Table 1 shows data for final body and organs weights and average intakes of water and diet. Final body weights in DMBDD groups 1–3 were not significantly different, and those in non-DMBDD groups 4 and 5 were also not significantly different. Relative liver, kidney and spleen weights also did not significantly vary among groups 1–3, or between groups 4 and 5. Therefore, PJJ-34 did not affect body and major organ weights. In addition, water intake and food consumption data did not differ among groups 1–3, or between groups 4 and 5. All these findings indicate that PJJ-34 did not show apparent toxicity and not affect survival of rats.

3.2. Chemopreventive effects of PJJ-34 on lung tumorigenesis

Macroscopically, all of the DMBDD-treated rats (groups 1–3) had many whitish nodular lesions on the surfaces of their lungs, most diagnosed as alveolar hyperplasias (100% incidence). The incidences (%) of neoplastic and pre-neoplastic lesions in the major targeted organs are summarized in Table 2. The majority of lung carcinomas were adenocarcinomas but a few included apparent squamous components (diagnosed as adeno-squamous carcinoma). The incidence of lung adenomas and carcinomas was significantly decreased by 5 mg/kg b.w. of PJJ-34 (group 2) as compared to the DMBDD-control (group 1) (adenoma, 100 → 75%; carcinoma, 63 → 30%; $P < 0.05$), but reduction in incidence was not apparent in the 10 mg/kg PJJ-34 case (group 3).

Fig. 2 shows the data for multiplicity (average number of lesions/rat) of neoplastic and/or pre-neoplastic lesions in the lung, colon and urinary bladder. Multiplicity of alveolar epithelial hyperplasias (Fig. 2a) was significantly decreased in both PJJ-34-treated rats relative to the non-treated controls ($P < 0.0001$). Multiplicity of adenomas was also significantly decreased ($P < 0.05$) in the 5 mg/kg (1.4 ± 1.1) and 10 mg/kg (1.7 ± 1.0) groups as compared to the DMBDD-alone group (2.5 ± 1.1). Multiplicity of carcinomas was significantly decreased only in group 2 (0.35 ± 0.59) as compared to group 1 (1.21 ± 1.65). Multiplicity of total tumors were significantly decreased in both groups 2 (1.8 ± 1.4, $P < 0.005$) and 3 (2.3 ± 1.4, $P < 0.05$) as compared to group 1 (3.7 ± 2.2). Thus, lung tumorigenesis was obviously suppressed by administration of PJJ-34 at both doses without a dose-dependency.

3.3. Chemopreventive effects of PJJ-34 on colorectal and bladder tumorigenesis

In the colorectum (Table 2), total tumor incidence was significantly decreased in group 3 (28%, $P < 0.05$) as compared to group 1 (63%). Although statistical significance was not detected, this was largely due to decrease in the adenocarcinoma incidence (47 → 17%). Similarly, as shown in Fig. 2b, multiplicities of adenocarcinomas (0.17 ± 0.38 vs. 0.47 ± 0.51) and total tumors (0.28 ± 0.46 vs. 0.68 ± 0.58) were signifi-

Table 1
Final body and relative organ weights, and daily intakes of water and diet.

Groups (treatment)	Effective o. of rats	Final body eights (g)	Relative organ weights (% of body weight)			Daily intakes (g/rat/day)[a]	
			Liver	Kidney	Spleen	Water	Diet
G1 (DMBDD → 0 mg/kg PJJ-34)	19	293 ± 20[b]	2.4 ± 0.4	0.63 ± 0.05	0.28 ± 0.09	16.7	11.6
G2 (DMBDD → 5 mg/kg PJJ-34)	20	295 ± 14	2.3 ± 0.2	0.63 ± 0.05	0.24 ± 0.04	15.9	11.5
G3 (DMBDD → 10 mg/kg PJJ-34)	18	296 ± 15	2.3 ± 0.3	0.65 ± 0.06	0.27 ± 0.12	16.7	11.8
G4 (Vehicle → 0 mg/kg PJJ-34)	5	361 ± 6	2.2 ± 0.1	0.54 ± 0.03	0.18 ± 0.01	18.7	13.2
G5 (Vehicle → 10 mg/kg PJJ-34)	5	348 ± 23	2.1 ± 0.1	0.55 ± 0.02	0.18 ± 0.01	18.3	12.8

PJJ-34 dissolved in 0.5% CMC-Na (carboxymethyl cellulose sodium salt) was intragastrically (i.g.) administered to rats 5 times per week in the post-initiation period. Body weights, water intake and food consumption were measured weekly until the 30-week termination.

[a] Data for average daily intakes of water and diet were reflected the measurements within PJJ-34-administered period.

[b] Means ± SD.

Table 2
Incidences of neoplastic and pre-neoplastic lesions in the target organs.

Organs	Pathological findings	G1: DMBDD → 0 mg/kg	G2: DMBDD → 5 mg/kg	G3: DMBDD → 10 mg/kg
Lung	Hyperplasia	100% (19/19)	100% (20/20)	100% (18/18)
	Adenoma	100% (19/19)	75% (15/20)*	94% (17/18)
	Carcinoma[a]	63% (12/19)	30% (6/20)*	50% (9/18)
	Total tumors	100% (19/19)	85% (17/20)	100% (18/18)
Duodenum	Adenocarcinoma	37% (7/19)	30% (6/20)	39% (7/18)
Jejunum	Adenocarcinoma	11% (2/19)	0% (0/20)	5.6% (1/18)
Colorectum	Adenoma	11% (2/19)	25% (5/20)	11% (2/18)
	Adenocarcinoma	47% (9/19)	30% (6/20)	17% (3/18)
	Mucinous carcinoma	0% (0/19)	5% (1/20)	0% (0/18)
	Signet-ring cell carcinoma	11% (2/19)	0% (0/20)	0% (0/18)
	Total tumors	63% (12/19)	50% (10/20)	28% (5/18)*
Liver	Hepatocellular adenoma	0% (0/19)	5% (1/20)	0% (0/18)
	Metastasis (mesenchymal tumor)	5.3% (1/19)	0% (0/20)	0% (0/18)
Kidney	Renal cell adenoma	0% (0/19)	10% (2/20)	5.6% (1/18)
	Nephroblastoma	42% (8/19)	35% (7/20)	56% (10/18)
Bladder	PN[b] hyperplasia	63% (12/19)	60% (12/20)	61% (11/18)
	Papilloma	16% (3/19)	10% (2/20)	5.6% (1/18)
	Transitional cell carcinoma	0% (0/19)	5% (1/20)	0% (0/18)
	Total tumors	16% (3/19)	15% (3/20)	5.6% (1/18)
Thyroid	Follicular cell adenoma	16% (3/19)	0% (0/20)	17% (3/18)
	Follicular cell carcinoma	11% (2/19)	5% (1/20)	5.6% (1/18)
	C-cell carcinoma	0% (0/19)	0% (0/20)	11% (2/18)
	Total tumors	26% (5/19)	5% (1/20)	33% (6/18)
Skin/subcutaneous tumors[c]		5.3% (1/19)	10% (2/20)	17% (3/18)

[a] Carcinomas in the lung include adenocarcinoma and adeno-squamous carcinoma.

[b] PN, papillary or nodular.

[c] Skin/subcutaneous tumors were each diagnosed as squamous cell papilloma, basal cell adenoma or carcinoma, and keratoacanthoma.

* $P < 0.05$ vs. corresponding group 1 (χ^2-test or Fisher's test).

