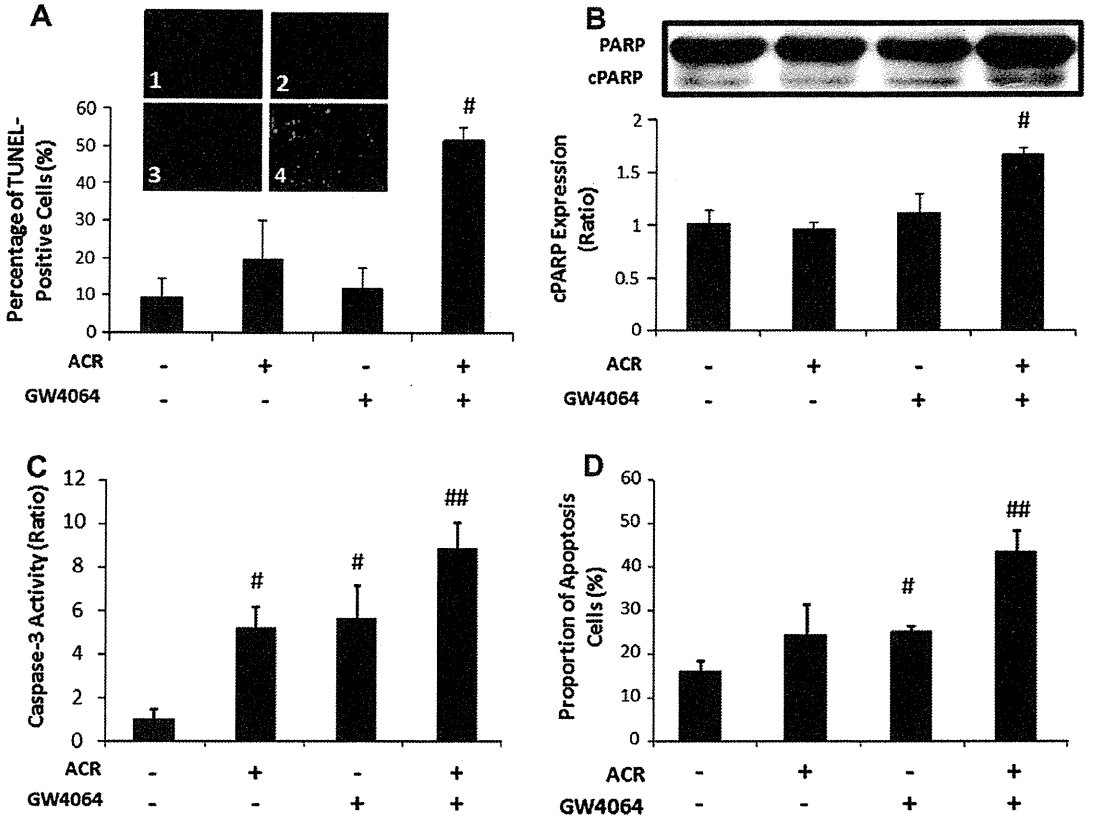

Fig. 3. Effects of the combination of ACR plus GW4064 on induction of apoptosis in HLE cells. The cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 48 h or 72 h. TUNEL assays (A) and Western blot analysis using a PARP-specific antibody (B, upper panel) were performed using cells treated with test drugs for 48 h. Caspase-3 activity assays (C) and Annexin V assays (D) were performed using samples treated for 72 h. (A) TUNEL-positive cells were counted and examined as the percentage of the DAPI-positive cell number (500 cells were counted in each flask). (B) The intensities of the cleaved PARP (c-PARP) blots were quantified using densitometry. Columns and lines indicate mean and SD of triplicate assays (lower panel). (C) Caspase-3 activity was performed with a fluorometric system. (D) Cultured cells were incubated with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were then analyzed by flow cytometry. Annexin V-FITC-positive and PI-negative cells were counted as apoptotic cells. #: $P < 0.05$, compared with vehicle treated cells. ##: $P < 0.05$, compared with vehicle, ACR alone, or GW4064 alone treated cells.

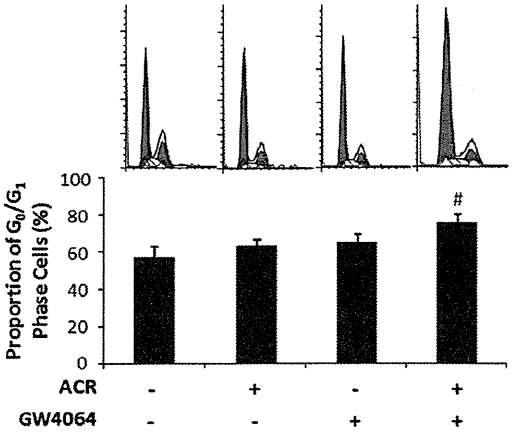

Fig. 4. Effects of the combination of ACR plus GW4064 on induction of the G_0/G_1 cell cycle arrest in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 72 h. The cells were then stained with propidium iodide to analyze their cell cycle progression. The distributions of cells in the G_0/G_1 of the cell cycle were calculated using a FACScan. *Error Bars*, SE of triplicate assays. #: $P < 0.05$, compared with vehicle treated cells.

in the levels $p21^{CIP1}$, and a decrease in the levels of c-*myc* and cyclin D1 mRNA expression (Fig. 6A–C). In addition, the expression level of SHP mRNA, which is one of the target genes of FXR [17,25,30,31], was also significantly increased by the combination treatment with ACR plus GW4064 (Fig. 6D).

3.7. ACR enhances the induction of FXRE promoter activities by GW4064

FXR and RXRs modulate the expression of target genes by interacting with FXRE elements located in the promoter regions of such genes [13]. Therefore, we next examined whether ACR might enhance the transcriptional activity of the FXRE promoter induced by GW4064 using transient transfection luciferase reporter assays. As shown in Fig. 6E, 1 μM GW4064 significantly increased the FXRE reporter activity in comparison with control HLE cells which were not treated with either ACR or GW4064. Moreover, when the cells were treated with a combination of 1 μM GW4064 plus 1 μM ACR, there was a significant increase in the transcriptional activity of the FXRE reporter, thus suggesting that treatment with these agents might cooperatively enhance the FXRE reporter activity.

4. Discussion

The prognosis for patients with HCC is poor and more effective strategies for the chemoprevention and chemotherapy of this malignancy are urgently required. The present study provides the

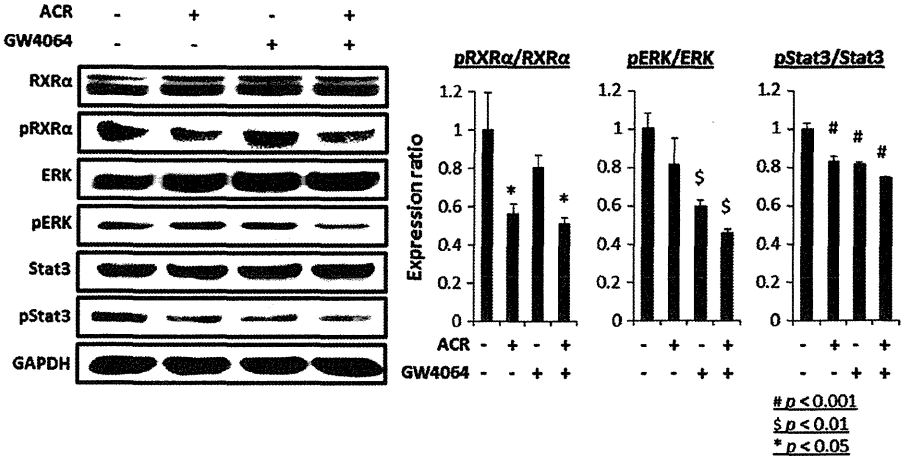

Fig. 5. Effects of the combination of ACR plus GW4064 on the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 12 h. The extracted proteins were examined by a Western blot analysis using the respective antibodies (left panels). The intensities of the blots were quantified using densitometry. Columns and lines indicate means and SD of triplicate assays (right panels). Repeat Western blots gave similar results. *: $P < 0.05$, $: $P < 0.01$, #: $P < 0.001$, compared with vehicle treated cells.

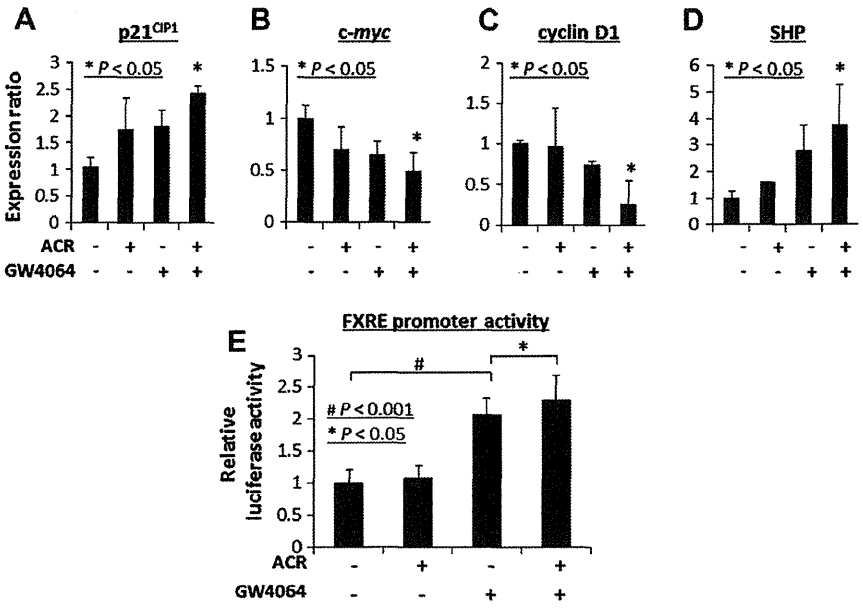

Fig. 6. Effects of the combination of ACR plus GW4064 on the expression of $p21^{CIP1}$, *c-myc*, cyclin D1, and SHP mRNA and on the transcriptional activity of the FXRE promoter in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 24 h. The extracted mRNAs were examined by a quantitative real-time RT-PCR analysis using the $p21^{CIP1}$ (A), *c-myc* (B), cyclin D1 (C), and SHP (D) specific primers. The expression levels of each mRNA were normalized to the level of β-actin mRNA. Values represent the means ± SD of triplicate analyses. *: $P < 0.05$. (E) A transient transfection reporter assay was performed with the FXRE luciferase reporter in the presence of vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064. Relative luciferase activity was determined after 24 h. Columns and lines indicate the means and SD of triplicate assays. #: $P < 0.001$, **: $P < 0.05$.

first evidence that GW4064, a synthetic ligand for FXR, preferentially inhibits the growth of HCC cells compared with Hc normal hepatocytes. This study also clearly indicates that the combination of GW4064 plus ACR, which is expected as a HCC chemopreventive agent [8–10], cause a synergistic inhibition of growth in HLE human HCC cells and that this is associated with the induction of apoptosis. This combination also acted cooperatively to induce the arrest of the cell cycle in the G_0/G_1 phase and the expression of $p21^{CIP1}$ and SHP mRNA, but suppress the expression levels of *c-myc* and cyclin D1 mRNA.

As illustrated in Fig. 7, we presume that the synergism generated by the combination of ACR plus GW4064 is mainly associated with the enhancement of the FXRE reporter activity. FXR regulates the expression of target genes by binding either as a monomer or as a heterodimer with RXR to the FXRE [13]. Therefore, in the present study, ACR and GW4064 cooperatively enhanced the binding of FXR to the FXRE promoter, thereby enhancing the expression of its target genes (Fig. 7, as indicated by A). Among the FXR target genes, SHP is considered to play a role in the inhibition of cell growth because it is a pivotal cell death receptor that targets the mitochondria, leading to the induction of apoptosis and inhibition of tumor growth [32]. GW4064 suppresses the growth of cancer cells through the activation of FXR targeted genes, including SHP [17,25,30,31]. SHP has also been shown to be a direct negative

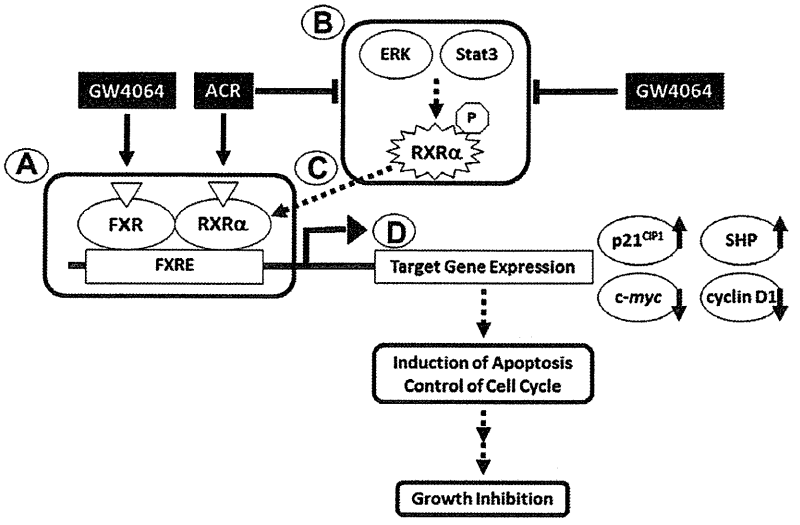

Fig. 7. A hypothetical schematic representation of the effects of the combination of ACR plus GW4064 on growth inhibition in HCC cells. ACR and GW4064 can bind to their receptors, RXRα and FXR, as ligands, and subsequently activate the FXRE promoter activity (A). ACR and GW4064 also inhibit RXRα phosphorylation, which is involved in liver carcinogenesis, by inhibiting ERK and Stat3 phosphorylation (B). The inhibition of RXRα phosphorylation by these agents might restore the function of this nuclear receptor as a heterodimeric partner for FXR (C), thus resulting in the activation of the FXRE promoter activity. Cooperative activation of this promoter activity by ACR and GW4064 regulates the expression of target genes, such as $p21^{CIP1}$, *c-myc*, cyclin D1, and SHP, which play a critical role in the induction of apoptosis, control of cell cycle progression, and inhibition of cancer cell growth (D). For additional details see Section 4.

regulator of cyclin D1 gene transcription [30]. Treatment with 1 μM GW4064 alone did not significantly increase the expression levels of SHP mRNA in the present study, whereas its expression was clearly increased by combined treatment with ACR plus GW4064. These findings may indicate that the concentration of 1 μM is insufficient to increase the levels of SHP mRNA in HCC cells and, therefore, an appropriate partner, such as ACR, is required for GW4064 to exert a synergistic effect on growth inhibition in HCC cells (Fig. 7).

