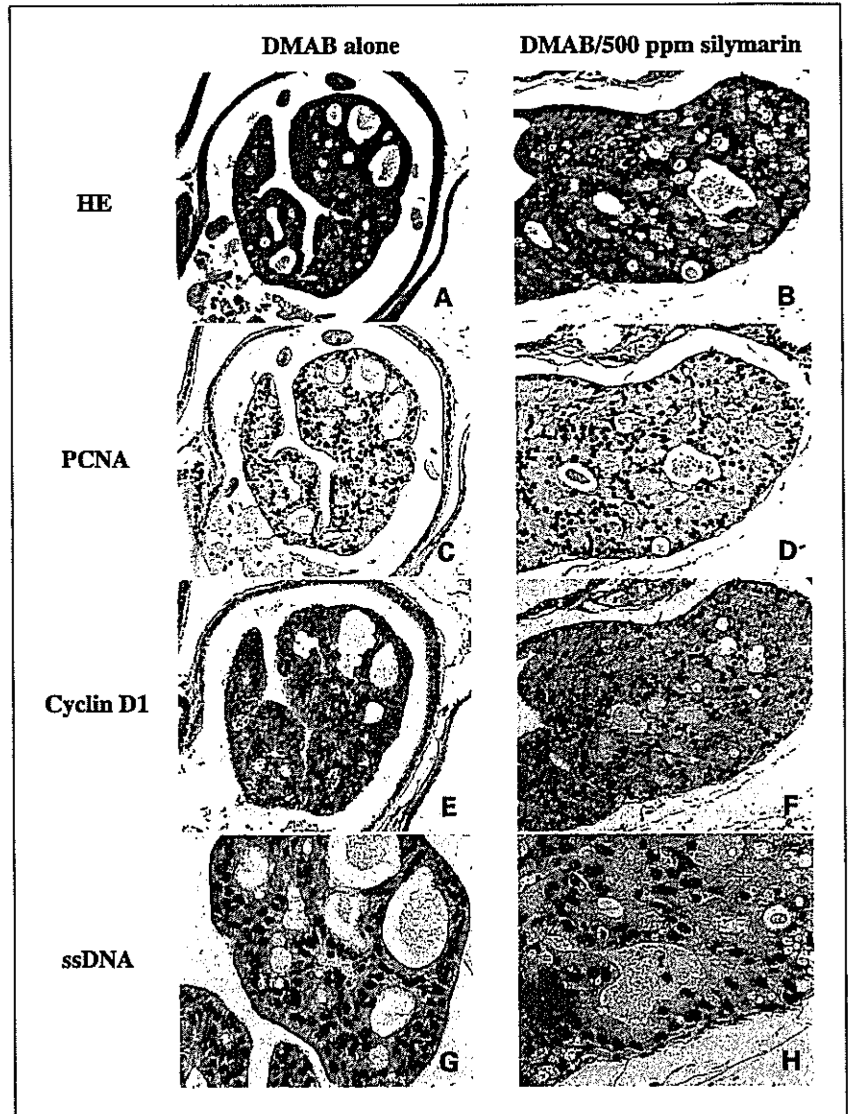

Fig. 1. Histopathology of adenocarcinomas and their immunohistochemistry of PCNA, cyclin D1, and ss DNA. An adenocarcinoma (*A, C, E, G*) from a rat given DMAB alone (group 1) and that (*B, D, F, H*) from a rat given DMAB and 500 ppm silymarin (group 3). H&E stain (*A, B*) and immunohistochemistry for PCNA (*C, D*), cyclin D1 (*E, F*), and ssDNA (*G, H*). Original magnification, ×20 (*A-F*) and ×40 (*G, H*).

in group 2 was lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the apoptotic indices of all groups were comparable.

Discussion

In the present study, dietary administration of 500 ppm silymarin during the promotion phase of DMAB-induced prostatic carcinogenesis significantly inhibited the incidence of prostatic adenocarcinoma. Silymarin is known to inhibit chemically induced carcinogenesis in skin (13), tongue (23), urinary bladder (24), and colon (25). Furthermore, Singh et al. (30) reported that the preventive and therapeutic efficacy of dietary feeding of silibinin on human prostate carcinoma DU145 tumor xenograft in athymic nude mice. These results indicate that silymarin might be a candidate chemopreventive agent against carcinogenesis in multiple organs including prostate.

Several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis could be considered. Cell proliferation plays an important role in multistage carcinogenesis and involves multiple genetic alterations (31, 32). Silymarin and silibinin are reported to suppress the growth of different cancer cells (17, 33-35). Other studies with human prostate cancer cells showed that silymarin and silibinin inhibit the cell growth of androgen-dependent and androgen-independent human prostate carcinomas LNCaP and DU145 cells, respectively (15, 17). Tyagi et al. (18) showed that silymarin and silibinin induce growth inhibition and apoptotic cell death in rat prostate cancer cells. Such effects are considered to occur through perturbation of cell cycle progression, leading to G_1 arrest in a dose- and time-dependent manner, and inhibiting DNA synthesis, possibly because of an effect of G_1 arrest (17, 33, 36, 37). Cyclin D1 is involved in cell cycle during early G_1 phase (38). As the major events leading to cell proliferation occur in the G_1 phase, altered expression of cyclin D1 and their cyclin-dependent kinases might be an important step in carcinogenesis (39). Cyclin D1 overexpression was reported in human cancers (40, 41) and in murine chemically induced carcinogenesis (24, 42). Cyclin D1, which is found to be overexpressed in major of human cancers, has been regarded as a