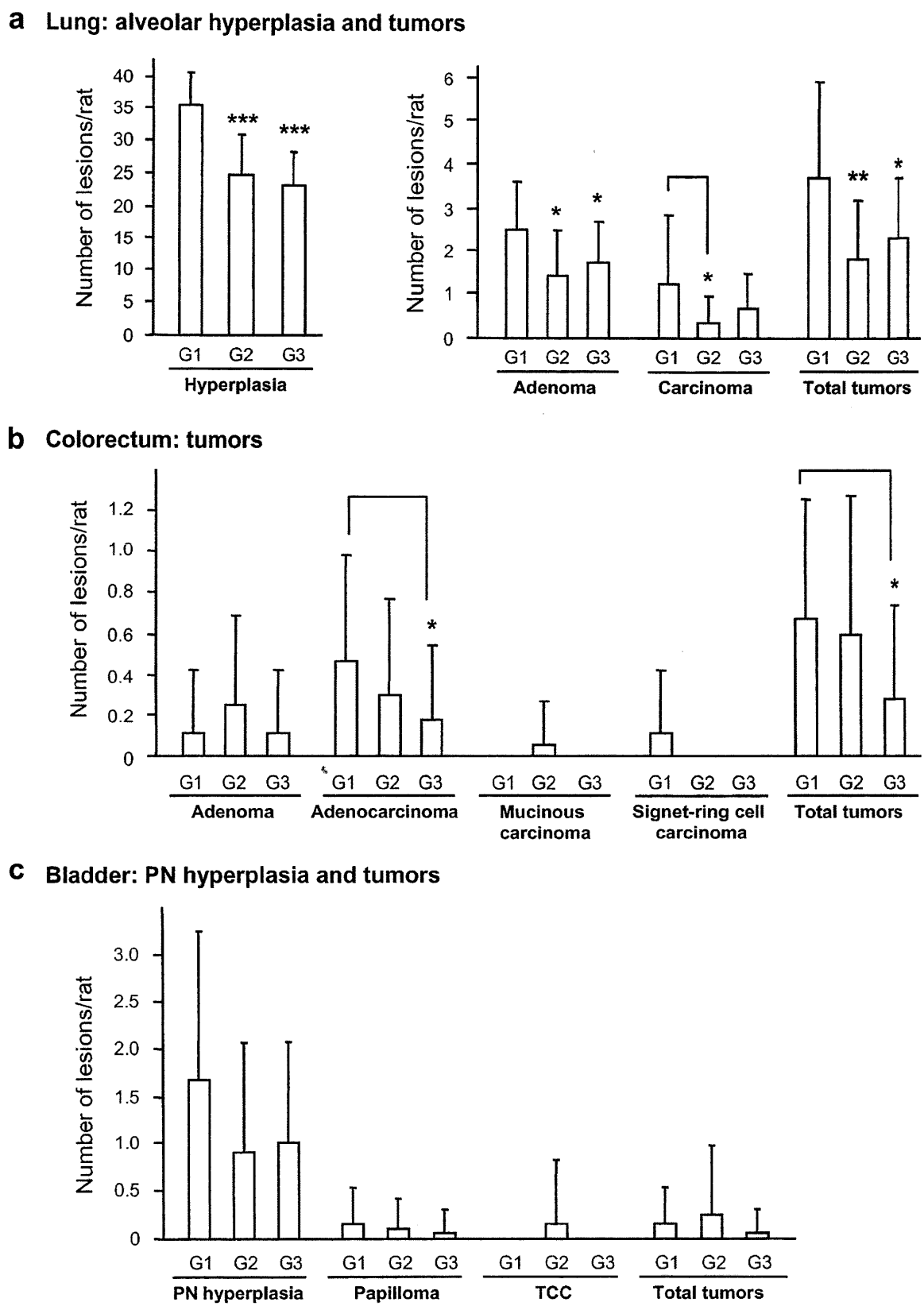

Fig. 2. Multiplicity (average number of lesions/rat) of neoplastic and pre-neoplastic lesions in the lung, colorectum and bladder (bars are SDs). (a) In the lung, multiplicities of alveolar hyperplasias, adenomas and total lung tumors were significantly decreased in groups 2 and 3, while that of carcinomas was only suppressed in group 2 as compared to group 1. (b) Total numbers of colorectal tumors were significantly decreased in group 3, as well as adenocarcinomas. (c) Among the bladder proliferative lesions, multiplicity of papillary or nodular (PN) hyperplasias tended to be decreased in groups 2 and 3 as compared to group 1. *$P < 0.05$ vs. G1; **$P < 0.005$ vs. G1; ***$P < 0.0001$ vs. G1.

cantly decreased in group 3 as compared to group 1 ($P < 0.05$) with a dose-dependency. On the other hand, incidences of bladder proliferative lesions (Table 2) were not significantly influenced by administration of PJJ-34. However, multiplicity of papillary or nodular (PN) hyperplasias tended to be decreased in both 5 and 10 mg/kg groups (Fig. 2c), without statistical significance.

3.4. Tumor incidences in the other organs

As shown in Table 2, the treatment of DMBDD can induce tumors at multiple-site at the whole-body level. Incidences of tumors in the major target organs (thyroid, kidney, liver, small intestine, skin/subcutis) other than the lung, colon and bladder were not apparently changed by administration of PJJ-34 as compared to the DMBDD-alone case.

3.5. Effect of PJJ-34 on cell proliferation in the lung and colon

Since tumor incidence and multiplicity of the lung and colon were significantly suppressed by PJJ-34, we further conducted immunohistochemical analysis of cell proliferation. As shown in Fig. 3a, PCNA-positive indices (%) in normal alveolar epitheliums of the lung were 6.9 ± 1.9, 3.7 ± 0.7 and 2.9 ± 0.4 in groups 1–3, respectively, and those for cyclin D1 were 3.8 ± 1.0, 2.7 ± 0.8 and 2.4 ± 1.0 in groups 1–3, respectively. Both indices were significantly suppressed by PJJ-34 (groups 2 and 3) as compared to group 1. On the other hand (Fig. 3b), PCNA-positive indices in normal colonic epitheliums were 33.9 ± 4.0, 30.7 ± 5.5 and 31.2 ± 4.0 in groups 1–3, respectively. Values were again significantly decreased in groups 2 and 3 as compared to group 1 but the differences were very small.

3.6. Quantitative analysis for GST-P foci in the liver

Fig. 4 shows quantitative data for GST-P foci (⩾0.2 mm in diameter) per liver area (/cm^2). The numbers were 8.4 ± 2.3 (group 1), 9.6 ± 4.4 (group 2) and 9.2 ± 2.9 (group 3), respectively, and the areas were 0.60 ± 0.22 (group 1), 0.70 ± 0.40 (group 2) and 0.65 ± 0.30 (group 3), respectively. There were no statistical significances among groups 1–3.

4. Discussion

The present study demonstrated a strong chemopreventive action of PJJ-34 against lung carcinogenesis and a relatively potent anti-rumor effect against colon carcinogenesis in rats. Suppression of cell proliferation appears to be an important chemopreventive action by PJJ-34. Regarding dose-efficacy, with an average human body weight of 60 kg, chemopreventive effect could be achieved at the relatively low levels of 300 mg/day/person (5 mg/kg b.w.) or 600 mg/day/person (10 mg/kg b.w.) and anti-carcinogenic effects of PJJ-34 noted in this study might not necessarily be dose-dependent. Interestingly, one natu-

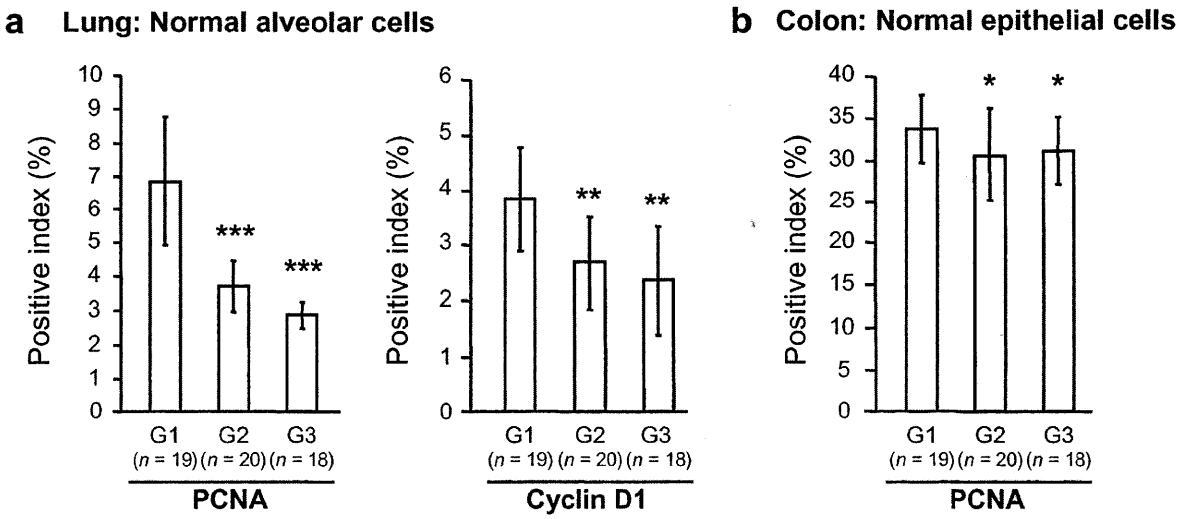

Fig. 3. Immunohistochemically demonstrated proliferation marker-positive indices (%) in the lung and colon (means ± SDs). (a) In normal alveolar epithelium of the lung, both PCNA- and cyclin D1-positive indices were significantly suppressed by PJJ-34 (5 and 10 mg/kg) as compared to the control. (b) In normal colonic epithelium, PCNA-positive indices were modestly but significantly suppressed by PJJ-34 (5 and 10 mg/kg). *P < 0.05 vs. G1; **P < 0.0005 vs. G1; ***P < 0.0001 vs. G1.

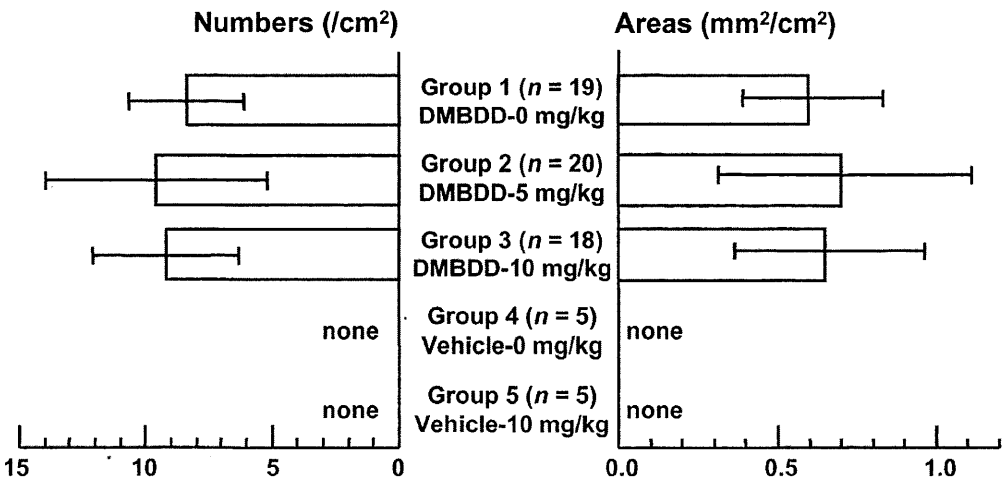

Fig. 4. Quantitative data for GST-P foci in the liver (means ± SDs). The numbers and the areas of GST-P foci 0.2 mm or more in diameter were quantitatively analyzed per liver area (/cm^2) and there was no significant variation in DMBDD-treated groups 1–3.

rally-occurring serratane-type triterpenoid, 3α-methoxyserrat-14-en-21β-ol (PJ-1), recently exhibited a similar inhibitory effect limited to lung carcinogenesis in the DMBDD model, without affecting other organs [20].

Until now, many naturally-occurring or synthetic serratane-type triterpenoids derived from the cuticle or stem bark of *P. jezoensis* Carr. var. *jezoensis* or *hondoensis* have proved to be potentially chemopreventive from EBV-EA testing assessed by Trypan-Blue staining (see Refs. by Tanaka et al.). In summary, at least 15 compounds out of above screened candidates have been evaluated for in vivo anti-tumor promoting effects in female ICR mice, including: PJJ-34 (5) and 3β-methoxy-21α-hydroxyserrat-14-en-29-al [5]; 14β,15β-epoxy-3β-methoxy-serratan-21β-ol [10]; 21-episerratenediol [11]; PJJ-1-13-en (4) and PJJ-43 (6) [12]; 13α,14α-epoxy-21α-methoxyserratan-3-one, 21α-methoxyserrat-13-en-3-one and 21α-hydroxy-3β-methoxyserrat-14-en-30-al [13]; PJJ-1 (1), 13α, 14α-epoxyserratan-3β,21β-diol, 13α,14α-epoxyserratan-3α,21β-diol, 13α,14α-epoxy-3α,21β-dimethoxyserratane, 13α,14α-epoxy-3α,21β-diethoxyserratane and 14β-H-3α-methoxyserratan-15β,21β-diol [14]. All compounds demonstrated relatively potent anti-tumor effects in this established bioassay with a 20-week regimen in mouse. Among them, PJJ-34 most strongly reduced the incidence (100 → 20%) and multiplicity (9 → 0.8/mouse) of skin papillomas [5], which was therefore, subjected to the whole-body examination with the DMBDD bioassay.