In addition, recent studies have revealed that activating FXR suppresses the expression of cyclin D1 and *c-myc*, but induces the expression of $p21^{CIP1}$, by targeting the Wnt/β-catenin signaling pathway [17,31]. These findings seem to be significant because the Wnt/β-catenin pathway plays a critical role in liver carcinogenesis, and thus may be a promising target for the treatment of HCC [33]. In FXR knockout mice, sustained activation of this pathway was shown to be involved in the development of HCC [17]. On the other hand, ACR has been shown to exert growth inhibitory effects in HCC cells by targeting the Wnt/β-catenin pathway [12]. ACR also induces apoptosis and cell cycle arrest in the G_0/G_1 phase in HCC cells by regulating the expression of $p21^{CIP1}$, cyclin D1, and *c-myc* [12,19,34,35]. Therefore, the activation of FXR by GW4064 may act cooperatively with ACR to inhibit the activation of the Wnt/β-catenin pathway, subsequently decreasing the expression of cyclin D1 and *c-myc*, but increasing the expression of $p21^{CIP1}$, as was demonstrated in the present study.

In addition to chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis virus, increased evidence has indicated that a malfunction of RXRα due to phosphorylation is profoundly involved in the development of HCC [4–8]. In HCC cells, the Ras/MAPK signaling pathway is highly activated, leading to phosphorylation of RXRα, which indicates that the Ras/MAPK pathway and p-RXRα are potential targets for inhibiting the growth of HCC cells [4–8]. Indeed, ACR dephosphorylates RXRα, ERK, and Stat3 proteins, and restores the function of RXRα, thus inhibiting the growth of HCC cells and suppressing liver tumorigenesis in obese mice [4,11,20,36]. The combinations of ACR plus valproic acid or vitamin K_2 also synergistically suppressed the growth of HCC cells by inhibiting RXRα phosphorylation [11,20]. Similar to these previous studies [11,20], in the present study, inhibition of RXRα phosphorylation by the combination of ACR plus GW4064 may also have restored the function of RXRα as a master regulator of nuclear receptors, thus contributing to synergistic growth inhibition in HCC cells (Fig. 7, as indicated by B). Dephosphorylation of RXRα by this combination treatment may play a role in the observed enhancement of the FXRE promoter activity because phosphorylation of RXRα abolishes its ability to form heterodimers with other nuclear receptors, but inhibition of this phosphorylation can restore its heterodimeric activity [7]. The combination of ACR plus GW4064 may also promote RXRs homodimerization and thus enhance the promoter activity of retinoid X response element, which is associated with the anticancer mechanisms of ACR [11,37].

One of the major questions that arose was how the combination of ACR plus GW4064 could inhibit the phosphorylation of ERK and Stat3 proteins. One of the mechanisms which might explain this phenomenon is that the effects of ACR and GW4064 inhibit the activation of specific receptor tyrosine kinases (RTKs). ACR has been shown to reduce HCC development and inhibit cancer growth by targeting growth factors and their corresponding RTKs, such as the epidermal growth factor (EGF) receptor (EGFR), and downstream signaling pathways, including the Ras/MAPK and Jak/Stat3 pathways [29,38]. The activation of FXR by its ligand also reduces the expression of HER2, a member of the EGFR family of RTKs, and inhibits EGF-mediated HER2 and ERK phosphorylation in human breast cancer cells [24]. Therefore, GW4064 may increase the inhibitory effects of ACR on certain types of RTKs by activating FXR, which results in the inhibition of ERK and Stat3 phosphorylation and subsequent RXRα phosphorylation. Future studies are required to clarify whether both ACR and GW4064 synergistically exert inhibitory effects on the activation of specific RTKs.

Finally, it should be noted that, in a clinical trial showing the chemopreventive effects of ACR on the recurrence of secondary HCC [9,10], the plasma concentration of this agent (which ranged from 1 to 5 μM) was approximately the same as the concentration used in the present study (1 μM). In phase II clinical trials, a FXR

ligand also ameliorated the increase in the alkaline phosphatase levels in patients with primary biliary cirrhosis and improved the insulin sensitivity in patients with diabetes and liver steatosis, although some unfavorable events that might be associated with FXRE reporter overactivity were observed [39]. The combination of ACR plus GW4064 may resolve such problems because this combination permits the administration of lower doses of both agents for treatment. Future pharmacokinetic studies are required to determine whether the dose of GW4064 used in this study is clinically relevant and pilot studies confirming are thus called for to clarify the safety of this agent.

In conclusion, the observation that a combination of appropriate concentrations of ACR plus GW4064 can inhibit the growth of human HCC cells without affecting the growth of normal hepatocytes should encourage further clinical studies using these agents to investigate their potential for HCC chemoprevention and chemotherapy. The results of our present study suggest that combining ACR with GW4064 might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects.

References

[1] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.

[2] P. Chambon, A decade of molecular biology of retinoic acid receptors, Faseb. J. 10 (1996) 940–954.

[3] L. Altucci, M.D. Leibowitz, K.M. Ogilvie, A.R. de Lera, H. Gronemeyer, RAR and RXR modulation in cancer and metabolic disease, Nat. Rev. Drug Discov. 6 (2007) 793–810.

[4] R. Matsushima-Nishiwaki, M. Okuno, Y. Takano, S. Kojima, S.L. Friedman, H. Moriwaki, Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid, Carcinogenesis 24 (2003) 1353–1359.

[5] R. Matsushima-Nishiwaki, M. Okuno, S. Adachi, T. Sano, K. Akita, H. Moriwaki, S.L. Friedman, S. Kojima, Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma, Cancer Res. 61 (2001) 7675–7682.

[6] S. Adachi, M. Okuno, R. Matsushima-Nishiwaki, Y. Takano, S. Kojima, S.L. Friedman, H. Moriwaki, Y. Okano, Phosphorylation of retinoid X receptor suppresses its ubiquitination in human hepatocellular carcinoma, Hepatology 35 (2002) 332–340.

[7] K. Yoshimura, Y. Muto, M. Shimizu, R. Matsushima-Nishiwaki, M. Okuno, Y. Takano, H. Tsurumi, S. Kojima, Y. Okano, H. Moriwaki, Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta, Cancer Sci. 98 (2007) 1868–1874.

[8] M. Shimizu, K. Takai, H. Moriwaki, Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention, Cancer Sci. 100 (2009) 369–374.

[9] Y. Muto, H. Moriwaki, M. Ninomiya, S. Adachi, A. Saito, K.T. Takasaki, T. Tanaka, K. Tsurumi, M. Okuno, E. Tomita, T. Nakamura, T. Kojima, Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma prevention study group, New Engl. J. Med. 334 (1996) 1561–1567.

[10] Y. Muto, H. Moriwaki, A. Saito, Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma, New Engl. J. Med. 340 (1999) 1046–1047.

[11] H. Tatebe, M. Shimizu, Y. Shirakami, H. Sakai, Y. Yasuda, H. Tsurumi, H. Moriwaki, Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells, Cancer Lett. 285 (2009) 210–217.

[12] M. Suzui, M. Masuda, J.T. Lim, C. Albanese, R.G. Pestell, I.B. Weinstein, 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 (2002) 3997–4006.

[13] Y.D. Wang, W.D. Chen, D.D. Moore, W. Huang, FXR: a metabolic regulator and cell protector, Cell Res. 18 (2008) 1087–1095.

[14] W. Huang, K. Ma, J. Zhang, M. Qatanani, J. Cuvillier, J. Liu, B. Dong, X. Huang, D.D. Moore, Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration, Science 312 (2006) 233–236.

[15] F. Yang, X. Huang, T. Yi, Y. Yen, D.D. Moore, W. Huang, Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor, Cancer Res. 67 (2007) 863–867.

[16] I. Kim, K. Morimura, Y. Shah, Q. Yang, J.M. Ward, F.J. Gonzalez, Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice, Carcinogenesis 28 (2007) 940–946.

[17] A. Wolfe, A. Thomas, G. Edwards, R. Jaseja, G.L. Guo, U. Apte, Increased activation of the Wnt/{beta}-catenin pathway in spontaneous hepatocellular carcinoma observed in farnesoid x receptor knockout mice, J. Pharmacol. Exp. Ther. 338 (2011) 12–21.

[18] A. Obora, Y. Shiratori, M. Okuno, S. Adachi, Y. Takano, R. Matsushima-Nishiwaki, I. Yasuda, Y. Yamada, K. Akita, T. Sano, J. Shimada, S. Kojima, Y. Okano, S.L. Friedman, H. Moriwaki, Synergistic induction of apoptosis by acyclic retinoid and interferon-beta in human hepatocellular carcinoma cells, Hepatology 36 (2002) 1115–1124.

[19] M. Shimizu, M. Suzui, A. Deguchi, J.T. Lim, D. Xiao, J.H. Hayes, K.P. Papadopoulos, I.B. Weinstein, Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells, Clin. Cancer Res. 10 (2004) 6710–6721.

[20] T. Kanamori, M. Shimizu, M. Okuno, R. Matsushima-Nishiwaki, H. Tsurumi, S. Kojima, H. Moriwaki, Synergistic growth inhibition by acyclic retinoid and vitamin K_2 in human hepatocellular carcinoma cells, Cancer Sci. 98 (2007) 431–437.

[21] H. Tatebe, M. Shimizu, Y. Shirakami, H. Tsurumi, H. Moriwaki, Synergistic growth inhibition by 9-cis-retinoic acid plus trastuzumab in human hepatocellular carcinoma cells, Clin. Cancer Res. 14 (2008) 2806–2812.

[22] Y. Liu, Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis, J. Clin. Invest. 112 (2003) 1678–1687.

[23] I. Kim, S.H. Ahn, T. Inagaki, M. Choi, S. Ito, G.L. Guo, S.A. Kliewer, F.J. Gonzalez, Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine, J. Lipid Res. 48 (2007) 2664–2672.

[24] C. Giordano, S. Catalano, S. Panza, D. Vizza, I. Barone, D. Bonofiglio, L. Gelsomino, P. Rizza, S.A. Fuqua, S. Ando, Farnesoid X receptor inhibits tamoxifen-resistant MCF-7 breast cancer cell growth through downregulation of HER2 expression, Oncogene (2011).

[25] K.E. Swales, M. Korbonits, R. Carpenter, D.T. Walsh, T.D. Warner, D. Bishop-Bailey, The farnesoid X receptor is expressed in breast cancer and regulates apoptosis and aromatase expression, Cancer Res. 66 (2006) 10120–10126.

[26] J. Kaeding, E. Bouchaert, J. Belanger, P. Caron, S. Chouinard, M. Verreault, O. Larouche, G. Pelletier, B. Staels, A. Belanger, O. Barbier, Activators of the farnesoid X receptor negatively regulate androgen glucuronidation in human prostate cancer LNCAP cells, Biochem. J. 410 (2008) 245–253.

[27] L. Zhao, M.G. Wientjes, J.L. Au, Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses, Clin. Cancer Res. 10 (2004) 7994–8004.

[28] M. Shimizu, Y. Yasuda, H. Sakai, M. Kubota, D. Terakura, A. Baba, T. Ohno, T. Kochi, H. Tsurumi, T. Tanaka, H. Moriwaki, Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-db/db obese mice, BMC Cancer 11 (2011) 281.

[29] M. Shimizu, M. Suzui, A. Deguchi, J.T. Lim, I.B. Weinstein, 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 (2004) 1130–1140.

[30] Y. Zhang, P. Xu, K. Park, Y. Choi, D.D. Moore, L. Wang, Orphan receptor small heterodimer partner suppresses tumorigenesis by modulating cyclin D1 expression and cellular proliferation, Hepatology 48 (2008) 289–298.

[31] R.R. Maran, A. Thomas, M. Roth, Z. Sheng, N. Esterly, D. Pinson, X. Gao, Y. Zhang, V. Ganapathy, F.J. Gonzalez, G.L. Guo, Farnesoid X receptor deficiency in mice leads to increased intestinal epithelial cell proliferation and tumor development, J. Pharmacol. Exp. Ther. 328 (2009) 469–477.

[32] Y. Zhang, J. Soto, K. Park, G. Viswanath, S. Kuwada, E.D. Abel, L. Wang, Nuclear receptor SHP, a death receptor that targets mitochondria, induces apoptosis and inhibits tumor growth, Mol. Cell Biol. 30 (2010) 1341–1356.

[33] D.J. Mulholland, S. Dedhar, G.A. Coetzee, C.C. Nelson, Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know?, Endocr Rev. 26 (2005) 898–915.

[34] Y. Yamada, Y. Shidoji, Y. Fukutomi, T. Ishikawa, T. Kaneko, H. Nakagama, M. Imawari, H. Moriwaki, Y. Muto, Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines, Mol. Carcinog. 10 (1994) 151–158.

[35] M. Suzui, M. Shimizu, M. Masuda, J.T. Lim, N. Yoshimi, I.B. Weinstein, Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells, Mol. Cancer Ther. 3 (2004) 309–316.

[36] M. Shimizu, H. Sakai, Y. Shirakami, J. Iwasa, Y. Yasuda, M. Kubota, K. Takai, H. Tsurumi, T. Tanaka, H. Moriwaki, Acyclic retinoid inhibits diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BLKS/J- +(db)/+Lepr(db) mice, Cancer Prev. Res. 4 (2011) 128–136.

[37] H. Araki, Y. Shidoji, Y. Yamada, H. Moriwaki, Y. Muto, Retinoid agonist activities of synthetic geranyl geranoic acid derivatives, Biochem. Biophys. Res. Commun. 209 (1995) 66–72.

[38] T. Sano, M. Kagawa, M. Okuno, N. Ishibashi, M. Hashimoto, M. Yamamoto, R. Suzuki, H. Kohno, R. Matsushima-Nishiwaki, Y. Takano, H. Tsurumi, S. Kojima, S.L. Friedman, H. Moriwaki, T. Tanaka, Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells, Nutr. Cancer 51 (2005) 197–206.