It has been reported that anti-carcinogenic effects of triterpenoids are mainly due to anti-inflammatory, anti-proliferative and cytotoxic actions against tumor cells. For example, a steroid-like triterpenoid, oleanolic acid is a known chemopreventive agent used as a positive control in above-mentioned EBV-EA activation test and significantly reduced the numbers of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in male F344 rats with significant reduction in the number of AgNORs in nuclei of the colonic epithelium [21]. Many kinds of pentacyclic triterpenes (PTs) have a wide distribution in plants and have been used as anti-inflammatory remedies in folk medicine. For example, oleanolic acid also possesses a strong anti-inflammatory potential [22]. In the present study, colorectal tumor numbers were significantly decreased in 10 mg/kg PJJ-34-administered rats mainly due to reduction of adenocarcinomas, suggesting that PJJ-34 may have efficiently suppressed the progression process from benign to malignant tumors in multi-step colon carcinogenesis. For one of the mechanisms, inhibition of cell proliferation (Fig. 3b) would be an essential role, as well as oleanolic acid [21]. However, another mechanism might also be operating. In fact, colon tumor incidence itself was obviously decreased in the 10 mg/kg group (63 → 28%) which may indicate that chemopreventive action of PJJ-34 is effective also in pre-neoplastic stages. In this context, PJJ-34 administered during the initiation period showed potent inhibition of mouse skin tumorigenesis with ultraviolet-B (UVB) initiation and TPA promotion [23]. Thus, the compound may possess both anti-initiation and anti-promotion/progression activities.

Nevertheless, the most prominent action of PJJ-34 resided in the marked inhibition of cell proliferation in normal alveolar cells of the lung (Fig. 3a). Regarding mechanisms, one synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), was found to inhibit cyclooxigenase-2 (COX-2) expression and demonstrated anti-proliferative activity with many human cancer cell lines [24]. Moreover, a recent study demonstrated CDDO to significantly inhibit lung adenocarcinoma development in female A/J mice treated with vinyl carbamate, and anti-inflammatory actions such as induction of heme oxygenase-1 (HO-1) and suppression of phosphorylation of signal transducers and activators of transcription 3 (STAT3), as well as induction of apoptosis, could be demonstrated in vitro [25]. Indeed, it is possible that anti-inflammatory, anti-oxidative and apoptotic effects may also participate in the mechanisms underlying anti-lung carcinogenesis. In addition, a further possible mechanism was suggested in an analogue of PJJ-34, namely PJ-1 [20]. PJ-1 demonstrated the lung-specific inhibition on DMBDD-induced multi-organ carcinogenesis with a significant reduction in PCNA-positive indices. The DMBDD treatment significantly decreased mRNA expression for cytochrome P450 (CYP) 2B1/2 in the rat lung and PJ-1 treatment significantly recovered their expression levels by which might read to activation of the detoxification process of a potent lung-targeting carcinogen, DHPN. Therefore, a similar mechanism might also relate to the PJJ-34 case. Anyway, further study is needed to clarify the underlying mechanisms of PJJ-34 against lung carcinogenesis.

From accumulated findings of chemical structural examination, some points should be noted regarding anti-carcinogenic effects of triterpenoids. First, biosynthetic alteration of the serratane skeleton indicates that the 6–6–7–6–6 ring system (serratane-type) effectively suppresses EBV-EA induction, while rearranged abeo-serratane skeletons, such as 6–6–7–5–6 [10,13] and 6–6–6–7–6 [8] ring systems have only reduced potency. For example, a naturally-occurring chemical analogue of PJJ-34, PJJ-43 (compound 6 in Fig. 1a) carries a different bonding of the epoxy ring at C-13 and C-14 (the epoxy epimer of PJJ-34), and this compound showed only weak anti-tumor effects (about 1/5 of that of PJJ-34) in a two-stage carcinogenesis test using mouse skin papillomas as endpoints. Therefore, the 13α,14α-epoxyserratane framework seems to be important for anti-tumor activity of these triterpenoids [12].

There are a lot of studies reporting significant cytotoxic effects of triterpenoids against a variety of tumor cell lines. For example, ursolic acid demonstrated significant cytotoxicity against lymphocytic leukemia cells P-388 and L-1210 as well as other human tumor cell lines [26], while also inhibiting lipoxygenase activity and HL-60 leukemic cell proliferation [27]. Besides, DMBA-initiated, TPA-promoted mouse skin tumorigenesis was also significantly suppressed by treatment with ursolic acid in female CD-1 mice [28]. Other triterpenoids such as hederagenin and its 3-O-glycosides (kalopanaxsaponin A and I) were also found to be cytotoxic to various tumor cell lines, including P-388, L-1210, HL-60, U-937, HepG-2 and SNU-C5, and anti-mutagenic against aflatoxin B1 (AFB1), possibly through inhibition of mutagenic activation of the carcino-

gen [29]. In addition, a report has appeared indicating that the coumaroyl moiety at the C-3 position of lupine-type triterpenes (such as 3-*O*-p-coumaroylalphitolic acids) may play a key role in enhancing cytotoxic activity against tumor cell lines [30].

In conclusion, a novel serratane-type triterpenoid, PJJ-34, is a chemopreventive agent against lung and colon carcinogenesis in the post-initiation phase in DMBDD-treated rats, without any apparent toxicity at the body or organ levels. Suppression of cell proliferation could be an important mode of action of this compound and further studies to elucidate underlying mechanisms appear warranted.

Conflict of interest

The authors disclose no potential conflicts of interest to the present work.

Acknowledgments

The authors are grateful to Mr. Kiyoshi Matsubara (Green Ace Co. Ltd., Hidaka town, Hokkaido, Japan) for supply of the plant material. This study was supported in part by a grant from the Ministry of Health, Labor, and Welfare of Japan.

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TOXICOLOGICAL SCIENCES 113(2), 349–357 (2010)
doi:10.1093/toxsci/kfp256
Advance Access publication October 25, 2009

Effects of Pioglitazone, a Peroxisome Proliferator–Activated Receptor Gamma Agonist, on the Urine and Urothelium of the Rat

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Received September 14, 2009; accepted October 13, 2009

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor superfamily. Some PPARγ agonists, such as pioglitazone, and dual PPARγ/PPARα agonists, such as muraglitazar, induced urothelial bladder tumors in rats but not in mice. In this study, we investigated the early effects in the urine and bladder of rats treated with pioglitazone to evaluate the possible relation between urinary solids formation and urothelial cytotoxicity and regenerative proliferation. In a 4-week experiment, treatment of rats with 16 mg/kg pioglitazone induced cytotoxicity and necrosis of the urothelial superficial layer, with increased cell proliferation measured by bromodeoxyuridine labeling index and hyperplasia by histology. It also produced alterations in urinary solid formation, especially calcium-containing crystals and calculi. PPARγ agonists (pioglitazone and troglitazone) *in vitro* reduced rat urothelial cell proliferation and induced uroplakin synthesis, a specific differentiation marker in urothelial cells. Our data support the hypothesis that the bladder tumors produced in rats by pioglitazone are related to the formation of urinary solids. This strongly supports the previous conclusion in studies with muraglitazar that this is a rat-specific phenomenon and does not pose a urinary bladder cancer risk to humans treated with these agents.

Key Words: peroxisome proliferator–activated receptor; urinary bladder; urothelial cell cytotoxicity; urinary solids; differentiation.

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor superfamily (Michalik *et al.*, 2004; Tachibana *et al.*, 2008; Yki-Jarvinen, 2004). Three major PPAR receptors have been identified, alpha, gamma, and delta (beta), with differing tissue distributions and effects (Berger and Moller, 2002). PPAR alpha (PPARα) is expressed predominantly in the liver, kidney, heart, and skeletal muscle and enhances free fatty acid oxidation, controls expression of multiple genes regulating lipoprotein concentration, and has anti-inflammatory effects (Berger and Moller, 2002; Tachibana *et al.*, 2008). PPAR delta (PPARδ) (also referred to as PPAR beta) is expressed ubiquitously, is required for placental development, and is involved in the control of lipid metabolism (Berger and Moller, 2002; Michalik *et al.*, 2004). PPAR gamma (PPARγ) has two isoforms, PPARγ1 and PPARγ2. PPARγ2, which contains an additional 28 amino acids at the N-terminal compared to PPARγ1, is expressed exclusively in adipose tissue, whereas PPARγ1 is expressed in heart, skeletal muscle, kidney, pancreas, and several epithelial tissues, such as urothelium and intestine. PPARγ induces adipocyte differentiation and is involved in the control of inflammatory reactions and in glucose metabolism through enhanced insulin sensitivity (Berger and Moller, 2002; Tachibana *et al.*, 2008). Agonists have been developed for each of these receptors, with differing pharmacologic and toxicologic effects (Berger and Moller, 2002; Yki-Jarvinen, 2004).

In a tabulation of PPAR agonists under development as pharmaceuticals, El-Hage (2005) reported that five of six dual PPARγ/PPARα agonists and pioglitazone, a PPARγ agonist, induced urothelial bladder tumors in male rats but not in mice. Lubet *et al.* (2008) also reported that another PPARγ agonist, rosiglitazone, enhanced bladder tumors in rats pretreated with N-(4-hydroxybutyl)-N-(butyl)nitrosamine (BBN), a known DNA-reactive bladder carcinogen in several species. Muraglitazar, one of the five dual PPARγ/PPARα agonists listed by El-Hage (2005) that induced rat urinary bladder carcinogenesis in a 2-year bioassay, caused bladder tumors, which occurred predominantly in male rats compared to females and did not occur in mice (Tannehill-Gregg *et al.*, 2007). Evidence was presented that the mechanism of rat bladder carcinogenesis induced by muraglitazar involved a mode of action involving increased formation of urinary solids resulting in urothelial cytotoxicity and increased cell proliferation (Cohen, 2005; Dominick *et al.*, 2006; Tannehill-Gregg *et al.*, 2007). In contrast, naveglitazar, another dual PPARγ/PPARα agonist, induced bladder tumors but urinary tract solids were not detected (Long *et al.*, 2008). However, detection of urinary solids can be problematic because of methodological issues (Cohen *et al.*, 2007).