[39] S. Fiorucci, S. Cipriani, A. Mencarelli, F. Baldelli, G. Bifulco, A. Zampella, Farnesoid X Receptor Agonist for the Treatment of Liver and Metabolic Disorders: Focus on 6-ethyl-CDCA, Mini Rev. Med. Chem. 11 (2011) 753–762.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Suppression of azoxymethane-induced colonic preneoplastic lesions in rats by 1-methyltryptophan, an inhibitor of indoleamine 2,3-dioxygenase

Kengo Ogawa,[1] Takeshi Hara,[1] Masahito Shimizu,[1] Soranobu Ninomiya,[1] Junji Nagano,[1] Hiroyasu Sakai,[1] Masato Hoshi,[2] Hiroyasu Ito,[2] Hisashi Tsurumi,[1,5] Kuniaki Saito,[3] Mitsuru Seishima,[2] Takuji Tanaka[4] and Hisataka Moriwaki[1]

[1]First Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu; [2]Department of Informative Clinical Medicine, Gifu University Graduate School of Medicine, Gifu; [3]Human Health Sciences, Graduate School of Medicine and Faculty of Medicine, Kyoto University, Kyoto; [4]The Tohkai Cytopathology Institute, Cancer Research and Prevention (TCI-CaRP), Gifu, Japan

(Received November 29, 2011/Revised February 3, 2012/Accepted February 4, 2012/Accepted manuscript online February 9, 2012/Article first published online March 6, 2012)

The escape of preneoplastic cells from the immune system, which is caused by immune tolerance, occurs during the development of several types of tumors. Indoleamine 2,3-dioxygenase (IDO) plays a critical role in the induction of immune tolerance. In the present study we investigated the effects of 1-methyltryptophan (1-MT), an IDO inhibitor, and (–)-epigallocatechin gallate (EGCG), the major catechin in green tea, on the development of azoxymethane (AOM)-induced colonic preneoplastic lesions by focusing on the inhibition of IDO. To induce colonic premalignant lesions, male F344 rats were injected with AOM (20 mg/kg body weight, s.c.) once a week for 2 weeks. They also received 0.2% 1-MT or 0.1% EGCG in their drinking water for 4 weeks, starting 1 week before the first dose of AOM. Both 1-MT and EGCG significantly decreased the total number of aberrant crypt foci and β-catenin-accumulated crypts, which overexpressed IDO protein. Treatment with EGCG decreased IDO mRNA expression in both the colonic epithelium and stroma of rats induced by AOM. The AOM-induced increase in *cyclooxygenase-2* mRNA expression in the colonic stroma was significantly decreased by EGCG. Furthermore, AOM-induced increases in IDO activity in the serum and stroma were significantly inhibited by 1-MT and EGCG. Inhibition of IDO activity by 1-MT and EGCG was also observed in cell-free assays. These findings suggest that upregulation of IDO activity is observed in the early stages of colon carcinogenesis and that the use of IDO inhibitors, such as 1-MT and EGCG, which suppress the occurrence of colonic preneoplastic lesions, could be a novel strategy for the chemoprevention of colon cancer. (*Cancer Sci* 2012; 103: 951–958)

The immune system recognizes preneoplastic cells and, in most cases, eliminates these cells before they expand into clinically detectable tumors. Therefore, the escape of precancerous cells from the immune system, which is closely associated with immune tolerance, is involved in the development of several types of tumors.[1] Recent studies have suggested that indoleamine 2,3-dioxygenase (IDO) plays a crucial role in the induction of immune tolerance.[2] Indoleamine 2,3-dioxygenase is an intracellular enzyme that catalyses the first and rate-limiting steps in the catabolism of the essential amino acid tryptophan along the kynurenine pathway.[3] In the tumor microenvironment, increased IDO activity inhibits the proliferation of T and natural killer cells and induces apoptosis through tryptophan depletion and the production of toxic tryptophan catabolites.[4] Overexpression of IDO has been shown to be correlated with poor clinical outcome in patients with ovarian carcinoma, endometrial carcinoma, and colorectal carcinoma.[5–7] We have recently reported that, in diffuse large B-cell lymphoma, IDO expression in tumor cells and serum concentrations of L-kynurenine, which reflect IDO activity, are useful indicators of a poor prognosis.[8,9] Several preclinical studies using rodent cancer models have demonstrated that IDO inhibitors, such as 1-methyltryptophan (1-MT), are therapeutically beneficial, especially when combined with different types of cytotoxic chemotherapeutic agents.[10,11] These reports suggest that targeting IDO, and therefore regulating tryptophan catabolism, may be an effective strategy for the treatment of certain types of human malignancies.[12] However, the possibility of cancer chemoprevention by inhibiting IDO expression and/or activity has not been considered.

(–)-Epigallocatechin gallate (EGCG), one of the major catechins in green tea, is the most biologically active component of green tea. It has been shown to exert its cancer chemopreventive and anti-carcinogenic effects in various organs, including the colon.[13,14] Previously, we demonstrated that EGCG can inhibit the growth of and induce apoptosis in human colorectal cancer cells.[15–17] The inhibitory effects of EGCG on both inflammation- and obesity-related colon carcinogenesis have also been demonstrated.[18,19] In addition, green tea polyphenols in the drinking water have been shown to inhibit the development of putative preneoplastic lesions called aberrant crypt foci (ACF) in rats treated with azoxymethane (AOM), which induces ACF.[20–22]

Recently, it was been reported that EGCG administration suppresses the expression of IDO in interferon (IFN)-γ-stimulated murine dendritic cells[23] and human oral cancer cell lines.[24] Although the mechanisms underlying the role of IDO in carcinogenesis have not yet been clarified, we hypothesized that the inhibitory effect of EGCG on IDO expression may contribute to the anti-carcinogenic properties of EGCG. To confirm our hypothesis, we examined the effects of 1-MT and EGCG on the development in the colon of AOM-induced preneoplastic lesions, namely ACF[21] and β-catenin-accumulated crypts (BCAC),[25] in male F344 rats by focusing on the inhibition of IDO expression and activity.

Materials and Methods

Animals, chemicals, and diets. Male F344 rats, aged 4 weeks (Charles River Japan, Tokyo, Japan), were maintained at the Gifu University Animal Facility according to Institutional Animal Care Guidelines. All rats were housed in plastic cages with free access to drinking water and a pelleted basal diet

[5]To whom correspondence should be addressed.
E-mail: htsuru@gifu-u.ac.jp

doi: 10.1111/j.1349-7006.2012.02237.x

(CRF-1; Oriental Yeast, Tokyo, Japan). Both 1-MT and AOM were purchased from Sigma (St Louis, MO, USA), whereas EGCG was obtained from Mitsui Norin (Tokyo, Japan). For 1-MT and EGCG treatment of rats, 1-MT (0.2%) and EGCG (0.1%) solutions were prepared in tap water and administered to the rats in their drinking water *ad libitum*. Fresh test solutions were prepared three times a week. The concentration of EGCG (0.1%) used in the present study was chosen on the basis of the results of previous chemopreventive studies[19,26] and was within the physiologic range of the daily intake of green tea catechins in humans on a per unit body weight basis.[27]

Experimental procedure

As shown in Figure 1, 60 male F344 rats were quarantined for the first 7 days and then randomized into one of three groups to receive either 0.2% 1-MT, 0.1% EGCG, or no test compounds. One week later, the rats in each group were further grouped to receive subcutaneous injections of AOM (20 mg/kg body weight) or saline (200 μL) once a week for 2 weeks. Rats were given control and test drinking water for 4 weeks, starting 1 week before the first AOM injection. All measurements, including the large bowel excision and the collection of blood samples from the inferior vena cava, were performed from rats that had been killed by CO_2 asphyxiation at Week 4 (9 weeks of age). One-quarter of the excised colons (cecum side) was used for crypt isolation, whereas the remainder was used to determine the number of colonic ACF and BCAC (see below). After the number of ACF had been counted, the colon was rolled like a "Swiss roll"[28] and paraffin-embedded sections were prepared using routine procedures for subsequent histopathologic and immunohistochemical examinations.

Counting colonic ACF and BCAC. The number of ACF and BCAC was determined as described previously.[25,29,30] Briefly, buffered formalin-fixed colons were stained with 0.5% methylene blue solution for 20 s and then placed on microscope slides to count the number of ACF and to determine their size. The number of ACF in the colon was recorded along with the number of crypts in each focus and the data are expressed as the total number of ACF per colon, total number of aberrant crypts (ACs) per colon, number of ACs per focus, and total number of large ACF (i.e. ACF with four or more aberrant crypts) per colon.[29] After the number of ACF had been counted, the rectal mucosa (2.0 cm from the anus) was cut and embedded in paraffin to identify BCAC intramucosal lesions, with 4-μm sections obtained from an *en face* preparation. The number of BCAC on histological sections stained with β-catenin was counted and is expressed as the number of BCAC per cm^2 mucosa.

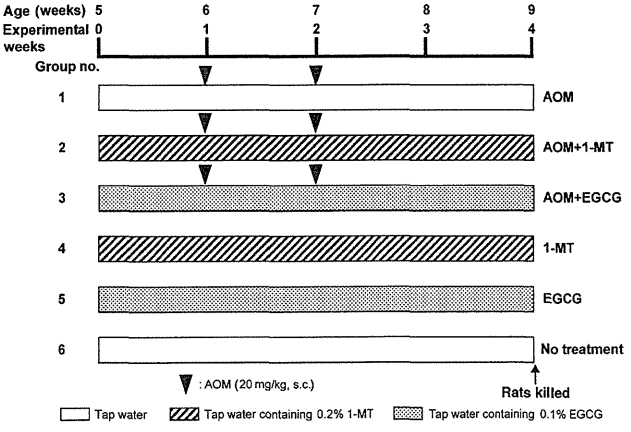

Fig. 1. Experimental protocol. Rats (5 weeks old) were allocated to one of six groups and treated over a period of 4 weeks, as indicated. AOM, azoxymethane; 1-MT, 1-methyltryptophan; EGCG, (−)-epigallocatechin gallate.

Immunohistochemical analysis. After endogenous peroxidase activity had been blocked with H_2O_2, sections were incubated overnight at 4°C with primary antibodies: anti-β-catenin (1:1000; BD Biosciences PharMingen, San Diego, CA, USA), anti-IDO (1:1000; LYFESPAN, Seattle, WA, USA), and anti-L-kynurenine (1:1000; Abnova, Taipei City, Taiwan). Subsequently, sections for the immunohistochemistry of β-catenin and IDO were incubated with biotinylated secondary antibodies against the primary antibodies (DAKO, Carpinteria, CA, USA), followed by incubation with avidin-coupled peroxidase. The sections for L-kynurenine immunohistochemistry were incubated with peroxidase-labeled polymer-conjugated secondary antibodies against the primary antibodies. They were then developed with 3,3′-diaminobenzidine using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and counterstained with hematoxylin.

Crypt isolation. Colonic tissue was washed twice with 1 × Hank's balanced salt solution (HBSS; Sigma) and then incubated with 1 × HBSS containing 30 mM EDTA at 37°C for 15 min. The tissue was dispersed in 1 × HBSS solution by vortexing and separated into epithelial crypts and stromal tissues as described previously.[31]

Quantitative real-time RT-PCR. Total RNA was extracted from isolated epithelial crypts and stromal tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was used for the synthesis of first-strand cDNA. Quantitative real-time RT-PCR was performed using specific primer/probe sets that amplified the *IDO*, *tryptophan 2,3-dioxygenase* (*TDO*), *cyclooxygenase* (*COX*)-*2*, *IFN-γ*, and *GAPDH* genes (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA) and TOYOBO Real-time PCR Master Mix (TOYOBO, Osaka, Japan). Each sample was analyzed on a LightCycler 1.0 (Roche Diagnostics, Mannheim, Germany), as described previously.[32] The expression of each gene was normalized against that of *GAPDH* using the standard curve method.

Determination of IDO activity. Indoleamine 2,3-dioxygenase activity was determined by calculating the ratio of L-kynurenine/L-tryptophan in serum and colonic tissues.[33] Serum samples were deproteinized with 3% perchloric acid. Isolated epithelial crypt and stromal samples were homogenized in 2 μL of 3% perchloric acid per mg tissue. After centrifugation at 4°C and 20 000 *g* for 10 min, aliquots of the supernatant were collected for HPLC determination of L-tryptophan and L-kynurenine concentrations, as described previously.[34]

The enzymatic activity of IDO was also measured using cell-free assays. An aliquot of recombinant human IDO (R&D Systems, Minneapolis, MN, USA) was diluted in 50 mM 2-(*N*-morpholino)ethanesulfonic buffer (pH 6.5). The reaction mixture contained 50 μL enzyme preparation and 50 μL substrate solution, which consisted of 100 mM potassium phosphate buffer (pH 6.5), 50 μM methylene blue, 20 μg catalase, 50 mM ascorbate, 0.4 mM L-tryptophan, and 2000 μM 1-MT or 200 μM EGCG. After incubation of the reaction mixture at 37°C for 1 h, the concentrations of the enzymatic products were measured by HPLC.[35] Enzymatic activity is expressed as the product content per hour.

Statistical analysis. All data are expressed as the mean ± SD. Differences between groups were analyzed by two-way ANOVA and, when statistical significance was found, individual

doi: 10.1111/j.1349-7006.2012.02237.x

differences were evaluated using the Tukey–Kramer multiple comparison test. $P < 0.05$ was considered significant.

Results

General observations. All rats remained healthy and none died during the experimental period. There were no significant differences in the consumption of food (data not shown) and drinking water (Table 1) between the different groups. Body, liver, and relative liver weights, as well as the length of the large bowel, at the end of the study are given in Table 1. The mean body weight of the AOM + 1-MT group was only significantly less than that of the 1-MT group ($P < 0.05$). This decrease may have been due to AOM toxicity, as observed in previous studies,[19,36,37] because 1-MT alone did not reduce body weight in the absence of AOM. Other measurements did not differ significantly among the groups. Histopathologically, there were no findings suggesting toxicity of 1-MT or EGCG in the liver, kidney, or spleen of rats (data not shown).

Effects of 1-MT and EGCG on AOM-induced ACF and BCAC in F344 rats. All rats in the AOM, AOM + 1-MT, and AOM + EGCG groups (i.e. all those treated with AOM) developed ACF and BCAC. In the 1-MT, EGCG, and untreated groups, there were no microscopically observable changes, including ACF or BCAC, in the colon. Compared with the group treated with AOM alone, daily oral administration of 1-MT and EGCG in the drinking water significantly reduced the frequency of ACF ($P < 0.001$ for each comparison). The reduction in the frequency of ACF was significantly greater following EGCG administration than after 1-MT administration ($P < 0.05$). We also noticed a significant reduction in the percentage of large ACF, consisting of four or more aberrant crypts, in the AOM + 1-MT and AOM + EGCG groups compared with the AOM group ($P < 0.001$ for each comparison; Fig. 2a). In addition, the number of BCAC per cm^2 in the AOM + 1-MT and AOM + EGCG groups was significantly less than that in the AOM group ($P < 0.001$ for each comparison; Fig. 2b).