Since urothelial cells have PPARγ receptors, it has been suggested that a direct effect of the agonist on the urothelial receptor might be the cause of bladder carcinogenesis by these non–DNA reactive agents, possibly, in the case of dual PPARγ/PPARα agonists, by an interaction between the PPARα and PPARγ receptors (Varley and Southgate, 2008). However, PPARγ agonists inhibit cell proliferation or induce differentiation in various cancer cell lines, including urothelial cell carcinoma lines (Tachibana *et al.*, 2008). Additionally, the PPARγ agonist, troglitazone, inhibits cell proliferation and induces differentiation in human urothelial cells in culture (Varley *et al.*, 2004, 2009) rather than increasing cell proliferation as would be expected for a non–DNA reactive chemical's carcinogenic mode of action.

In this study, we investigated the early effects on the bladder and on the urine of rats treated with pioglitazone, a PPARγ agonist, to evaluate the possible relation between urinary solids formation and urothelial cytotoxicity and proliferation.

MATERIALS AND METHODS

Chemicals. Pioglitazone (purity: > 99%) and troglitazone (purity: 99.5%) were kindly provided by Bristol-Myers Squibb (Mount Vernon, IN). Pioglitazone was stored in the dark at ~4°C. Troglitazone was stored in the dark at room temperature. For the *in vitro* study, stock solutions of pioglitazone or troglitazone were prepared by dissolving the agonist in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). Working solutions of pioglitazone and troglitazone were prepared by diluting the stock solution in medium. The DMSO concentration of the working solutions was 0.1%.

Animal experiments. Five-week-old male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Kingston, NY). On arrival, the animals were placed in a level-4 barrier facility accredited by the American Association for Accreditation of Laboratory Animal Care, in a room with a targeted temperature of 22°C, humidity of 50%, and a 12-h light/dark cycle (0600/1800 h). The level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1986). The animals were housed three per cage in polycarbonate cages, on dry corncob bedding, and fed basal diet (Certified Purina 5002; Dyets Inc., Bethlehem, PA). Food and tap water were available *ad libitum* throughout the study. Nylabones (Nylabone Products, Neptune, NJ) were added to the cages for environmental enrichment. Fresh diet was supplied to the animals at least once weekly. Food consumption and water consumption were measured during study week 3. Body weights of all animals were measured the day after arrival, once per week, and on the day of sacrifice. Detailed clinical observations of each animal were performed on day 0 and on the last day of the consumption period, including behavior and movement, respiratory function, ocular appearance, condition around the ears and mouth, condition of coat, and abdominal palpation.

Rats were ~6 weeks of age at the beginning of treatment. Following quarantine, animals were randomized using a weight stratification method (Martin *et al.*, 1984) into two groups of 15 rats each: group 1 was gavaged daily between 0800 h and 1000 h with 0.5% methyl cellulose in distilled water as vehicle and group 2 was similarly gavaged with pioglitazone (16 mg/kg body weight) in 0.5% methyl cellulose. All animals were sacrificed after 4 weeks of treatment by an overdose of Nembutal (150 mg/kg of body weight, ip). One hour prior to sacrifice, all rats were injected with 100 mg/kg bromodeoxyuridine (BrdU). The urinary bladder from 10 rats from each group was inflated *in situ* with Bouin's fixative, and after removal, the bladders were placed in Bouin's fixative. Following fixation, the bladders were rinsed in 70% ethanol, bisected longitudinally, and weighed. The entire surface of one half of the bladder was examined by scanning electron microscopy (SEM) and classified in one of five categories as previously described (Cohen *et al.*, 1990). Briefly, class 1 bladders have flat polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; and class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2 or occasionally class 3. The other half of the bladder was cut longitudinally into strips and with a slice of intestinal tissue removed at the time of necropsy, was embedded in paraffin, stained with hematoxylin and eosin, and examined histopathologically (Cohen, 1983; Cohen *et al.*, 1990, 2007). A diagnosis of mild simple hyperplasia was made when there were four to five cell layers in the bladder epithelium and a diagnosis of severe simple hyperplasia was made when nine or more cell layers were present. Unstained slides of the bladder and intestinal tissue were used for immunohistochemical detection of BrdU (Cohen *et al.*, 2007). The intestinal tissue served as a positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:200. The number of BrdU-labeled cells in at least 3000 urothelial cells (all layers) was counted to determine a labeling index. Unstained slides of the bladder and intestinal tissue were used for periodic acid-Schiff (PAS) staining (Luna, 1968). The urinary bladder from the remaining five rats from each group was excised, and the epithelial cell layer was collected by scraping with a scapel blade. The epithelial cells were immediately immersed in TRIzol Reagent (Invitrogen, Carlsbad, CA) and stored at −80°C until processed for RNA extraction.

Evaluation of crystals in urine. After treatment for 14 and 22 days, fresh void urine samples were collected separately from each rat between 7:00–9:00 A.M. All urine samples were centrifuged at approximately 6400 *g* for 10 min. After removal of most of the supernatant, the urine sediment was resuspended in the remaining urine and filtered through a 0.22μM Millipore filter (Millipore, Billerica, MA) by vacuum. The crystals remaining on the filter were characterized morphologically by SEM, and their composition was determined by attached energy dispersive X-ray spectroscopy.

In vitro *experiments.* The MYP3 urinary rat bladder epithelial cell line was provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL). The MYP3 cell line was obtained from a small benign nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (Kawamata *et al.*, 1993). The cell line has retained the characteristics of epithelial cells in culture, expresses keratin 5 mRNA, does not exhibit anchorage-independent growth, and does not cause the development of tumors when inoculated sc in nude mice. The cells were grown in Ham's F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10μM nonessential amino acids, 10 ng/ml epidermal growth factor, 10 μg/ml insulin, 5 μg/ml transferrin, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco), and 2.7 mg/ml dextrose and 1 μg/ml hydrocortisone (from Sigma). All cells were grown in an atmosphere of 95% air and 5% CO_2 at 37°C.

For determination of PPARγ agonist effects on the urothelial cells *in vitro*, cells were seeded at a concentration of 5.0×10^3 cells per well in eight-well Lab-Tek Chamber Slides (NUNC, Inc., Naperville, IL). Twenty-four hours later, treatment with pioglitazone (5μM) or troglitazone (5μM) was begun and continued for 3 days without changing the medium. For immunohistochemistry, after fixation with 10% formalin, sections were autoclaved in 10mM sodium citrate buffer (pH 6.0) at 120°C for 5 min. The sections were then treated with anti-Ki-67 antibody (MIB-5; Dako, Carpinteria, CA) at a dilution of 1:50 or anti-asymmetrical unit membrane (AUM) antibody kindly provided by Dr. T. T. Sun, which consisted of rabbit antiserum made against highly purified bovine AUM (Wu *et al.*, 1990), at a dilution of 1:200. The number of Ki-67–labeled cells in at least 500 urothelial cells was counted to determine a labeling index.

To determine expression of RNA, cells were seeded at a concentration of 1.0×10^4 cells per well in a 24-well plate. Twenty-four hours later, treatment with 5μM pioglitazone or 5μM troglitazone was begun and continued for

TABLE 1
Effects of Treatment with Pioglitazone on Body, Bladder, and Heart Weights and on the Bladder Urothelium

	Control	Pioglitazone
BW, g $(n)^a$	393 ± 6 (14)	387 ± 6 (15)
Bladder weight $(n)^a$		
Absolute, g	0.111 ± 0.013 (9)	0.108 ± 0.006 (10)
Relative, mg/g BW	0.28 ± 0.03 (9)	0.28 ± 0.02 (10)
Heart weight $(n)^a$		
Absolute, g	1.26 ± 0.03 (14)	1.32 ± 0.02 (15)
Relative, mg/g BW	3.2 ± 0.1(14)	3.4 ± 0.1 $(14)^b$
Bladder histopathology		
Normal	9	5
Hyperplasia	0	$5^{b,c}$
Bladder labeling index, % $(n)^a$	0.16 ± 0.03 (9)	0.43 ± 0.07 $(10)^b$
Bladder SEM classification[d]		
1	1	3
2	4	—
3	3	2
4	—	—
5	—	1

Note. BW, body weight; (n), number of rats.
[a]Values expressed as the mean ± SE.
[b]Significantly different from control group, $p < 0.05$.
[c]Four of five bladders with mild simple hyperplasia; one of five bladders with severe simple hyperplasia.
[d]Unable to classify one bladder in the control group and four bladders in the pioglitazone-treated group by SEM due to the presence of an unknown substance on the bladder surface.

3 days without a change of medium. After the treatment, cells were treated with TRIzol Reagent (Invitrogen) and stored at −80°C until processed for RNA extraction.

RNA extraction and detection of RNA expression. Total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions. Sequence-specific primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc. (Foster City, CA). β-Actin was employed as an internal control. Briefly, complementary DNA (cDNA) synthesis was performed with 600 ng of RNA using an Advantage RT-for-PCR kit (Takara Bio, Inc., Shiga, Japan) and then cDNA solutions were diluted to a final volume of 100 μl by adding 80 μl diethylpyrocarbonate-treated H_2O. PCRs were performed in a 20 μl reaction mixture containing 5 μl cDNA, 1 μl of Taqman Gene Expression Assay Mix, and 10 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Inc.) under the following conditions: 95°C for 20 s, then 40 cycles at 95°C for 3 s, and 60°C for 30 s using a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Tokyo, Japan). Serially diluted standard cDNA was included in each Taqman PCR to create a standard curve. The amount of gene products in the test samples was estimated relative to the respective standard curves. Values for target genes were normalized to those for β-actin.

Statistics. For the *in vivo* studies, group means for body weights, consumptions, tissue weights, and labeling indices were evaluated using analysis of variance followed by Duncan's multiple range test for group-wise comparisons. Histopathology was compared using the two-tailed Fisher's exact test. SEM data were analyzed using one-way nonparametric procedures followed by a chi square test. p Values < 0.05 were considered significant. These statistical analyses were performed using SAS for Windows (Version 9.1).

For the *in vitro* studies, the difference between control and treatment group was compared by the unpaired t-test. For multiple groups, differences between control and treated were compared by ANOVA, which, when significant ($p < 0.05$), was followed by Dunnett's test (GraphPad Prism 5; GraphPad Software, Inc., La Jolla, CA).

RESULTS

Body and Heart Weights

Administration of pioglitazone to male rats caused little or no depression in body weight gain. However, it induced a significant increase in the relative weight of the heart (Table 1), a characteristic effect of PPARγ and dual agonists (El-Hage, 2005). It had no effect on food and water consumption (control and pioglitazone-treated rats [mean ± SE]: water consumption, 40.3 ± 1.9 and 41.3 ± 1.7 g/rat/day and food consumption, 26.9 ± 1.2 and 28.5 ± 1.0 g/rat/day, respectively).

Urothelial Effects

Administration of pioglitazone caused no effects on the absolute and relative bladder weights (Table 1). By light microscopy, simple hyperplasia of the bladder urothelium was significantly increased in pioglitazone-treated rats (Table 1) and was not present in control rats (Fig. 1A). Additionally, one of five pioglitazone-treated rats had severe simple hyperplasia (Fig. 1C), whereas the other instances of hyperplasia were mild simple hyperplasia (Fig. 1B). Administration of pioglitazone also caused a significant increase in the BrdU labeling index of the urothelium compared to the control group (Table 1). Administration of pioglitazone tended to induce cytotoxicity and necrosis in the bladder epithelium (Figs. 1E and 1F), although the SEM classification in the group-administered pioglitazone was not statistically significantly different from the control group (Table 1). The pioglitazone-treated rat with severe urothelial hyperplasia was also the rat diagnosed as class 5 by SEM. Examination by SEM showed that the bladder surface of the rats in one of the control and four of the pioglitazone-treated rats was covered with a coarse substance (Fig. 2A), which made it difficult to visualize the bladder surface in many areas. The morphology of the substance by SEM was similar to mucin (Balish *et al.*, 1982). An eosinophilic substance was also detected by light microscopy on the luminal surface of these same bladders (Fig. 2B) and it stained positive by PAS stain (Fig. 2C). Therefore, this covering likely corresponds to the glycosaminoglycan layer described for the normal urinary bladder (Soler *et al.*, 2008), which is usually lost during routine processing for histology or SEM examination.

Evaluation of Urinary Sediments

In the urine, the normally present $MgNH_4PO_4$ crystals (Fig. 3A) were observed in both control and pioglitazone-treated groups in similar amounts (Table 2). Aggregates of

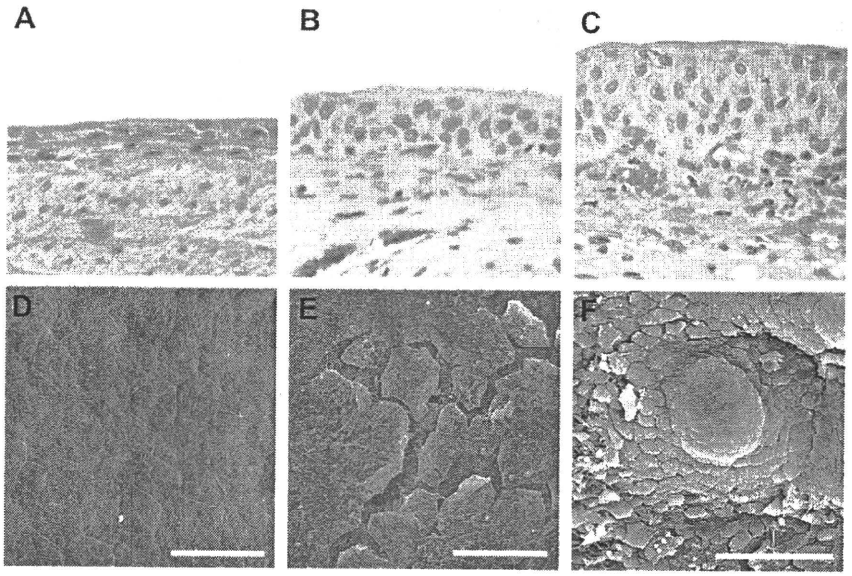

FIG. 1. Histopathology, SEM of rat bladder. (A–C) hematoxylin and eosin. (D–F) SEM. Normal epithelium in control rat (A and D). Mild (B) and severe (C) simple hyperplasia in pioglitazone-treated rats. Abrasion of superficial cells (E) and piling up of rounded urothelial cells (F) in pioglitazone-treated rats. White bar: 100 μm.

$MgNH_4PO_4$ crystals occurred in similar amounts, sizes, and number of rats in the two groups. Thin rod-like crystals were only detected in one control group rat at day 14, and they appeared to be $MgNH_4PO_4$. Calcium phosphate–containing amorphous precipitate was not observed in the urinary sediment from any of the rats from either group at either time point. Calcium-containing crystals (Figs. 3B and 3C) were only detected in pioglitazone-treated rats at day 14. However, they were detected in both control and pioglitazone-treated rats at day 22. At day 14, the calcium-containing crystals also contained oxygen in the sediment from three rats, most likely representing calcium oxalate crystals, and one rat had calcium phosphate crystals. Carbon and hydrogen are not clearly distinguished by our instrument. At day 22, similar calcium oxalate crystals were observed in controls and in the pioglitazone-treated rats, but pioglitazone-treated rats also had calcium phosphate crystals and crystals containing calcium, oxygen, and sulfur. Furthermore, a calcium phosphate calculus was detected in one pioglitazone-treated rat at day 22 (the rat with severe hyperplasia).

PPARγ Effects on Rat Urothelial Cells In Vitro

Treatment with pioglitazone (5μM) induced enlargement of cytoplasm and binucleated and multinucleated cells (Figs. 4B and 4D) compared to control cells (Figs. 4A and 4C). Additionally, treatment with pioglitazone significantly ($p < 0.001$) reduced the Ki-67 labeling index (24.7 ± 2.1%; Fig. 4F) compared to controls (49.5 ± 3.3%; Fig. 4E). Anti-AUM antibody diffusely stained the nucleus and cytoplasm of large cells present in both control and treated wells but small cells present in the wells did not stain. In the control wells, the number of small cells and large cells was similar. In pioglitazone-treated wells, the ratio of large cells to small cells was higher than in the control wells. Therefore, the ratio of AUM-positive staining cells to negative cells in pioglitazone-treated wells (Fig. 4H) was higher than

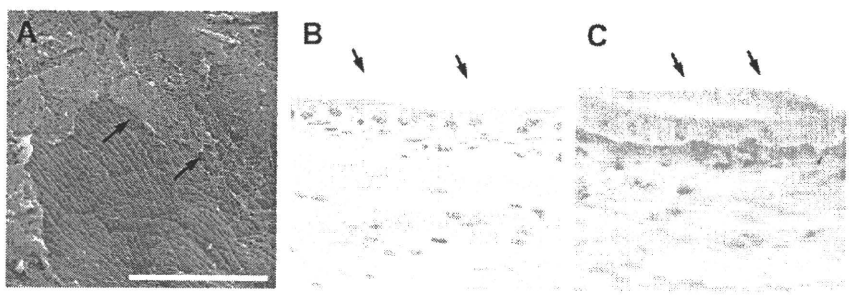

FIG. 2. Rough substance on superficial layer of urothelium. (A) SEM, (B), and hematoxylin and eosin. (C) PAS staining. Rough substance was covered on superficial cells (A and B) and stained with PAS staining (C). White bar: 100 μm.

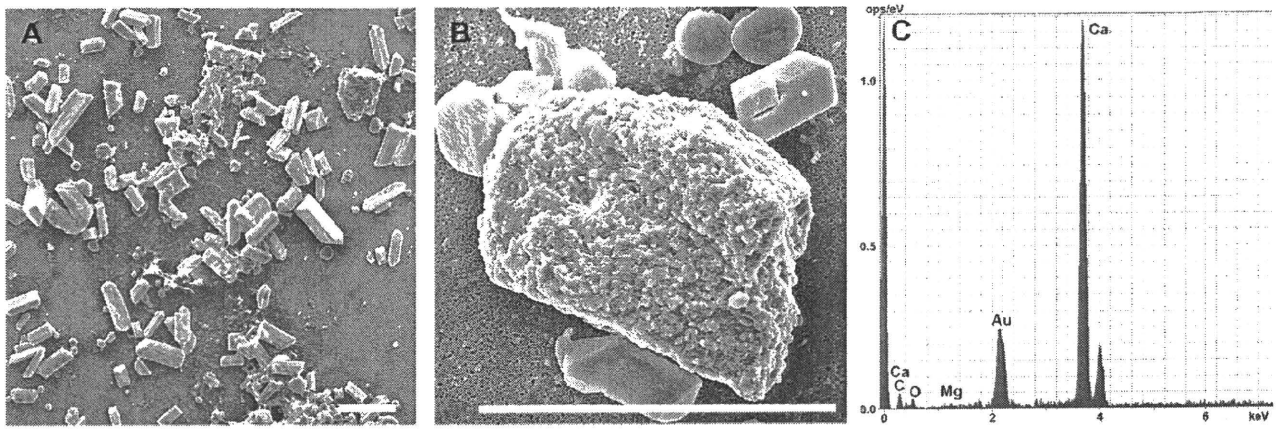

FIG. 3. Crystals in rat urine. (A and B) SEM. (C) energy dispersive X-ray spectroscopy (EDS). $MgNH_4PO_4$ crystals (A) and calcium-containing crystal (B) in urine of pioglitazone-treated rat. Composition of calcium-containing crystal (B) by EDS (C). White bar: 100 μm.

in control wells (Fig. 4G). Treatment with troglitazone (5μM) induced the same morphology and significant reduction ($p < 0.001$) of the Ki-67 labeling index (23.3 ± 2.4%) compared to control (45.2 ± 4.7%). The effects of both pioglitazone and troglitazone on MYP3 cells were the same in this study.