Immunohistochemical analysis of IDO and L-kynurenine in the colonic mucosa. The expression of IDO and L-kynurenine was determined in colonic crypts and preneoplastic lesions (i.e. ACF and BCAC) using immunohistochemical analysis. Compared with colonic crypt cells in untreated control rats, which exhibited only weak positive cytoplasmic staining to IDO, there was a significant increase in IDO staining in the atypical cell cytoplasm of the ACF and BCAC that had developed in AOM-treated rats. Furthermore, L-kynurenine expression, which was very weak in normal crypts of untreated control rats, was slightly increased in the ACF and BCAC of AOM-treated rats (Fig. 3a). Neither EGCG nor 1-MT treatment significantly altered the AOM-induced increases in IDO and L-kynurenine staining (Fig. 3b,c).

Effects of 1-MT and EGCG on IDO and TDO expression in isolated epithelial crypts and stromal cells. In cancer tissues, IDO is overexpressed in both tumor epithelial cells and antigen-presenting cells in the stroma.[38] Tryptophan 2,3-dioxygenase, a hepatic enzyme that catalyses the first step of tryptophan degradation, is also expressed in many tumors.[39] Therefore, after crypt isolation, we determined whether there was increased expression of IDO and TDO in both epithelial crypts and stromal tissues in the colon of AOM-treated rats. As indicated in Figure 4(a,b), quantitative RT-PCR analysis revealed a significant increase in IDO expression in both the crypts and stromal cells of the AOM-treated group compared with the untreated control group ($P < 0.05$ for each comparison). Furthermore, these increases were significantly inhibited by EGCG treatment ($P < 0.05$ for each comparison). Although 1-MT treatment tended to decrease IDO expression in crypts and stromal tissues, the difference failed to reach statistical significance. In the absence of AOM treatment, *IDO* mRNA expression was not affected by the administration of either 1-MT or EGCG. In contrast with IDO, AOM did not induce an increase in *TDO* mRNA expression and neither EGCG nor 1-MT had any effect on TDO expression in crypts and stromal tissues (Fig. 4c,d).

Effects of 1-MT and EGCG on IDO activity. We next examined the enzymatic activity of IDO in serum and colon tissues of AOM-treated rats by measuring the concentrations of L-kynurenine and L-tryptophan. The L-kynurenine/L-tryptophan ratios in the serum (Fig. 5a) and stromal cells (Fig. 5c) of the AOM-treated group were significantly higher than in the untreated control group ($P < 0.05$ for each comparison). Treatment of rats with 1-MT and EGCG resulted in a significant decrease in these ratios in AOM-treated rats ($P < 0.05$ for each comparison), suggesting that 1-MT and EGCG significantly inhibit both the systemic (serum) and focal (colonic stromal) AOM-induced increases in IDO activity. In epithelial cells, there were no significant differences in the L-kynurenine/L-tryptophan ratios between the different groups (Fig. 5b). In the absence of AOM treatment, neither 1-MT nor EGCG alone had any effect on the L-kynurenine/L-tryptophan ratios (Fig. 5a–c).

To further investigate whether 1-MT and EGCG directly influence IDO activity, we measured IDO enzyme kinetics (kynurenine production) using recombinant human IDO in a cell-free system. As shown in Figure 6, levels of L-kynurenine produced by IDO were significantly inhibited by 1-MT and EGCG treatment ($P < 0.001$ for each comparison). These findings suggest that both 1-MT and EGCG act directly to inhibit IDO activity.

Effects of 1-MT and EGCG on COX-2 and IFN-γ expression in stromal cells. We next assessed the inhibitory effects of 1-MT and EGCG on COX-2 and IFN-γ expression, because both are regulated by inflammatory cells in the stroma and are implicated in the induction of IDO.[40–43] Using quantitative RT-PCR, we found that the expression of *COX-2* mRNA in stromal tissues was markedly upregulated in the AOM-treated group, but this upregulation was significantly inhibited by

Table 1. General parameters

Treatment	No. rats examined	Drinking water intake (g/day)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Length of the large bowel (cm)
AOM alone	14	27.3 ± 1.3	203 ± 11	10.2 ± 1.0	5.0 ± 0.5	19.0 ± 1.8
AOM + 0.2% 1-MT	14	25.2 ± 2.3	198 ± 13*	10.1 ± 0.7	5.1 ± 0.4	19.6 ± 1.1
AOM + 0.1% EGCG	14	26.1 ± 2.9	202 ± 8	9.8 ± 0.8	4.9 ± 0.5	19.0 ± 0.9
0.2% 1-MT	6	25.3 ± 4.3	217 ± 12	10.6 ± 0.8	4.9 ± 0.2	20.3 ± 1.6
0.1% EGCG	6	26.5 ± 1.4	212 ± 16	10.2 ± 0.9	4.8 ± 0.6	19.5 ± 1.1
No treatment	6	26.8 ± 0.8	208 ± 9	9.6 ± 0.8	4.6 ± 0.4	19.0 ± 2.1

Data are given as the mean ± SD. *$P < 0.05$ compared with 0.2% 1-methyltryptophan (1-MT) alone. AOM, azoxymethane; EGCG, (−)-epigallocatechin gallate.

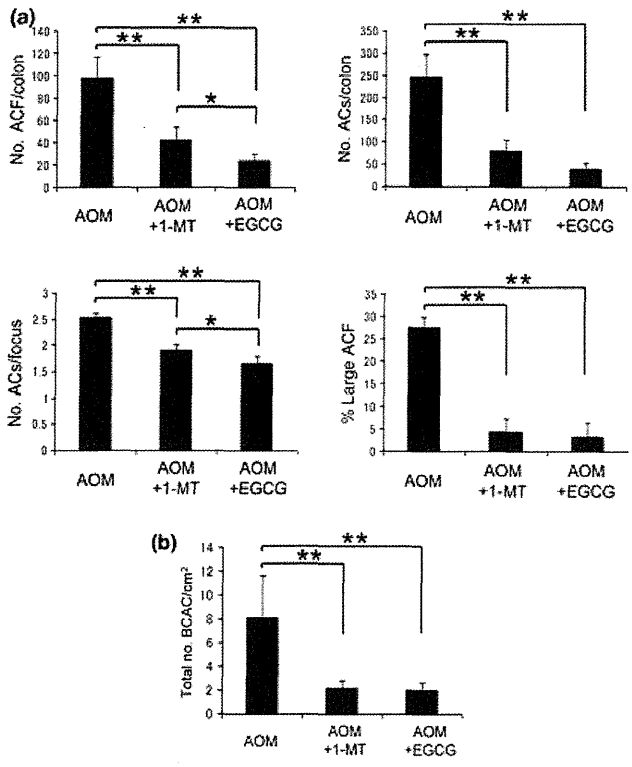

Fig. 2. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on azoxymethane (AOM)-induced formation of aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCAC). (a) Number of ACF per colon, total number of aberrant crypts (ACs) per colon, the number of ACs in each focus, and the percentage of large ACF (i.e. those with four or more ACs). (b) Number of BCAC per cm^2. Data are the mean ± SD (n = 6). *P < 0.05, **P < 0.001.

EGCG treatment (Fig. 7a). In addition, although AOM increased *IFN-γ* mRNA expression in stromal cells, this increase was not inhibited by EGCG (Fig. 7b). Treatment of rats with 1-MT did not have any significant effect on AOM-induced increases in the expression of *COX-2* or *IFN-γ* mRNA. In the absence of AOM treatment, neither 1-MT nor EGCG alone had any effect on *COX-2* or *IFN-γ* mRNA levels (Fig. 7a,b).

Discussion

The results of the present study suggest that upregulation of IDO is possibly involved in colon carcinogenesis, as evidenced by higher IDO expression (Figs 3a,4a,b) and activity (Fig. 5c) in the colonic mucosa of AOM-treated rats compared with the untreated controls, which did not receive any carcinogen. The results of the present study also provide the first evidence that treatment with the IDO inhibitor 1-MT effectively suppresses the development of colonic preneoplastic lesions (ACF and BCAC) induced by AOM (Fig. 2). This inhibition is considered to be associated with the inhibition of IDO activity, which is increased in AOM-treated rats (Fig. 5a,c), because IDO-mediated immune tolerance plays a critical role in tumor development and progression.[2,3] Therefore, 1-MT may correct IDO-mediated immune escape and thus suppress AOM-induced colorectal carcinogenesis. These results suggest that upregulation of IDO, and most likely subsequent immune tolerance caused by this enzyme, is involved in the early phase of colon carcinogenesis and that targeting IDO may, therefore, be an effective strategy to prevent colorectal carcinogenesis.

The chemopreventive and anti-cancer effects of green tea and EGCG are partially attributed to their anti-oxidative properties, their anti-angiogenic and anti-mutagenic effects, and their anti-inflammatory activities, all of which act in combination to suppress carcinogenesis. Thus, these activities are considered to be the main mechanisms underlying the anti-cancer effects of EGCG.[13,14] Furthermore, earlier studies showed

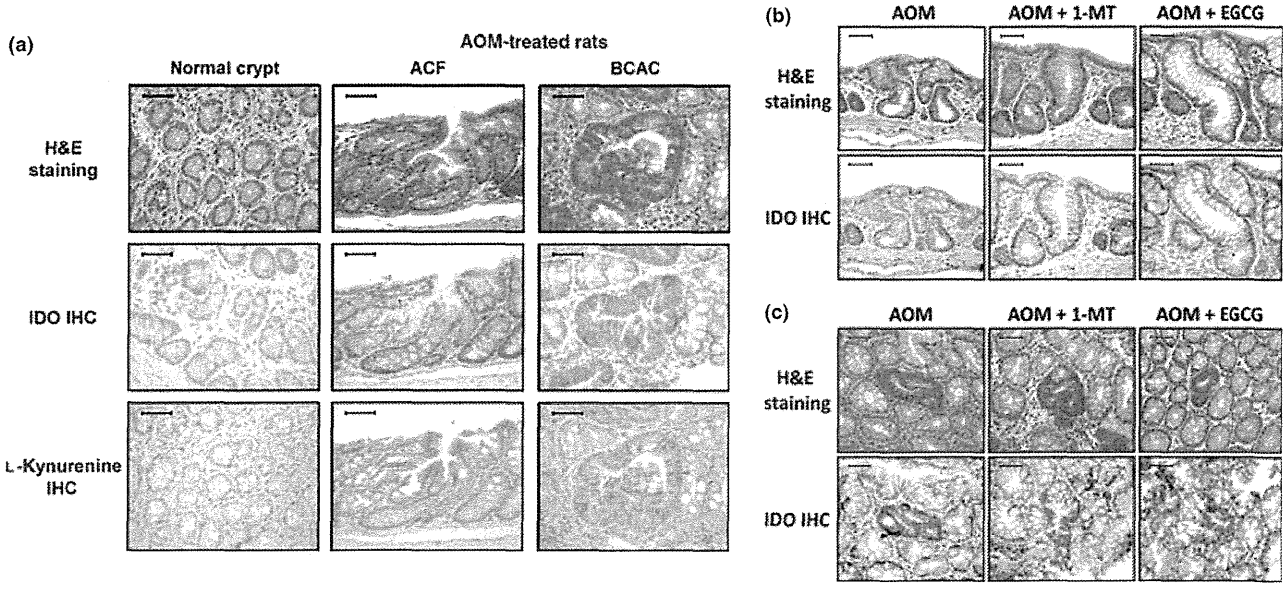

Fig. 3. *Immunohistochemical evaluation of the expression of indoleamine 2,3-dioxygenase (IDO) and L-kynurenine in normal crypts from untreated rats and aberrant crypt foci (ACF), and β-catenin-accumulated crypts (BCAC) in the colonic mucosa of rats treated with azoxymethane (AOM).* (a) Expression of IDO and L-kynurenine in representative samples of colonic mucosa, as evidenced by H&E staining and immunohistochemistry (IHC). (b,c) Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of IDO in ACF (b) and BCAC (c), as determined by IHC. Scale lines, 50 μm.

doi: 10.1111/j.1349-7006.2012.02237.x

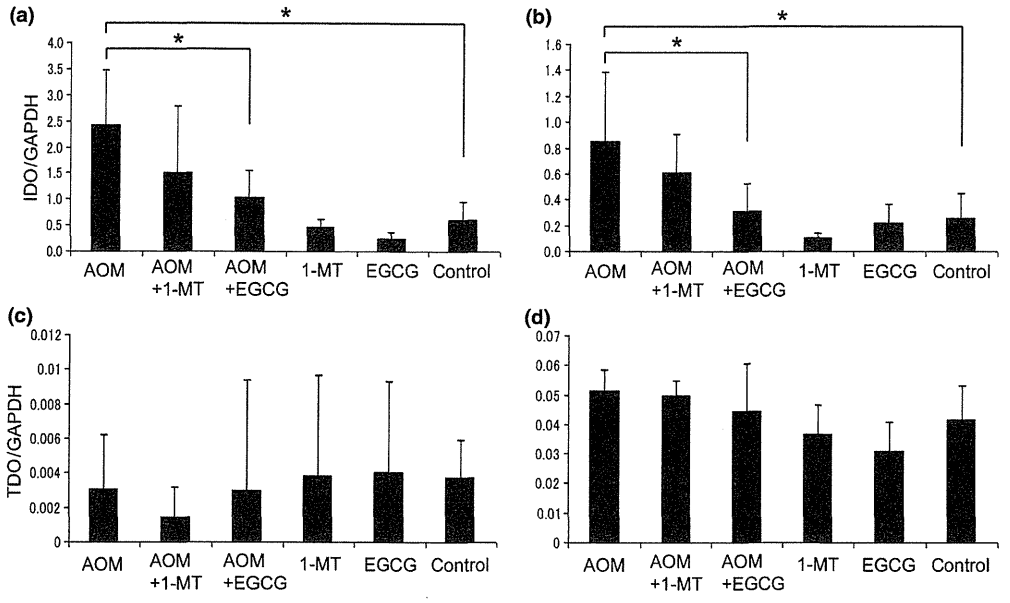

Fig. 4. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of (a,b) indoleamine 2,3-dioxygenase (IDO) and (c,d) tryptophan 2,3-dioxygenase (TDO) in the epithelium (a,c) and stroma (b,d). Total RNA was extracted from epithelial crypts and stromal tissues and IDO and TDO mRNA expression evaluated by quantitative RT-PCR. Expression is normalized against that of GAPDH. AOM, azoxymethane. Data are the mean ± SD (n = 6). *P < 0.05.