Gene Expression Differences in Urothelium of PPARγ Agonist–Treated Rats and Urothelial Cells

In vivo, there was no difference in the mRNA expression of PPARγ in the urothelium of pioglitazone-treated rats compared to control rats (Fig. 5A). *In vitro*, PPARγ mRNA in both pioglitazone- and troglitazone-treated cells was significantly reduced compared to control (Fig. 5B). The mRNA expression of cyclin D1 in both pioglitazone- and troglitazone-treated cells was also reduced compared to control (Fig. 5C).

DISCUSSION

PPARγ and dual PPARα/γ agonists frequently increase the incidence of bladder cancer in rats in 2-year bioassays but not in mice (El-Hage, 2005). Pioglitazone, a thiazolidinedione, is a PPARγ agonist that induced a relatively low incidence of bladder tumors in the 2-year bioassay and only in male rats (El-Hage, 2005; Physicians Desk Reference, 2008). Rosiglitazone, another thiazolidinedione PPARγ agonist, has not been reported to produce bladder tumors in a 2-year bioassay but did produce an increased incidence of bladder tumors in rats pretreated with BBN, a known DNA-reactive bladder carcinogen in several species (Lubet *et al.*, 2008). Troglitazone, a third PPARγ agonist of the thiazolidinedione class, has not been reported to induce effects on the urothelium of rats or mice (Herman *et al.*, 2002). In a summary of the carcinogenic effects of various PPAR agonists under development as

TABLE 2
Effects of Treatment with Pioglitazone on Urinary Sediments

Treatment (*n*)	Normal $MgNH_4PO_4$ crystals			Aggregates of $MgNH_4PO_4$ crystals < 100 μm			Aggregates of $MgNH_4PO_4$ crystals > 100 μm			Calcium-containing crystals			Calcium phosphate calculi		
	0	+1	+2	0	+1	+2	0	+1	+2	0	+1	+2	0	+1	+2
A: Day 14															
Control (10)	3	7	–	7	3	–	8	2	–	10	–	–	10	–	–
Pioglitazone (9)	5	4	–	7	2	–	7	2	–	5	4[a]	–	9	–	–
B: Day 22															
Control (5)	–	4	1	1	4	–	4	1	–	1	4[b]	–	5	–	–
Pioglitazone (11)	2	8	1	5	6	–	9	2	–	7	4[c]	–	10	1	–

[a]Ca- and O-containing crystals on three filters and two Ca- and P-containing crystals on one filter.
[b]One to two Ca- and O-containing crystals per filter.
[c]Ca- and O-containing crystals on two filters; Ca- and P-containing crystals on one filter; and Ca-, O-, and S-containing crystal on one filter.

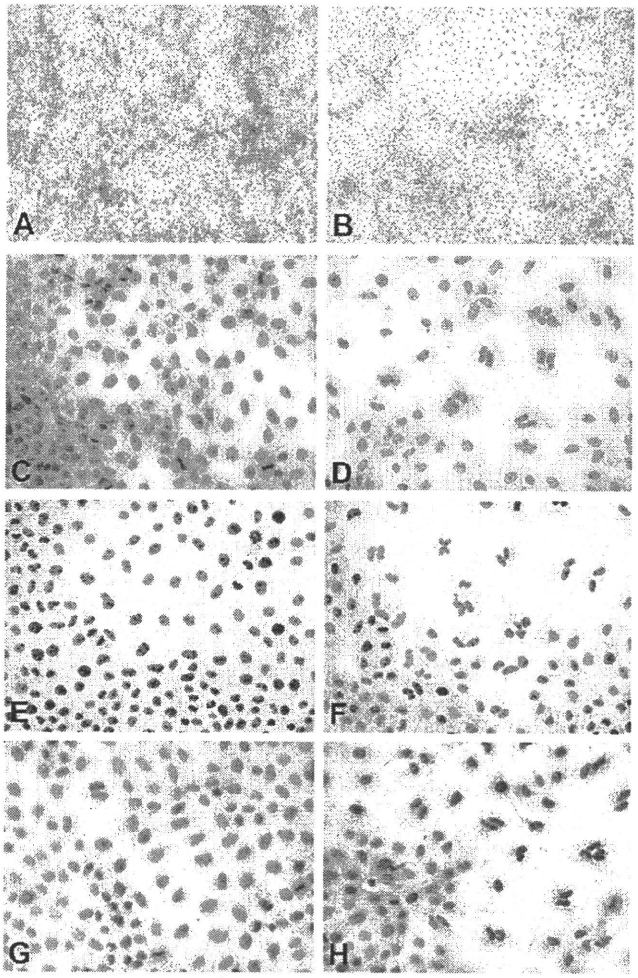

FIG. 4. Histology and immunohistochemistry of rat bladder cell line. (A–D) hematoxylin and eosin. (E and F) Ki-67 antibody. (G and H) AUM antibody. Control cells (A, C, E, and G) and pioglitazone-treated cells (B, D, F, and H).

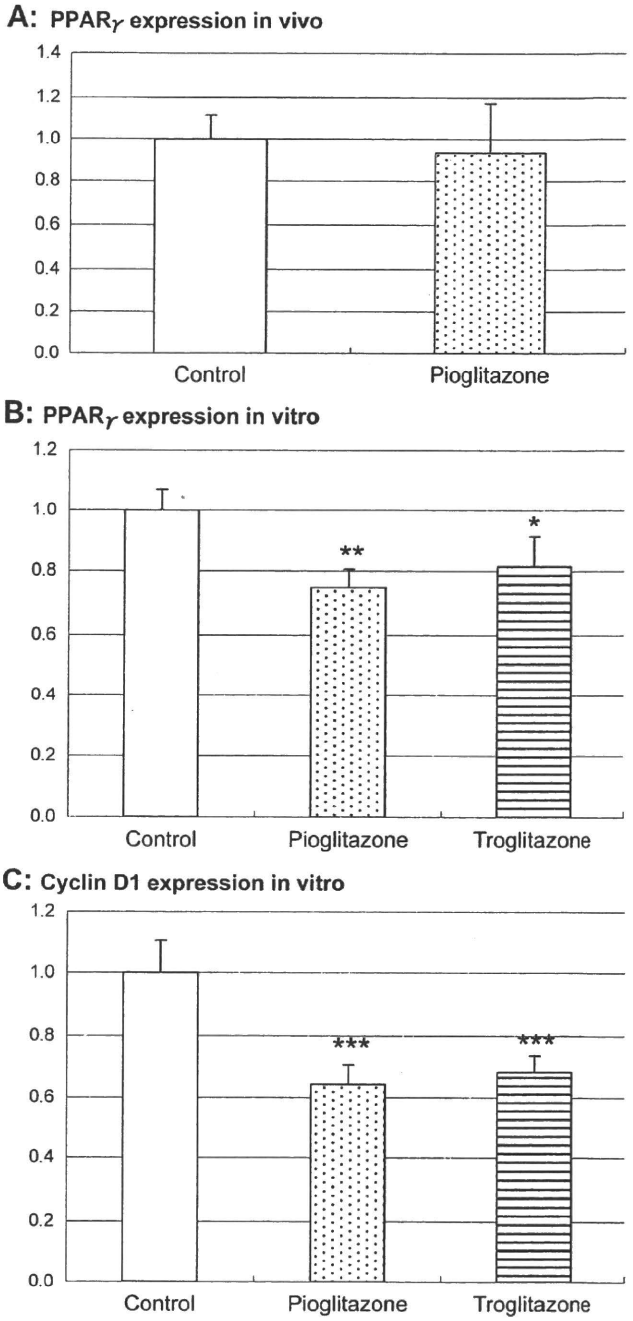

FIG. 5. Gene expression analysis of PPARγ and cyclin D1. PPARγ expression *in vivo* (A) and *in vitro* (B) in rat urothelial cells. Cyclin D1 expression *in vitro* (C). The expression data were adjusted to each control as 1.0. *, **, ***Significantly different from each control at $p < 0.05, 0.01, 0.001$, respectively.

pharmaceuticals, El-Hage (2005) reported that five of six dual PPARα/γ agonists induced bladder tumors in rats but again were without effect in mice. Those that have been reported specifically to cause bladder tumors include muraglitazar (Dominick *et al.*, 2006), naveglitazar (Long *et al.*, 2008), and ragaglitazar (Egerod *et al.*, 2005). Since rats treated with these agents at doses that produce bladder tumors have blood levels that frequently are similar to the blood levels achieved in patients treated with these drugs, bladder tumors in rats have become a significant issue for the potential approval of these agents by regulatory agencies for clinical use.

Two modes of action have been hypothesized for the possible induction of bladder tumors in rats by PPAR agonists, with very different implications for potential human cancer risk. Since PPARγ receptors are plentiful in the urothelium, one hypothesis is based on the direct interaction of the agonist with the receptor, producing an effect that ultimately leads to the induction of cancer. Although this possibility has not been completely excluded, there are several arguments against it (Cohen, 2005). Foremost among these are the observation that

the PPARγ receptor is expressed at similar levels in rat and mouse urothelium and yet the agents produce bladder tumors only in the rat not in the mouse (Cohen, 2005). Most importantly, the effects of PPARγ agonists *in vitro* appear to be an inhibition of cell proliferation and potentiation of differentiation when the epidermal growth factor receptor has been inhibited (Varley and Southgate, 2008; Varley *et al.*, 2004). These studies utilized human urothelial cell lines, and the decreased proliferation along with urinary differentiation was corroborated in the present experiment using a rat urothelial cell line. Thus, a direct mitogenic effect to the urothelium by the PPAR agonist is unlikely. Biologically, this is the opposite effect of what would be expected for a non–DNA reactive carcinogen. Furthermore, these agonists have frequently been shown to inhibit the proliferation of a variety of cancer cell lines, including urothelial carcinoma cell lines (Berger and Moller, 2002; Tachibana *et al.*, 2008). An additional factor is the small percentage of administered drug being excreted in the urine since PPAR agonists are highly lipophilic.

Previous studies with the thiazolidinedione class of drugs have suggested that they induce differentiation and inhibit proliferation of urothelial cells in culture. These studies investigated the effects of troglitazone and rosiglitazone on human cell lines derived from distal ureters obtained from kidney transplant patients (Varley and Southgate, 2008; Varley *et al.*, 2004). Pioglitazone is the only thiazolidinedione so far reported, which actually produced bladder tumors by itself in a 2-year bioassay (El-Hage, 2005). Thus, we examined the effect of pioglitazone *in vitro* to determine if it also had cell-differentiating properties, using a rat urothelial cell line since that is the target species for bladder carcinogenicity. Troglitazone was used as a positive control based on its reported effects on human urothelial cells. We were able to show that treatment with either pioglitazone or troglitazone *in vitro* utilizing a rat urothelial cell line reduced cell proliferation and there was evidence of cell differentiation in this culture system (Fig. 4). Our observations in the rat cell line (Fig. 4) support the previously reported observations utilizing human urothelial cells. Although we did not prove that this was a PPARγ-specific mechanism molecularly, we detected no difference in gene expression of PPARγ between control and pioglitazone-treated rats *in vivo* and found reduction of PPARγ in both pioglitazone- and troglitazone-treated cells *in vitro*. Reduction of PPARγ expression by muraglitazar *in vivo* was reported by Achanzar *et al.* (2007). For detection of PPARγ activity, it is necessary to use another methodology, which would also detect phosphorylation of PPARγ. Again, our results provide support for the previous observations utilizing troglitazone in human urothelial cell culture systems. Pioglitazone and troglitazone had the same effects on the rat urothelial cells *in vitro* but only pioglitazone has been reported to produce bladder urothelial tumors in rats (Physicians Desk Reference, 2008), whereas troglitazone does not affect the lower urinary tract of the rat.

A second, indirect mode of action such as reaction to urinary solids has been postulated by Cohen (2005) and has been demonstrated for the dual PPARγ/PPARα agonist muraglitazar (Dominick *et al.*, 2006). This involves alteration of the urine resulting in abnormal urinary solids leading to cytotoxicity, necrosis, and regenerative proliferation of the bladder epithelium. Alteration of the urine composition appears to be due to an inhibition by muraglitazar of citrate synthesis leading to hypocitratemia and consequent hypocitraturia (Dominick *et al.*, 2006). Citrate is the major chelating substance for divalent cations, such as calcium, in the urine keeping them in solution. When the citrate level is lowered significantly, the calcium salts, which are at supersaturated levels in the urine, precipitate. This ultimately induces tumors. Cytotoxicity followed by regenerative hyperplasia as a mode of action has been described for a wide variety of agents in the rat and occasionally in the mouse (Cohen, 1998). For reasons that are not entirely clear, the rat appears to be more susceptible to this effect. Most importantly, humans appear to be completely resistant to the urothelial cytotoxic effects of urinary amorphous precipitate and crystals but do have a toxic and regenerative response to the presence of calculi. Calculi, when formed, are present in the human urinary bladder for brief periods of time because of their propensity to cause obstruction and consequent severe pain leading to their removal clinically (DeSesso, 1995).

For muraglitazar, the sequence of key events has been demonstrated in extensive detail (Achanzar *et al.*, 2007; Dominick *et al.*, 2006; Tannehill-Gregg *et al.*, 2007). In these experiments, muraglitazar induced urinary bladder carcinomas and also induced calcium-containing solids and reduced citrate and soluble calcium concentrations in the urine. Also, coadministration of ammonium chloride with muraglitazar produces an acidified urine, which inhibits the formation of the calcium-containing crystals and consequently inhibits the bladder toxicity, regenerative proliferation, and tumorigenicity of muraglitazar. In contrast, studies with naveglitazar and ragaglitazar have reported that increased urinary solids have not been found in rats administered these drugs (Egerod *et al.*, 2005; Long *et al.*, 2008). However, there are several potential technical difficulties that must be addressed when examining urine for the presence of these solids. Foremost is the requirement that the animals not be fasted prior to collection of the urine. It is also best for fresh void collections to be used rather than using overnight or 24-h collections. The technical difficulties of urine collection and examination for solids have been described in detail elsewhere (Cohen *et al.*, 2007). The time of day of urine collection, the strain of the rat, where the rat was purchased, the type of diet used, and a variety of other details appear critical in the detection of the formation of urinary solids in response to PPAR agonists and also in response to other agents.

In the present series of experiments, we were able to demonstrate that pioglitazone produced calcium-containing

urinary solids, although limited, which were associated with increased urothelial cytotoxicity, necrosis, and regenerative proliferation (Table 2). The more extensive the urinary solids formation was, the more extensive the toxic and proliferative response was. In one of the rats we examined, calculi were present, which were associated with severe simple hyperplasia, even in as short a period as 4 weeks. Overall, there were considerably less urinary solids detected in these rats administered pioglitazone than previously observed for muraglitazar (Dominick *et al.*, 2006). Correspondingly, muraglitazar induced a significantly higher incidence of bladder tumors than the small number induced by pioglitazone. Furthermore, the amount of calcium-containing crystals varied between times of collection, even for this short experiment. Experiments with other agents have demonstrated the variability in formation of urinary solids over time, even with the continued administration of the agent (Clayson *et al.*, 1995). The reversibility and relatively low amount of crystals seen with pioglitazone is likely directly related to its weak overall effect on the rat bladder in contrast to the more plentiful and frequent urinary solids associated with muraglitazar, which also had more urothelial proliferation and neoplastic lesions. The sporadic nature of these urinary crystals in rats administered pioglitazone might partly explain the difficulty of detecting the crystals in rats administered pioglitazone or other PPARγ or dual agonists, and this intermittent nature might also explain the difficulty in detecting hyperplasia in short-term studies with pioglitazone and some of the other PPAR agonists.

Most commonly with these agents, the extent of cytotoxicity involves only the superficial urothelial cell layer, which can be difficult to observe by light microscopy but is readily observable by SEM. Although we did observe superficial cytotoxicity in some of the rats administered pioglitazone in the present study, several of the bladders could not be examined by SEM because of the overlying apparent glycosaminoglycan layer, which had adhered to the luminal surface of the epithelium (Fig. 2). This precludes the examination of the surface characteristics of the epithelium. Nevertheless, the combination of findings by light microscopy, SEM, and increased BrdU labeling index is supportive of the mode of action of cytotoxicity with consequent regenerative proliferation. The evidence from our urinalysis examinations supports the hypothesis that the cytotoxicity is produced by the formation of urinary solids, namely calcium-containing crystals and calculi.

In summary, we have demonstrated evidence that pioglitazone, a PPARγ agonist which induced bladder tumors in male rats, produces alterations in urinary solid formation, albeit in small amounts, especially calcium-containing crystals and calculi, which could lead to cytotoxicity and consequent regenerative proliferation. We have also confirmed that the thiazolidinediones, including troglitazone and pioglitazone, inhibit urothelial cell proliferation and potentiate differentiation of rat urothelial cells in culture. Our data support but do not prove the hypothesis that the bladder tumors produced in rats by pioglitazone are related to the formation of urinary solids. The quantitative levels of the crystals appear to correlate with the extent and incidence of urothelial lesions occurring in rats administered PPAR agonists, few solids and few lesions with pioglitazone in contrast to numerous solids and lesions associated with muraglitazar. Since clinical trials with PPARγ and dual agonists have not been associated with urinary calculus formation (Dominick *et al.*, 2006; Dormandy *et al.*, 2005), the findings in the present experiment with pioglitazone strongly support the previous conclusion from studies with muraglitazar that this is a rat-specific phenomenon and does not pose a urothelial cancer risk to humans treated with these agents.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous contribution of pioglitazone and troglitazone by Bristol-Myers Squibb and the excellent assistance of Connie Winters in the preparation of this manuscript. Conflict of interest: Dr Cohen has consulted for several pharmaceutical firms that have developed PPAR agonists.

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Fukushima J. Med. Sci.,
Vol. 56, No. 1, 2010

[Original Article]

NO INHIBITORY EFFECTS OF (−)-EPIGALLOCATECHIN GALLATE AND LYCOPENE ON SPONTANEOUS HEPATOTUMORIGENESIS IN C3H/HeN MICE

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(Received March 11, 2009, accepted March 3, 2010)

Abstract: Although several studies have indicated that (−)-epigallocatechin gallate (EGCG) and lycopene, representative dietary antioxidants, inhibit chemically induced animal tumorigenesis, only a few studies have examined the inhibitory effects of these compounds on spontaneous liver tumorigenesis in rodents. In this study, we investigated the inhibitory effects of these compounds on the formation of spontaneous liver tumors in C3H/HeN mice. We used xeroderma pigmentosum group A (XPA) gene-deficient mice to simultaneously examine whether the knockout mice could be used as a sensitive animal model. In addition, we examined the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) — a marker of reactive oxygen species-induced DNA injury — in liver tissue. Male *XPA* +/+, *XPA* +/−, and *XPA* −/− mice with a C3H/HeN genetic background were divided into 3 groups: control, EGCG, and lycopene. Autopsy at 18 months of age revealed that EGCG and lycopene did not exhibit obvious suppressive effects on the development of liver tumors in any XPA genotype; further, the XPA genotype did not influence any susceptibility to liver tumors. With regard to 8-OHdG levels in non-tumorous liver tissue at 8 months of age, EGCG showed no significant inhibitory effects and lycopene showed significant inhibitory effects only in *XPA* +/− mice. The present study demonstrates that contrary to previous reports of the inhibitory effects of EGCG and lycopene on the development of various carcinogen-induced animal tumors, these compounds exert no chemopreventive effects on spontaneous liver tumorigenesis in C3H/HeN mice. EGCG and lycopene may inhibit carcinogen-induced tumors through properties other than their antioxidant abilities.

Key words: (−)-Epigallocatechin gallate, Knockout mice, Lycopene, Spontaneous liver

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tumor, Xeroderma pigmentosum group A

INTRODUCTION

Liver cancer is a major cause of death in African and Asian countries, including Japan[1]. More than 30,000 Japanese die of liver cancer annually, and 95% of these deaths are due to hepatocellular carcinoma (HCC)[2]. Moreover, the number of HCC patients in Japan is increasing.

Reactive oxygen species (ROS) are considered to be one of the main causes of carcinogenesis in various organs. ROS induce cancer-causing mutations, oxidize lipids and proteins, and alter signal transduction pathways, resulting in increased cancer risk[3,4]. The relationship between ROS and carcinogenesis is supported by the fact that dietary and endogenous antioxidants inhibit carcinogenesis in animal models[5]. Epidemiological studies have revealed that high consumption of antioxidant-rich fruits and vegetables is inversely correlated with the incidence of cancer[6-8].