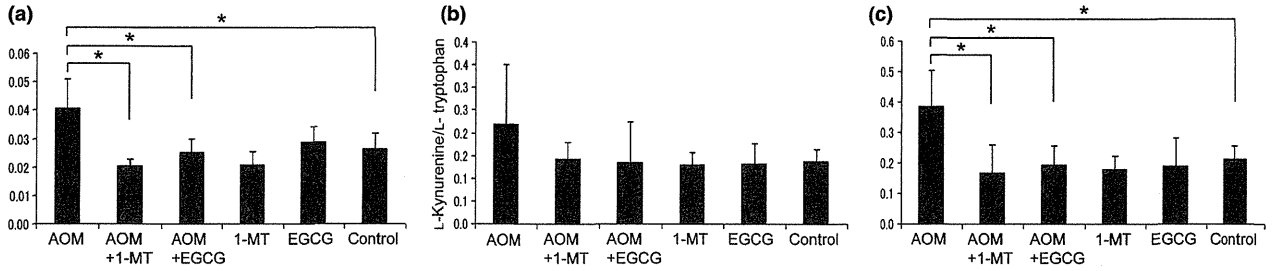

Fig. 5. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on indoleamine 2,3-dioxygenase (IDO) activity in the (a) serum and colonic (b) epithelium and (c) stroma. Functional IDO activity was determined by measuring the concentrations of L-kynurenine and tryptophan using HPLC. The L-kynurenine/L-tryptophan ratio indicates IDO activity. AOM, azoxymethane. Data are the mean ± SD (n = 6). *P < 0.05.

that EGCG suppresses the induction of IDO *in vitro*.[23,24] In the present study, EGCG inhibited the functional enzyme activity of IDO in AOM-treated rats (Fig. 5a,c). In addition, the inhibitory effects of EGCG against AOM-induced increases in *IDO* mRNA expression in the colonic mucosa were greater than those of 1-MT (Fig. 4a,b). This may be associated with the observation that EGCG caused a greater inhibition of the total number of ACF that did 1-MT (Fig. 2a). Therefore, these results suggest that, in addition to the previously reported multiple critical mechanisms of action underlying tumor suppression,[13,14] EGCG may prevent the early phase of colon carcinogenesis, at least in part, by inhibiting the expression and activity of IDO and thus mediating an immune response. The results of a recent study indicating that green tea catechins exert anti-cancer effects by regulating the expression and function of both T and natural killer cells[44] may also strengthen the case for EGCG modulating immune tolerance.

A recent study has revealed the possible roles of toxic tryptophan catabolites produced by IDO in cancer.[45] Of these metabolites, L-kynurenine is considered to play a critical role in the immune escape of malignant cells that occurs within the tumor and its surrounding microenvironment.[4] Conversely, IDO inhibitors can impede the growth of IDO-expressing tumors by reducing the amount of kynurenine present in the microenvironment.[46] Therefore, in addition to inhibiting IDO expression, EGCG has a direct effect in inhibiting IDO enzyme activity (Fig. 6), which may have contributed to its prevention of the development of colonic preneoplastic lesions in the present study.

In the present study, *IDO* mRNA levels in both the epithelium and stroma decreased in rats treated with 1-MT or EGCG; however, the ratio of L-kynurenine/L-tryptophan decreased only in the stroma (Fig. 4a,b,5c). These findings suggest that IDO-induced metabolic conversion of tryptophan to kynurenine occurs mainly in the stroma. For example, in human dendritic cells constitutively expressing IDO protein, the functional activity of this enzyme is tightly regulated and requires additional triggering signals supplied during antigen presentation by $CD4^+$ T cells.[47] Many important immunoregulatory pathways, such as the IFN/JAK/signal transducer and activator of transcription (STAT) pathway and the non-canonical nuclear factor-κB pathway, which are controlled by

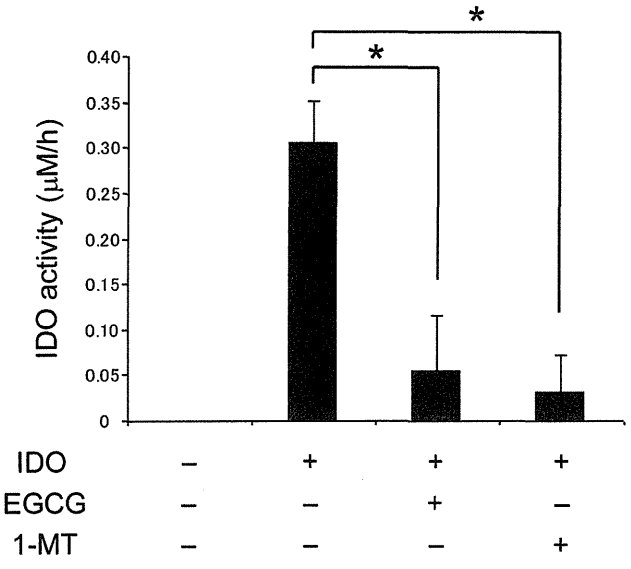

Fig. 6. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on indoleamine 2,3-dioxygenase (IDO) activity in cell-free assays. Functional activity of recombinant human IDO enzyme in response to 1-MT and EGCG was determined by measuring the concentrations of enzymatic products (L-kynurenine) using HPLC. Enzymatic activity is expressed as the product content per hour (μM/h). Data are the mean ± SD. *$P < 0.001$.

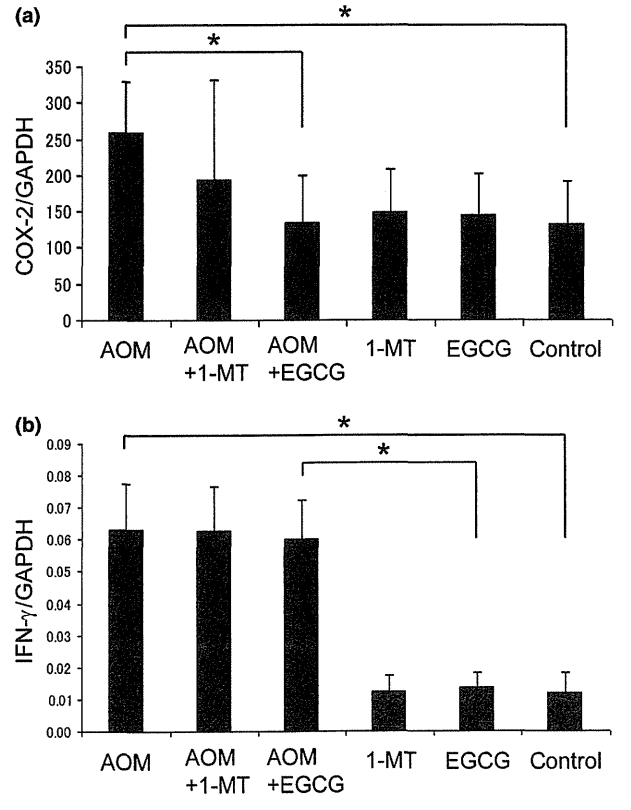

Fig. 7. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of (a) cyclooxygenase (COX)-2 and (b) interferon (IFN)-γ in stromal cells, as determined by quantitative RT-PCR. Expression is normalized against that of GAPDH. AOM, azoxymethane. Data are the mean ± SD ($n = 6$). *$P < 0.05$.

immune cells in the stroma, are related to IDO expression.[38] In addition, several other immune regulatory factors have been implicated as inducers of IDO, including COX-2, which is regarded as one of the most critical inflammatory mediators in the regulation of IDO expression.[42] In the present study, the AOM-induced upregulation of COX-2 in the colonic stroma of rats was significantly inhibited by the administration of EGCG (Fig. 7a). These findings, together with those of Basu *et al.*,[43] who reported a suppressive role of a COX-2 inhibitor against IDO expression in the tumor microenvironment, suggest that EGCG inhibits the expression of IDO, possibly by preventing the induction of COX-2, although further investigations are required to clarify the effects of EGCG. Thus, combination treatment using an IDO inhibitor plus a COX-2 inhibitor may be an effective regimen for the chemoprevention of colorectal cancer because this combination will synergistically inhibit the expression and activity of IDO.

Interferon-γ is also thought to be a major stimulator of IDO,[40,41] and EGCG has been reported to suppress IDO expression by inhibiting STAT-1 activation in response to IFN-γ *in vitro*.[23,24] However, in the present study the expression of IFN-γ in the colonic stroma was not affected by EGCG in the drinking water (Fig. 7b). Other novel mechanisms by which EGCG modulates the expression of IDO may exist; therefore, further studies are needed to clarify the effects of EGCG on the immunoregulatory pathways related to IDO expression.

Aberrant crypt foci have attracted attention as putative precancerous lesions of the colon in experimental models.[48] Numerous molecular abnormalities, including increased expression of K-*ras* and *APC* gene mutations, have been demonstrated in human ACF.[49] In addition, BCAC, which accumulate β-catenin protein in the nucleus and cytoplasm, are regarded as putative precursors to colorectal adenomas.[50] Several rodent studies have shown that both these lesions are useful as biomarkers to evaluate the chemopreventive properties of specific agents.[19,36,37,51] Therefore, our findings, namely that both 1-MT and EGCG markedly inhibit the development of ACF and BCAC, appear to be significant when considering the chemoprevention of colorectal cancer. In particular, a significant reduction of large ACF by 1-MT and EGCG should be emphasized, because large ACF are known to have a strong correlation with the incidence of colonic adenocarcinoma.[20,21]

Finally, it should be mentioned that one limitation of the present study was that the L-kynurenine/L-tryptophan ratio may not directly reflect IDO activity because kynurenine can be metabolized further and TDO can also produce kynurenine from tryptophan.[12] However, in the present study we presumed that TDO exerted little effect on L-kynurenine levels because the expression of TDO was not affected by AOM treatment (Fig. 4c,d). Systemic IDO activity is currently estimated by the serum L-kynurenine/L-tryptophan ratio,[33] as in the present study, because IDO is an intracellular enzyme and circulating IDO concentrations are barely detectable.[3] In fact, a method for analyzing serum IDO protein itself has not been established in experimental animals and there is only one report, published in 2012, of its detection in humans.[52] This limitation needs to be addressed in future studies.

In conclusion, the escape of precancerous cells from the immune system caused by immune tolerance is involved in certain types of carcinogenesis and, therefore, may be an effective target for the implementation of chemoprevention. The results of the present study support the notion that IDO upregulation, which induces immune tolerance, contributes to the early phase of colon carcinogenesis. Furthermore, the present study is the first to provide evidence that the anti-carcinogenic properties of 1-MT and EGCG may be related to inhibition of

doi: 10.1111/j.1349-7006.2012.02237.x

IDO activity, suggesting that targeting IDO and correcting IDO-mediated immune tolerance with EGCG or an IDO inhibitor could be a promising strategy for the prevention of colorectal cancer development in the future. Further experiments using IDO-knockout mice would strengthen the connection between IDO activity and the development of colorectal cancer, and may prove useful in the exploration of IDO inhibitors as chemopreventive agents for colorectal cancer.

Acknowledgment

The authors thank Mitsui Norin (Tokyo, Japan) for providing the EGCG.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006; **6**: 715–27.
2 Uyttenhove C, Pilotte L, Theate I *et al.* Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med* 2003; **9**: 1269–74.
3 Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004; **4**: 762–74.
4 Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med* 2002; **196**: 459–68.
5 Okamoto A, Nikaido T, Ochiai K *et al.* Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. *Clin Cancer Res* 2005; **11**: 6030–9.
6 Ino K, Yoshida N, Kajiyama H *et al.* Indoleamine 2,3-dioxygenase is a novel prognostic indicator for endometrial cancer. *Br J Cancer* 2006; **95**: 1555–61.
7 Brandacher G, Perathoner A, Ladurner R *et al.* Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells. *Clin Cancer Res* 2006; **12**: 1144–51.
8 Ninomiya S, Hara T, Tsurumi H *et al.* Indoleamine 2,3-dioxygenase in tumor tissue indicates prognosis in patients with diffuse large B-cell lymphoma treated with R-CHOP. *Ann Hematol* 2010; **90**: 409–16.
9 Yoshikawa T, Hara T, Tsurumi H *et al.* Serum concentration of L-kynurenine predicts the clinical outcome of patients with diffuse large B-cell lymphoma treated with R-CHOP. *Eur J Haematol* 2010; **84**: 304–9.
10 Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy. *Nat Med* 2005; **11**: 312–9.
11 Hou DY, Muller AJ, Sharma MD *et al.* Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. *Cancer Res* 2007; **67**: 792–801.
12 Lob S, Konigsrainer A, Rammensee HG, Opelz G, Terness P. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat Rev Cancer* 2009; **9**: 445–52.
13 Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol* 2002; **42**: 25–54.
14 Yang CS, Wang X, Lu G, Picinich SC. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer* 2009; **9**: 429–39.
15 Shimizu M, Deguchi A, Lim JT, Moriwaki H, Kopelovich L, Weinstein IB. (−)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin Cancer Res* 2005; **11**: 2735–46.
16 Shimizu M, Deguchi A, Joe AK, McKoy JF, Moriwaki H, Weinstein IB. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. *J Exp Ther Oncol* 2005; **5**: 69–78.
17 Shimizu M, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. *Biochem Biophys Res Commun* 2005; **334**: 947–53.
18 Shirakami Y, Shimizu M, Tsurumi H, Hara Y, Tanaka T, Moriwaki H. EGCG and polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM and DSS. *Mol Med Report* 2008; **1**: 355–61.
19 Shimizu M, Shirakami Y, Sakai H *et al.* (−)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-*db/db* mice. *Cancer Prev Res* 2008; **1**: 298–304.
20 Pretlow TP, O'Riordan MA, Somich GA, Amini SB, Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis* 1992; **13**: 1509–12.
21 Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett* 1995; **93**: 55–71.
22 Xiao H, Hao X, Simi B *et al.* Green tea polyphenols inhibit colorectal aberrant crypt foci (ACF) formation and prevent oncogenic changes in dysplastic ACF in azoxymethane-treated F344 rats. *Carcinogenesis* 2008; **29**: 113–9.
23 Jeong YI, Jung ID, Lee JS, Lee CM, Lee JD, Park YM. (−)-Epigallocatechin gallate suppresses indoleamine 2,3-dioxygenase expression in murine dendritic cells: evidences for the COX-2 and STAT1 as potential targets. *Biochem Biophys Res Commun* 2007; **354**: 1004–9.
24 Cheng CW, Shieh PC, Lin YC *et al.* Indoleamine 2,3-dioxygenase, an immunomodulatory protein, is suppressed by (−)-epigallocatechin-3-gallate via blocking of gamma-interferon-induced JAK-PKC-delta-STAT1 signaling in human oral cancer cells. *J Agric Food Chem* 2010; **58**: 887–94.
25 Yamada Y, Yoshimi N, Hirose Y *et al.* Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res* 2001; **61**: 1874–8.
26 Shimizu M, Sakai H, Shirakami Y *et al.* Preventive effects of (−)-epigallocatechin gallate on diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-*db/db* mice. *Cancer Prev Res* 2011; **4**: 396–403.
27 Wang ZY, Agarwal R, Bickers DR, Mukhtar H. Protection against ultraviolet B radiation-induced photocarcinogenesis in hairless mice by green tea polyphenols. *Carcinogenesis* 1991; **12**: 1527–30.
28 Moolenbeek C, Ruitenberg EJ. The "Swiss roll": a simple technique for histological studies of the rodent intestine. *Lab Anim* 1981; **15**: 57–9.
29 Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987; **37**: 147–51.
30 Suzuki R, Kohno H, Yasui Y *et al.* Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male *db/db* mice. *Int J Cancer* 2007; **120**: 252–8.
31 Sakai H, Yamada Y, Shimizu M, Saito K, Moriwaki H, Hara A. Genetic ablation of Tnfalpha demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis. *Chem Biol Interact* 2010; **184**: 423–30.
32 Hoshi M, Saito K, Hara A *et al.* The absence of IDO upregulates type I IFN production, resulting in suppression of viral replication in the retrovirus-infected mouse. *J Immunol* 2010; **185**: 3305–12.
33 Suzuki Y, Suda T, Furuhashi K *et al.* Increased serum kynurenine/tryptophan ratio correlates with disease progression in lung cancer. *Lung Cancer* 2010; **67**: 361–5.
34 Fujigaki S, Saito K, Sekikawa K *et al.* Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism. *Eur J Immunol* 2001; **31**: 2313–8.
35 Fujigaki S, Saito K, Takemura M *et al.* The L-Tryptophan–L-kynurenine pathway metabolism accelerated by *Toxoplasma gondii* infection is abolished in gamma interferon-gene-deficient mice: cross-regulation between inducible nitric oxide synthase and indoleamine-2,3-dioxygenase. *Infect Immun* 2002; **70**: 779–86.
36 Shimizu M, Shirakami Y, Iwasa J *et al.* Supplementation with branched-chain amino acids inhibits azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-*db/db* mice. *Clin Cancer Res* 2009; **15**: 3068–75.
37 Yasuda Y, Shimizu M, Shirakami Y *et al.* Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice. *Cancer Sci* 2010; **101**: 1701–7.
38 Katz JB, Muller AJ, Prendergast GC. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. *Immunol Rev* 2008; **222**: 206–21.
39 Dolusic E, Larrieu P, Moineaux L *et al.* Tryptophan 2,3-dioxygenase (TDO) inhibitors. 3-(2-(pyridyl)ethenyl)indoles as potential anticancer immunomodulators. *J Med Chem* 2011; **54**: 5320–34.
40 Carlin JM, Borden EC, Sondel PM, Byrne GI. Interferon-induced indoleamine 2,3-dioxygenase activity in human mononuclear phagocytes. *J Leukoc Biol* 1989; **45**: 29–34.
41 Takikawa O, Tagawa Y, Iwakura Y, Yoshida R, Truscott RJ. Interferon-gamma-dependent/independent expression of indoleamine 2,3-dioxygenase. Studies with interferon-gamma-knockout mice. *Adv Exp Med Biol* 1999; **467**: 553–7.

42 von Bergwelt-Baildon MS, Popov A, Saric T *et al.* CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E_2 and expressed by tumor-associated dendritic cells *in vivo*: additional mechanisms of T-cell inhibition. *Blood* 2006; **108**: 228–37.
43 Basu GD, Tinder TL, Bradley JM *et al.* Cyclooxygenase-2 inhibitor enhances the efficacy of a breast cancer vaccine: role of IDO. *J Immunol* 2006; **177**: 2391–402.
44 Shimizu K, Kinouchi Shimizu N, Hakamata W, Unno K, Asai T, Oku N. Preventive effect of green tea catechins on experimental tumor metastasis in senescence-accelerated mice. *Biol Pharm Bull* 2010; **33**: 117–21.
45 Chung KT, Gadupudi GS. Possible roles of excess tryptophan metabolites in cancer. *Environ Mol Mutagen* 2011; **52**: 81–104.
46 Koblish HK, Hansbury MJ, Bowman KJ *et al.* Hydroxyamidine inhibitors of indoleamine-2,3-dioxygenase potently suppress systemic tryptophan catabolism and the growth of IDO-expressing tumors. *Mol Cancer Ther* 2010; **9**: 489–98.
47 Munn DH, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by human $CD4^+$ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. *J Immunol* 2004; **172**: 4100–10.
48 Raju J. Azoxymethane-induced rat aberrant crypt foci: relevance in studying chemoprevention of colon cancer. *World J Gastroenterol* 2008; **14**: 6632–5.
49 Gupta AK, Pretlow TP, Schoen RE. Aberrant crypt foci: what we know and what we need to know. *Clin Gastroenterol Hepatol* 2007; **5**: 526–33.
50 Mori H, Hata K, Yamada Y, Kuno T, Hara A. Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chem Biol Interact* 2005; **155**: 1–9.
51 Yasui Y, Suzuki R, Kohno H *et al.* 9*trans*,11*trans* conjugated linoleic acid inhibits the development of azoxymethane-induced colonic aberrant crypt foci in rats. *Nutr Cancer* 2007; **59**: 82–91.
52 Eleftheriadis T, Antoniadi G, Liakopoulos V, Stefanidis I, Galaktidou G. Plasma indoleamine 2,3-dioxygenase concentration is increased in hemodialysis patients and may contribute to the pathogenesis of coronary heart disease. *Renal Fail* 2012; **34**: 68–72.

doi: 10.1111/j.1349-7006.2012.02237.x

Nutrition and Cancer, 1–8, 2011

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2012.630554

Routledge
Taylor & Francis Group

Preventive Effects of Curcumin on the Development of Azoxymethane-Induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db/db* Obese Mice

Masaya Kubota, Masahito Shimizu, Hiroyasu Sakai, Yoichi Yasuda, Daishi Terakura, Atsushi Baba, Tomohiko Ohno, and Hisashi Tsurumi
Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu, Japan

Takuji Tanaka
Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

Hisataka Moriwaki
Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu, Japan

Obesity-related metabolic abnormalities include a state of chronic inflammation and adipocytokine imbalance, which increase the risk of colon cancer. Curcumin, a component of turmeric, exerts both cancer preventive and antiinflammatory properties. Curcumin is also expected to have the ability to reverse obesity-related metabolic derangements. The present study examined the effects of curcumin on the development of azoxymethane (AOM)-induced colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Feeding with a diet containing 0.2% and 2.0% curcumin caused a significant reduction in the total number of colonic premalignant lesions compared with basal diet-fed mice. The expression levels of tumor necrosis factor-α, interleukin-6, and cyclooxygenase-2 (COX-2) mRNAs on the colonic mucosa of AOM-treated mice were significantly decreased by curcumin administration. Dietary feeding with curcumin markedly activated AMP-activated kinase, decreased the expression of COX-2 protein, and inhibited nuclear factor-κB activity on the colonic mucosa of AOM-treated mice. Curcumin also increased the serum levels of adiponectin while conversely decreasing the serum levels of leptin and the weights of fat. In conclusion, curcumin inhibits the development of colonic premalignant lesions in an obesity-related colorectal carcinogenesis model, at least in part, by attenuating chronic inflammation and improving adipocytokine imbalance. Curcumin may be useful in the chemoprevention of colorectal carcinogenesis in obese individuals.

Submitted 28 June 2010; accepted in final form 20 June 2011.
Address correspondence to Masahito Shimizu, Department of Internal Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Phone: +81-(58) 230-6313. Fax: +81-(58) 230-6310. E-mail: shimim-gif@umin.ac.jp

INTRODUCTION

Colorectal cancer (CRC) is a serious global health care problem. There is increasing evidence that obesity and its related metabolic abnormalities are associated with colorectal carcinogenesis. Several biological mechanisms linking obesity to the development of CRC have been demonstrated, including the emergence of insulin resistance and alterations in the insulin-like growth factor (IGF)/IGF-1 receptor axis (1,2). Imbalance of adipocytokines (such as decreased adiponectin levels and increased leptin levels) and a state of chronic inflammation, both of which are highly correlated with the presence of excess adipose tissue, also play roles in obesity-related colorectal carcinogenesis (1,2). For instance, there is a close correlation between lower levels of serum adiponectin and the development of colorectal tumors (3,4). Increased leptin levels exert tumor-promoting effects in obesity- and inflammation-related CRC (5–9). Tumor necrosis factor (TNF)-α, a central mediator of chronic inflammatory diseases, has markedly increased expression levels in adipose tissue, and its dysregulation is associated with stimulation of tumor promotion and progression of carcinogenesis (10,11). These reports suggest that targeting adipocytokine imbalance and chronic inflammation may provide effective strategies for preventing the development of obesity-related CRC (6–8).

Curcumin, a yellow-colored pigment derived from turmeric (Curcuma longa), has been shown to exert numerous pharmacological effects, including antiinflammatory and chemopreventive properties (12–14). In rodent models, dietary curcumin was found to inhibit chemically induced colorectal carcinogenesis (see the Chemopreventive Database at http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html). Curcumin also inhibited cell growth and induced apoptosis in human CRC-derived cells

by suppressing the expression of cyclooxygenase-2 (COX-2) (15,16), which is one of the most critical targets of CRC chemoprevention (17). Curcumin showed benefits in the treatment of ulcerative colitis in a randomized clinical trial (18), and it was also found to attenuate chronic experimental colitis in rats by reducing the expression of TNF-α and COX-2 on the colonic mucosa (19). Therefore, curcumin may be capable of exerting chemopreventive and antiinflammatory effects on the colon through regulation of TNF-α and COX-2 expression.

In addition, recent studies have revealed that curcumin has the potential to improve obesity-related chronic inflammatory conditions and metabolic derangements. In rodent models of obesity and diabetes, administration of curcumin significantly ameliorates diabetes, increases adiponectin production by adipose tissue, and decreases serum levels of TNF-α and interleukin (IL)-6 (20,21). Therefore, curcumin may be a promising agent for the prevention of obesity-related pathogenesis including CRC development. In the present study, we investigated the effects of curcumin on the development of colonic premalignant lesions, aberrant crypt foci (ACF), and β-catenin accumulated crypts (BCAC) (22,23) in an animal model for obesity-related colorectal carcinogenesis (5–8), which was produced by administrating the colonic carcinogen azoxymethane (AOM) to obese and diabetic C57BL/KsJ-*db/db* (*db/db*) mice.

MATERIALS AND METHODS

Animals and Chemicals

Four-week-old male homozygous *db/db* mice were obtained from Japan SLC (Shizuoka, Japan) and were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. Curcumin and AOM were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Procedure

A total of 38 male *db/db* mice were divided into the following 5 experimental and control groups: untreated (Group 1, $n = 5$); 2% curcumin alone (Group 2, $n = 5$); AOM alone (Group 3, $n = 10$); AOM plus 0.2% curcumin (Group 4, $n = 9$); and AOM plus 2% curcumin (Group 5, $n = 9$). The curcumin concentrations (0.2% and 2%) were chosen based on the following 2 reasons: 1) the doses of dietary curcumin in the most published rodent experiments were between 0.05 and 2% from the Chemopreventive Database; and 2) the dose level of 0.2% curcumin in diet, which is equivalent to 300mg/kg/day (24), is relevant to the dose used in clinical trials. At 5 wk of age, all mice were given 4 weekly subcutaneous injections of saline (Groups 1 and 2) or AOM (15 mg/kg body weight; Groups 3, 4, and 5). The mice in Groups 1 and 3 were fed the basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), throughout the experiment (for 11 wk). Group 2 was fed the basal diet containing 2% curcumin throughout the experiment. Groups 4 and 5 were given the basal diet containing 0.2% and 2% curcumin, respectively, for 7 wk, starting 1 wk after the last injection of AOM. At the termination of the study (16 wk of age), all mice were sacrificed for analysis of ACF and BCAC. The animal experiment was approved by the Committee of the Institutional Animal Experiments of Gifu University.

Identification and Counting of ACF and BCAC

The numbers of ACF and BCAC were determined according to standard procedures (6–8). After fixing flat in 10% buffered formalin for 24 h, the colons were stained with methylene blue (0.5% in distilled water) to count the number of ACF. The distal parts of the colon (2 cm from anus; mean area, 0.7 cm^2/colon) were then cut and embedded in paraffin, and a total of 20 serial sections (each 4 μm thick) per mouse were created by an en face preparation to identify BCAC intramucosal lesions (6–8). For each case, 2 serial sections were used to analyze BCAC.

Histopathology and Immunohistochemical Analyses for β-Catenin and Nuclear Factor-κB

Two serial sections, prepared from the paraffin-embedded tissue blocks, were subjected to hematoxylin and eosin staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC (6–8). In addition, phospho-nuclear factor-κB (NF-κB) p65 immunohistochemistry was performed in vertical histological sections of colonic mucosa to estimate NF-κB activity in the crypt cells and mucosal interstitial cells (25). Immunohistochemistry for β-catenin and phospho-NF-κB p65 was performed using the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark). Anti-β-catenin antibody (1:1000 final dilution) was obtained from BD Transduction Laboratories (San Jose, CA). Antiphospho-NF-κB p65 antibody (Ser276; 1:50 final dilution) was from Cell Signaling Technology (Danvers, MA). In the phospho-NF-κB p65-immunostained sections, the cells with intensely stained nuclei were considered to be active in NF-κB. Cells with active NF-κB in both the colonic epithelium and interstitium were counted and expressed as a percentage of the total number of cells in the tissues. A positive cell index (%) was determined by counting at least 500 cells in the colonic epithelium and 300 cells in the interstitium of each mouse.

Protein Extraction and Western Blot Analysis

Total proteins were extracted from the scraped mucosa from the remaining colon portions of the AOM-treated mice (Groups 3 to 5), and equivalent amounts of proteins (20 μg/lane) were examined by a Western blot analysis (6–8). The primary antibodies for AMP-activated kinase (AMPK), phosphorylated form of AMPK, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (6–8). An antibody against GAPDH served as a loading control.

RNA Extraction and Quantitative Real-Time Reverse Transcription-PCR analysis

Total RNA was isolated from the scraped colonic mucosa of the AOM-treated mice using the RNAqueous-4PCR kit

(Ambion Applied Biosystems, Austin, TX). The cDNA was synthesized from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, San Diego, CA). A quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the TNF-α, IL-6, COX-2, and GAPDH genes, as previously described (8). Real-time RT-PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with the SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the TNF-α, IL-6, and COX-2 genes were normalized to the GAPDH gene expression levels.

Clinical Chemistry

The blood samples, which were collected at the time of sacrifice after 6 h of fasting, were used for chemical analyses. The serum concentrations of adiponectin and leptin (Shibayagi, Gunma, Japan) were determined by using an enzyme immunoassay according to the manufacturer's protocol.

Statistical Analyses

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program, v. 3.05 (GraphPad Software, San Diego, CA) for Macintosh. The differences among the groups were analyzed by 1-way analysis of variance (ANOVA) or by 2-way ANOVA if required. When ANOVA showed a statistically significant effect ($P < 0.05$), comparison of each experimental group with the control group was performed using the Tukey-Kramer multiple comparisons test. The differences were considered statistically significant when the 2-tailed P-value was less than 0.05.

RESULTS

General Observations

During the study, dietary feeding with curcumin did not cause clinical symptoms or evidence of toxicity (data not shown). The mean body weights of the AOM-injected groups (Group 3: 40.9 ± 5.5 g, Group 4: 35.0 ± 2.6 g, and Group 5: 39.1 ± 6.1 g) were lower than that of the saline-injected group (Group 1: 54.6 ± 8.8 g) at the termination of the experiment ($P < 0.01$ for each comparison). This might be caused by the toxicity of AOM, which has been observed in previous experiments (6–8). No significant differences were observed in the mean weights of the liver and kidney among the groups (data not shown). Histopathological examination also revealed that there were no alterations visible in the liver and kidney tissues. This provides further evidence of the lack of toxicity of curcumin with respect to the liver and kidney of the mice in Groups 3 to 5 (data not shown).

Effects of Curcumin on AOM-Induced ACF and BCAC Formations in *db/db* Mice

Table 1 summarizes the total numbers of ACF and BCAC in each group. ACF and BCAC were observed to develop in the colon of all mice that received AOM (Groups 3 to 5), but not in the control groups (Groups 1 and 2). The mean number of ACF in the AOM alone group (Group 3) was 134.0 ± 24.5 and dietary feeding with 0.2% (Group 4) and 2% (Group 5) curcumin significantly reduced the incidence of ACF by 27% ($P < 0.05$) and 43% ($P < 0.01$), respectively. Moreover, the number of BCAC in Group 5 was significantly lower than that in Group 3 (76% inhibition, $P < 0.01$).

Effects of Curcumin on the Expression Levels of TNF-α, IL-6, and COX-2 mRNAs in the Colonic Mucosa of AOM-Injected *db/db* Mice

Quantitative real-time RT-PCR analysis indicated that feeding with 2% of curcumin significantly decreased the expression levels of TNF-α (Fig. 1A, $P < 0.05$) and COX-2 (Fig. 1C, $P < 0.05$) mRNAs in the colonic mucosa of AOM-injected mice relative to those of the curcumin-untreated control mice. In addition, the expression levels of IL-6 mRNA were apparently decreased to a greater extent by administration of both doses of curcumin (Fig. 1B, $P < 0.01$).

Effects of Curcumin on the Activation of AMPK and the Expression of COX-2 Proteins in the Colonic Mucosa of AOM-Injected *db/db* Mice

A Western blot analysis indicated that administration of both concentrations of curcumin caused significant phosphorylation

TABLE 1
Effects of curcumin on AOM-induced ACF and BCAC formation in the colon of male *db/db* mice

Group no.	Treatment	No. of mice	Length of colon (cm)	Total no. ACFs/colon	Total no. BCACs/cm^2
1	Saline	5	11.1 ± 0.8[a]	0	0
2	Saline + 2% curcumin	5	10.5 ± 0.2	0	0
3	AOM alone	10	10.6 ± 0.8	134.0 ± 24.5	3.4 ± 1.8
4	AOM + 0.2% curcumin	9	9.6 ± 1.6	98.2 ± 36.6[b]	1.5 ± 1.1
5	AOM + 2% curcumin	9	10.4 ± 0.4	76.4 ± 13.6[c]	0.8 ± 1.4[c]

[a]Mean ± SD.
[b]Significantly different from Group 3 ($P < 0.05$).
[c]Significantly different from Group 3 ($P < 0.01$).

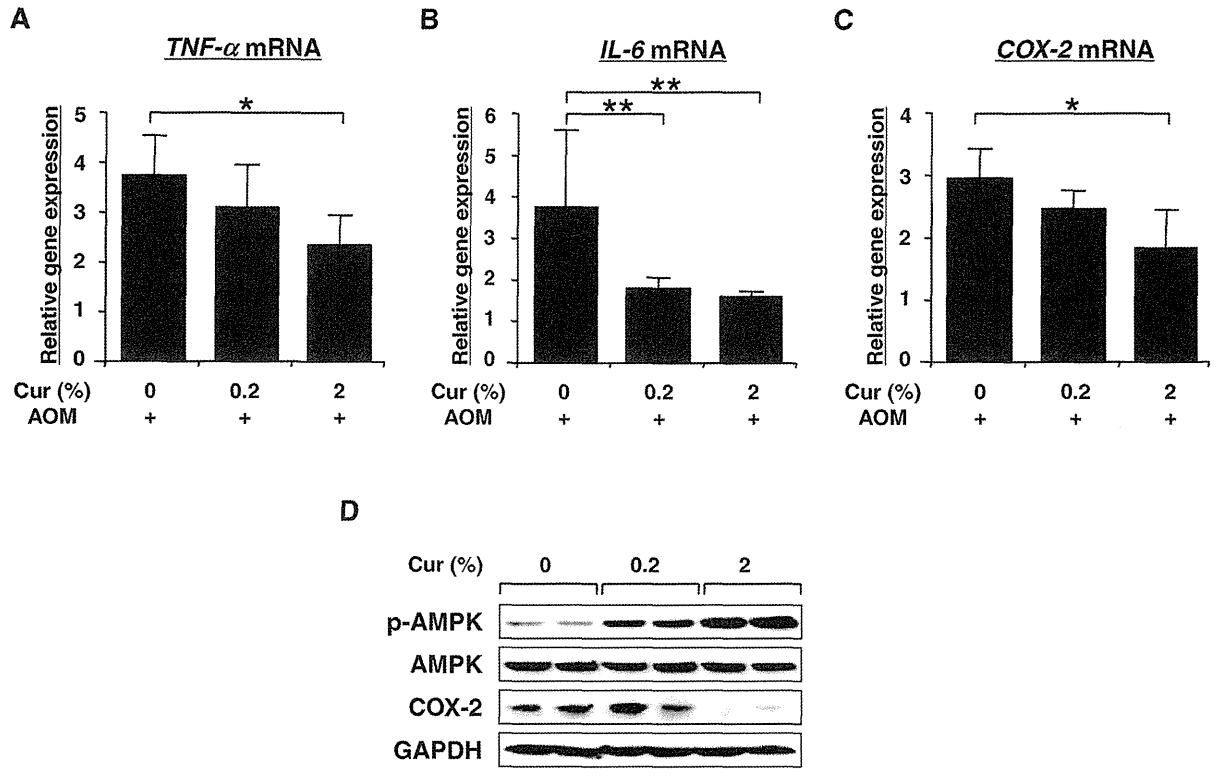

FIG. 1. Effect of curcumin (Cur) on the levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), and cyclooxygenase-2 (COX-2) (C) mRNAs and on the activation of AMP-activated kinase (AMPK) and expression of COX-2 proteins (D) in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. cDNA was synthesized from the colonic mucosa and real-time RT-PCR was performed using TNF-α (A), IL-6 (B), and COX-2 (C) specific primers. The expression levels of these genes were normalized to the level of the GAPDH gene. Each experiment was done in triplicate and the average was subsequently calculated. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. Total proteins were extracted from the scraped colonic mucosa and equivalent amounts of proteins were examined by a Western blot analysis using phosphorylated AMPK (p-AMPK), AMPK, and COX-2 specific antibodies (D). Two lanes represent protein samples from 2 different mice in each group (Groups 3 to 5). An antibody to GAPDH served as a loading control.

(activation) of AMPK protein in the colonic mucosa of AOM-treated mice. In addition, there was an apparent decrease in the expression levels of COX-2 proteins in the colonic mucosa of mice after feeding the diet containing 2% curcumin (Fig. 1D).

Effects of Curcumin on the NF-κB Activity in the Colonic Mucosa of AOM-Injected *db/db* Mice

Curcumin inhibits the activation of NF-κB and this is one of the critical mechanisms for the antiinflammatory effects of this agent (12,13). Therefore, the effects of curcumin on NF-κB activity were examined in the colonic mucosa of AOM-injected mice. As shown in Fig. 2, the indices of phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitium of curcumin-supplemented mice were significantly smaller than those of the curcumin-untreated mice ($P < 0.01$, for each comparison), thus indicating that curcumin supplementation significantly inhibits the NF-κB activity in the colonic mucosa, mainly crypt and inflammatory cells, of the AOM-treated *db/db* mice.

Effects of Curcumin on the Serum Levels of Adiponectin and Leptin and on the Relative Weights of White Adipose Tissue in *db/db* Mice

The serum concentrations of adiponectin in the 2% curcumin-treated groups were significantly higher than those of the groups that did not receive curcumin ($P < 0.01$ and $P < 0.05$), regardless of AOM-injection (Fig. 3A). In AOM-injected groups, administration of both doses of curcumin showed a significant decrease in the serum levels of leptin ($P < 0.05$ for each comparison) when compared to mice that did not receive curcumin (Fig. 3B). In addition, administration of curcumin also significantly reduced the relative weights of white adipose tissue (periorchis and retroperitoneum) compared with the untreated groups ($P < 0.05$ and $P < 0.01$), regardless of AOM-treatment (Fig. 3C).

DISCUSSION

Obesity-related physiological alterations, such as a state of chronic inflammation, are known to influence the risk of CRC development (1,2). This is the first study to report evidence

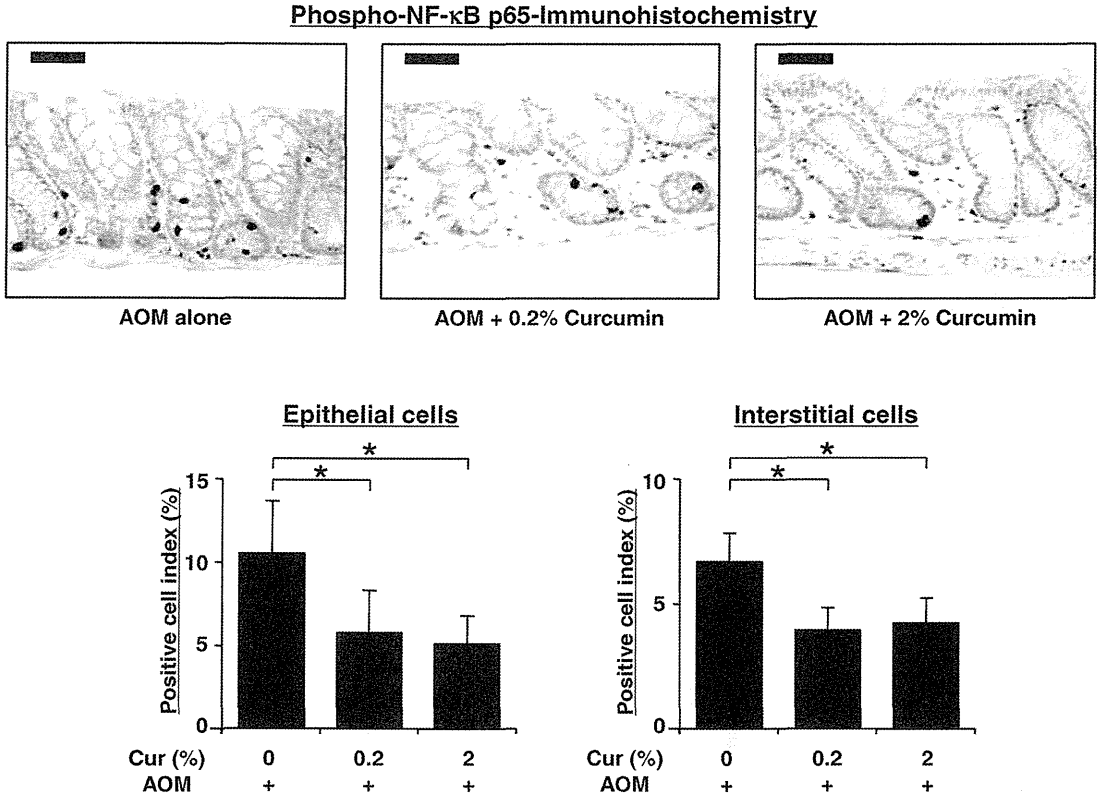

FIG. 2. Effects of curcumin (Cur) on the nuclear factor (NF)-κB activity in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. Sections of the colon were stained with antiphospho-NF-κB p65 antibody. Representative photographs from each group are shown in the upper panels. The positive cell indices, which were determined by counting the phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitial inflammatory cells, are shown in the lower panels. Bars: 50 μm. $^*P < 0.01$ compared to mice not fed with curcumin.

of curcumin effectively inhibiting the development of putative precursor lesions (ACF and BCAC) of colonic adenocarcinoma in the obese and diabetic *db/db* mice (Table 1). These suppressive effects are most likely associated with the decrease in expression of proinflammatory cytokines TNF-α and IL-6 in the colonic mucosa of AOM-treated *db/db* mice (Figs. 1A and 1B), although other anticancer mechanisms, such as modulation of cell proliferation and apoptosis, could be considered (12–14). The expression levels of COX-2, which represents an early response to proinflammatory mediators and a critical target for CRC chemoprevention (17), are also inhibited in the colonic mucosa of mice fed with curcumin (Figs. 1C and 1D). In addition, curcumin supplementation inhibited NF-κB activity, which is involved in regulation of COX-2 expression (26), in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2). These findings are consistent with those of a previous study indicating that curcumin inhibits the induction of COX-2 by TNF-α stimulus via the inhibition of NF-κB activation in human colon epithelial cells (27). Curcumin suppresses the growth of CRC-derived cells by inhibiting the expression of COX-2 (16). Curcumin also inhibits the secretion of TNF-α and IL-6 from monocytes cultured under high-glucose conditions, and lowers the serum levels of TNF-α and IL-6 in diabetic rats (21). Therefore, overexpression of inflammatory mediators, especially TNF-α, IL-6, and COX-2, which are relevant to excessive storage of lipid, might represent critical targets of curcumin to prevent the development of obesity-related CRC.

The present study also demonstrated the first evidence that administration of curcumin markedly enhances AMPK activation in the colonic mucosa of obese and diabetic mice (Fig. 1D). This finding is significant because AMPK, which functions as a major metabolic switch for maintenance of energy homeostasis, controls processes relevant to tumor development and therefore may be a promising target for cancer chemoprevention (28). For instance, the antidiabetic drug metformin, a pharmacological AMPK activator, exerts growth inhibitory effects on colon cancer xenografts as well as on human CRC cells (29,30). Pitavastatin, which is used to treat hyperlipidemia, inhibits development of AOM-induced BCAC in *db/db* mice by activating AMPK and decreasing the expression levels of TNF-α, IL-6, and COX-2 mRNAs in the colonic mucosa (8). Curcumin also inhibits proliferation and induces apoptosis in human CRC cells

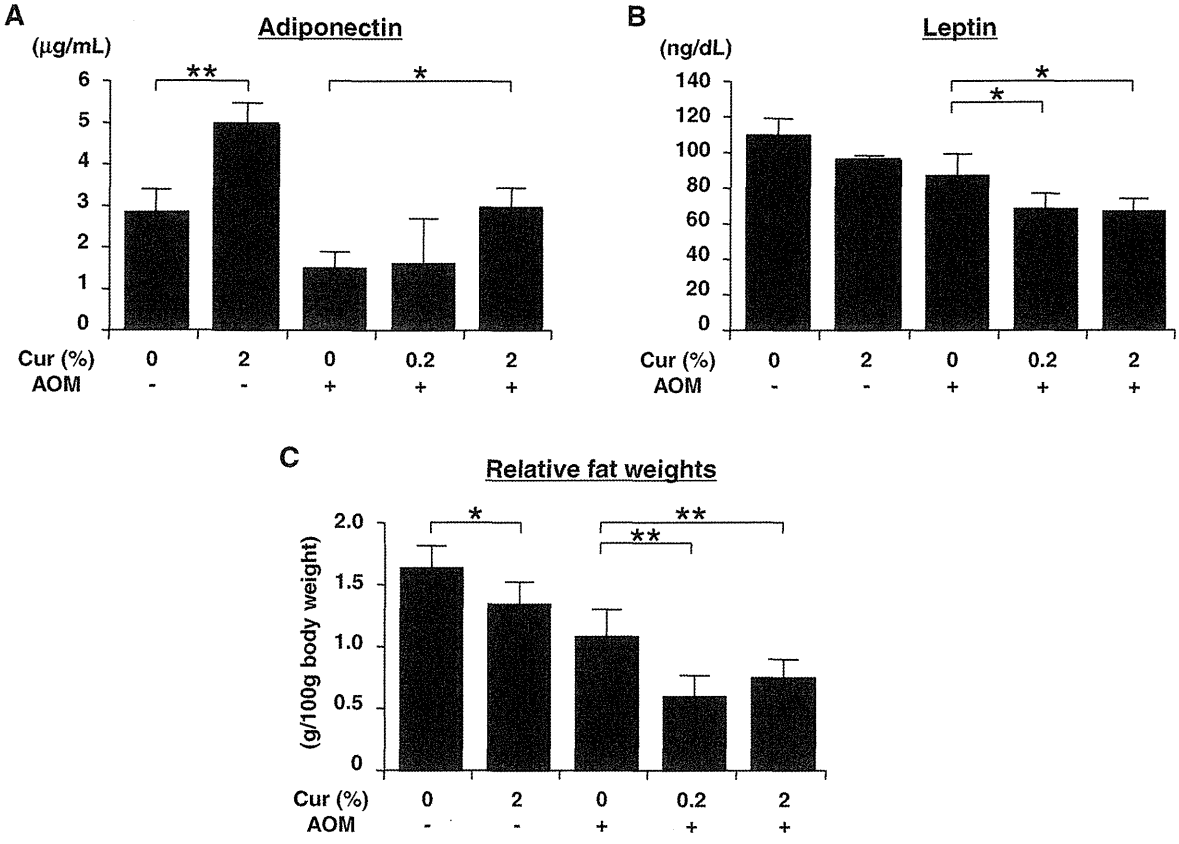

FIG. 3. Effect of curcumin (Cur) on the serum levels of adiponectin and leptin and on the relative weights of fat in experimental mice. The serum concentrations of adiponectin (A) and leptin (B) were measured by using an enzyme immunoassay. At sacrifice, white adipose tissue of the periorchis and retroperitoneum was excised and the relative weights (g/100 g body weight) were calculated (C). Values are expressed as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. AOM indicates azoxymethane.

by activating AMPK and decreasing COX-2 expression (15). These reports (8,15,29,30), together with our present findings (Table 1 and Fig. 1D), suggest that curcumin may prevent the development of obesity-related colorectal carcinogenesis, at least in part, through the activation of AMPK.

Dysregulation of adipocytokines is also involved in the link between obesity and colorectal carcinogenesis (1,2). In particular, a low level of serum adiponectin, which raises the risk of colorectal tumorigenesis (3,4), is associated with obesity-based chronic inflammation because adiponectin exerts antiinflammatory effects by downregulating the production of TNF-α and IL-6 (31). Adiponectin inhibits the growth of CRC cells through the activation of AMPK and suppresses colonic epithelial proliferation in mice that are fed a high-fat diet (32,33). On the other hand, a higher level of serum leptin, which is proportional to the amount of body fat stored, plays a role in enhancing the development of obesity- and inflammation-related CRC (5–9). Leptin also increases cell growth and promotes cell mobility and invasion in CRC-derived cells (34,35). These reports suggest that targeting the imbalance of adiponectin and leptin might be an effective strategy for preventing obesity-related CRC. Indeed, improvement of adipocytokine imbalance by certain agents may contribute to the inhibition of obesity-related tumorigenesis in the colorectum (6–8). Therefore, our findings that dietary curcumin significantly increased the levels of adiponectin as well as decreased the levels of leptin in the serum of *db/db* mice, which might be caused by the reduction in white adipose tissue (Fig. 3), is important to explain the chemopreventive effects of this agent on obesity-related colorectal carcinogenesis. Similarly, it was also reported that curcumin administration ameliorates diabetes and increases the expression of adiponectin both in serum and adipose tissue of leptin-deficient *ob/ob* mice (20). Therefore, in addition to the improvement of metabolic abnormalities, curcumin has the potential to suppress obesity-related colorectal carcinogenesis by affecting the serum levels of adiponectin and leptin.

The limitation of present study is that the dose of curcumin in Group 5 (2%) is relatively high when considering the use of this

agent in clinical practice. However, our histopathologic examination revealed no alterations in the vital organs of the mice that received the diet containing 2% curcumin. Our ongoing study on the safety and chemopreventive ability of different doses of curcumin may show dose-response efficacy in inhibiting colorectal tumorigenesis of mice, as suggested for ACF-inhibition in this study (see Group 4 in Table 1). Moreover, a recent clinical trial has shown a significant result indicating that a daily oral dose of 3.6 g curcumin, which is well tolerated at patients with advanced CRC (36), achieves pharmacologically efficacious levels in the colorectum with negligible distribution of this agent outside the gut (37). This finding suggests that regions of the gastrointestinal tract, such as the colorectum, may be more appropriate targets for curcumin to exert its cancer chemopreventive effects because such organs are directly exposed to high concentrations of this agent.

It should be emphasized again that obesity and obesity-related metabolic abnormalities represent serious global health care problems, and CRC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis, which includes chronic inflammation and adipocytokine imbalance, might be a promising strategy for treating obese individuals who are at an increased risk of developing CRC. Curcumin, which has been shown to exert various chemopreventive and anticancer properties (12–14), appears to be a potentially effective candidate for this purpose because this phytochemical can attenuate inflammation while improving the imbalance of adipocytokines and can, furthermore, prevent the development of colonic precancerous lesions in obese and diabetic *db/db* mice.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (No. 18790457 to M. S. and No. 17015016 to H. M.) and by Grant-in-Aid for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare of Japan.

REFERENCES

1. Gunter MJ and Leitzmann MF: Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes. *J Nutr Biochem* **17,** 145–156, 2006.
2. Huang XF and Chen JZ: Obesity, the PI3K/Akt signal pathway and colon cancer. *Obes Rev* **10,** 610–616, 2009.
3. Wei EK, Giovannucci E, Fuchs CS, Willett WC, and Mantzoros CS: Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *J Natl Cancer Inst* **97,** 1688–1694, 2005.
4. Otake S, Takeda H, Suzuki Y, Fukui T, Watanabe S, et al.: Association of visceral fat accumulation and plasma adiponectin with colorectal adenoma: evidence for participation of insulin resistance. *Clin Cancer Res* **11,** 3642–3646, 2005.
5. Hirose Y, Hata K, Kuno T, Yoshida K, Sakata K, et al.: Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-*db/db* mice. *Carcinogenesis* **25,** 821–825, 2004.
6. Shimizu M, Shirakami Y, Sakai H, Adachi S, Hata K, et al.: EGCG suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-*db/db* mice. *Cancer Prev Res* **1,** 298–304, 2008.
7. Shimizu M, Shirakami Y, Iwasa J, Shiraki M, Yasuda Y, et al.: Supplementation with branched-chain amino acids inhibits azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-*db/db* mice. *Clin Cancer Res* **15,** 3068–3075, 2009.
8. Yasuda Y, Shimizu M, Shirakami Y, Sakai H, Kubota M, et al.: Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice. *Cancer Sci* **101,** 1701–1707, 2010.
9. Miyamoto S, Yasui Y, Tanaka T, Ohigashi H, and Murakami A: Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice. *Carcinogenesis* **29,** 1057–1063, 2008.
10. Hotamisligil GS, Shargill NS, and Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259,** 87–91, 1993.
11. Szlosarek P, Charles KA, and Balkwill FR: Tumour necrosis factor-alpha as a tumour promoter. *Eur J Cancer* **42,** 745–750, 2006.
12. Aggarwal BB: Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. *Annu Rev Nutr* **30,** 173–199, 2010.
13. Aggarwal BB and Harikumar KB: Potential therapeutic effects of curcumin, the antiinflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int J Biochem Cell Biol* **41,** 40–59, 2009.
14. Rao CV: Regulation of COX and LOX by curcumin. *Adv Exp Med Biol* **595,** 213–226, 2007.
15. Lee YK, Park SY, Kim YM, and Park OJ: Regulatory effect of the AMPK-COX-2 signaling pathway in curcumin-induced apoptosis in HT-29 colon cancer cells. *Ann NY Acad Sci* **1171,** 489–494, 2009.
16. Goel A, Boland CR, and Chauhan DP: Specific inhibition of cyclooxygenase-2 (COX-2) expression by dietary curcumin in HT-29 human colon cancer cells. *Cancer Lett* **172,** 111–118, 2001.
17. Gupta RA, and Dubois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* **1,** 11–21, 2001.
18. Hanai H, Iida T, Takeuchi K, Watanabe F, Maruyama Y, et al.: Curcumin maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial. *Clin Gastroenterol Hepatol* **4,** 1502–1506, 2006.
19. Camacho-Barquero L, Villegas I, Sanchez-Calvo JM, Talero E, Sanchez-Fidalgo S, et al.: Curcumin, a Curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis. *Int Immunopharmacol* **7,** 333–342, 2007.
20. Weisberg SP, Leibel R, and Tortoriello DV: Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity. *Endocrinology* **149,** 3549–3558, 2008.
21. Jain SK, Rains J, Croad J, Larson B, and Jones K: Curcumin supplementation lowers TNF-alpha, IL-6, IL-8, and MCP-1 secretion in high glucose-treated cultured monocytes and blood levels of TNF-alpha, IL-6, MCP-1, glucose, and glycosylated hemoglobin in diabetic rats. *Antioxid Redox Signal* **11,** 241–249, 2009.
22. Bird RP and Good CK: The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicol Lett* **112–113,** 395–402, 2000.
23. Yamada Y and Mori H: Precancerous lesions for colorectal cancers in rodents: a new concept. *Carcinogenesis* **24,** 1015–1019, 2003.
24. Perkins S, Verschoyle RD, Hill K, Parveen I, Threadgill MD, et al.: Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* **11,** 535–540, 2002.
25. Larmonier CB, Uno JK, Lee KM, Karrasch T, Laubitz D, et al.: Limited effects of dietary curcumin on Th-1 driven colitis in IL-10 deficient mice suggest an IL-10-dependent mechanism of protection. *Am J Physiol Gastrointest Liver Physiol* **295,** G1079–G1091, 2008.

26. Wu KK: Control of cyclooxygenase-2 transcriptional activation by pro-inflammatory mediators. *Prostaglandins Leukot Essent Fatty Acids* **72,** 89–93, 2005.
27. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, et al.: Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signaling complex. *Oncogene* **18,** 6013–6020, 1999.
28. Fay JR, Steele V, and Crowell JA: Energy homeostasis and cancer prevention: the AMP-activated protein kinase. *Cancer Prev Res* **2,** 301–309, 2009.
29. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, et al.: Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res* **67,** 6745–6752, 2007.
30. Zakikhani M, Dowling RJ, Sonenberg N, and Pollak MN: The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. *Cancer Prev Res* **1,** 369–375, 2008.
31. Ouchi N and Walsh K: Adiponectin as an antiinflammatory factor. *Clin Chim Acta* **380,** 24–30, 2007.
32. Kim AY, Lee YS, Kim KH, Lee JH, Lee HK, et al.: Adiponectin represses colon cancer cell proliferation via AdipoR1- and -R2-mediated AMPK activation. *Mol Endocrinol* **24,** 1441–1452, 2010.
33. Fujisawa T, Endo H, Tomimoto A, Sugiyama M, Takahashi H, et al.: Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition. *Gut* **57,** 1531–1538, 2008.
34. Hardwick JC, Van Den Brink GR, Offerhaus GJ, Van Deventer SJ, and Peppelenbosch MP: Leptin is a growth factor for colonic epithelial cells. *Gastroenterology* **121,** 79–90, 2001.
35. Jaffe T and Schwartz B: Leptin promotes motility and invasiveness in human colon cancer cells by activating multiple signal-transduction pathways. *Int J Cancer* **123,** 2543–2556, 2008.
36. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, et al.: Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res* **10,** 6847–6854, 2004.
37. Garcea G, Berry DP, Jones DJ, Singh R, Dennison AR, et al.: Consumption of the putative chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. *Cancer Epidemiol Biomarkers Prev* **14,** 120–125, 2005.