Lycopene and (−)-epigallocatechin gallate (EGCG) are representative dietary antioxidants. EGCG is the most abundant polyphenolic compound present in green tea (more than 40% of the total polyphenolic mixture)[9], and it is the most powerful antioxidant among green tea catechins[10]. Lycopene is the most abundant carotenoid in tomatoes and the cause of their deep-red color[11]. Lycopene is known to possess high singlet oxygen-quenching capability[12]. Although EGCG and lycopene have been reported to inhibit the formation of carcinogen-induced tumors in various animal models[13-16], only a few studies have examined the inhibitory effects of these compounds on spontaneous liver tumorigenesis in rodents[17,18].

Male C3H mice exhibit high susceptibility to spontaneous and chemically induced hepatotumorigenesis ; these mice spontaneously develop liver tumors late in their life, with an incidence as high as 70%[19]. Genetic linkage analysis using C3H mice and hepatotumorigenesis-resistant strains have revealed that 6 different regions on chromosomes 2, 5, 7, 8, 12, and 19 showed significant linkage with hepatocellular tumor development ; these regions were named "hepatocarcinogen sensitivity (Hcs) loci"[20]. Thus, C3H mice are considered to be a good model of polygenic inheritance for predisposition to liver cancer.

Xeroderma pigmentosum (XP) is a hereditary disease characterized by impairment of the nucleotide excision repair (NER) pathway, which is one of the main DNA repair pathways[21,22]. XP is classified into 8 types. Previously, we established XP group A gene-deficient mice (*XPA*-deficient mice) on a C3H/HeN genetic background and confirmed that *XPA* −/− mice were more susceptible to spontaneous and carcinogen-induced liver tumorigenesis than *XPA* +/+ and *XPA* +/− mice[23]. This observation suggests that *XPA*-deficient C3H mice may be a more sensitive animal model than wild-type C3H mice in identifying liver carcinogens or liver tumor-preventing compounds.

In this study, we administered EGCG or lycopene to *XPA*-deficient mice with a C3H/HeN genetic background for a long period and examined the inhibitory effects of these compounds on the development of spontaneous liver tumors. We also examined the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) — a marker of ROS-induced DNA injury — in

liver tissue.

MATERIALS AND METHODS

Mice

XPA-deficient mice were originally produced by Tanaka *et al.*; these mice had a hybrid genetic background[24]. We established *XPA*-deficient congenic mice with a C3H/HeN genetic background by repeated back-crossing with inbred C3H/HeN mice[23]. In the present study, we used an F12 line because we maintained the mice of this line. The method of genotype analysis has previously been described[23]. The mice were maintained in the Laboratory Animal Center of Teikyo University School of Medicine at 21°C and 53% humidity in accordance with the rules of the animal center.

Effects of EGCG and lycopene on spontaneous liver tumor

The *XPA*-deficient mice with a C3H/HeN genetic background were divided into 3 groups : control, EGCG, and lycopene. Each group contained 15-20 male mice for each XPA genotype. Only male mice were used because male C3H mice are more susceptible to spontaneous and chemically induced hepatotumorigenesis than female C3H mice[25-27]. The mice in the control group were fed a CRF-1 diet (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum until the end of the experiment. The mice in the EGCG group were fed a CRF-1 diet and tap water containing 0.05% EGCG ad libitum from 6 weeks of age until the end of the experiment. Pure EGCG was extracted from green tea as previously reported[10], and stored in a refrigerator at 4°C. Tap water containing EGCG was administered from light-shielded, polyvinyl bottles, and the bottles were refilled with fresh solution twice a week. The mice in the lycopene group were fed a CRF-1 diet containing 0.005% lycopene and tap water ad libitum from 6 weeks of age until the end of the experiment. Lycopene was donated by LycoRed Natural Products Industries (Beer-Sheva, Israel) as a natural tomato extract containing 6% lycopene (Lyc-O-Mato). The Lyc-O-Mato and CRF-1 diet containing lycopene were stored in a refrigerator at 4°C. Feed containers for mice were refilled with fresh lycopene diet twice a week. The doses of EGCG and lycopene in the present study are comparable to those in previous studies in which these compounds inhibited chemically induced and spontaneous mouse tumorigenesis[16,17].

All the mice were killed by anesthesia at 18 months of age, and a complete autopsy was performed. At autopsy, the total body weight and weight of the major organs were measured. The surface of the livers was grossly examined, and tumor nodules were counted. After fixation in 10% formaldehyde solution, each liver lobe was completely sectioned into 2-mm-thick slices and internal tumor nodules were counted. The total number of tumor nodules was calculated as the sum of the surface and internal tumor nodules. All sections were mounted on slides for light microscopy. Hepatocellular adenomas (HCAs) and carcinomas were diagnosed microscopically on the basis of established diagnostic criteria[28]. Major organs, except the liver, were also grossly examined, and representative sections were

mounted on slides.

Quantification of 8-OHdG

The *XPA*-deficient mice with a C3H/HeN genetic background were divided into 3 groups as in the tumorigenesis experiment, and each group contained 6 male mice for each XPA genotype. The treatments were the same as those in the tumorigenesis experiment. All the mice were killed at 8 months of age, and approximately 1 g of non-tumorous liver tissue was excised and stored at −80°C. The mice were killed at that age because, in our experience, we have found that spontaneous liver tumors begin to occur around that age. DNA samples isolated from the frozen liver by phenol and chloroform were digested into deoxynucleosides by a combined treatment with nuclease P1 and alkaline phosphatase. The 8-OHdG levels were determined using high-performance liquid chromatography as described previously[29] and expressed as the number of 8-OHdG residues for every 10^5 deoxyguanosines.

The protocol for this research project was approved by the Ethics Committee of Teikyo University School of Medicine, and it conformed to the provisions of the 1995 Declaration of Helsinki (as revised in Edinburgh in 2000). The experiment was ethically acceptable and conformed to national guidelines for animal usage in research.

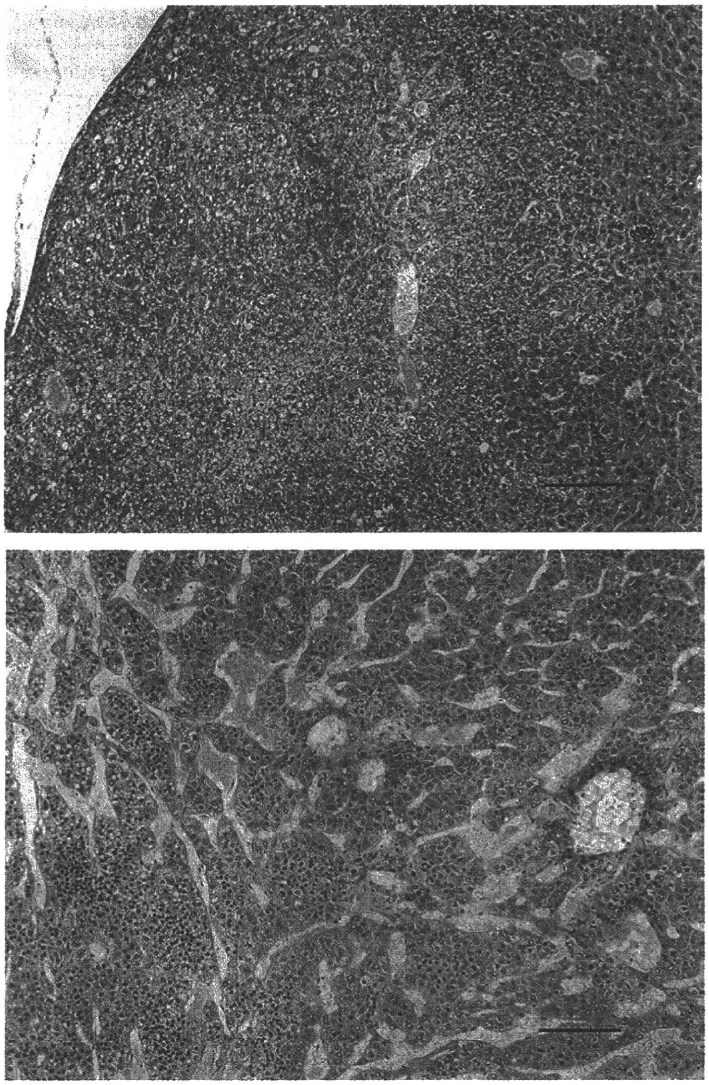
Statistical analysis

Group differences were assessed for statistical significance by using the χ^2 test for the incidence of tumors and the *t*-test for the number of tumors per mouse, diameter of tumors, and 8-OHdG levels. A p-value of <0.05 was considered to be significant.

RESULTS

General observations

No statistically significant differences were observed in the average body weight and average food or water consumption among the 3 experimental groups or the 3 XPA genotypes (data not shown). In addition, there were no statistically significant differences in the weight of the major organs among the 3 experimental groups or the 3 XPA genotypes (data not shown). Only a few mice died during the experiments, and in most cases, liver tumor was not the cause of death ; these mice were excluded from the study. On the basis of autopsy findings of these mice, we speculated that liver tumors began to occur at 8 or 9 months of age. Most liver tumors that occurred around this age were HCAs. In addition to liver tumors, lung adenomas were observed, although the incidence was low (14%, in total). With regard to the incidence of lung adenoma, there were no statistically significant differences among the 3 experimental groups or the 3 XPA genotypes. Previously, the incidence of spontaneous lung adenoma in C3H/HeN mice was reported to be 12% until 28 months of age[30]. Neither the liver nor the lung tumors had metastasized. Pathological examinations confirmed that no tumors occurred in other major organs.



1a

1b

Fig. 1. (a) Representative histological appearance of HCA. Small and monotonous tumor cells proliferate, forming a thin trabecular pattern. Scale bar represents 200 μm. (b) Representative histological appearance of HCC. Tumor cells show a thick trabecular growth pattern with dilated sinusoids. Scale bar represents 200 μm.

Liver tumors

Representative histological samples of HCA and HCC are shown in Figures 1a and 1b, respectively. Table 1 shows the occurrence of tumorigenesis in the 18-month-old mice. The incidence of liver tumor was 65-89%, and the average number of liver tumors per mouse was 1.2-2.9. In many mice, HCAs and HCCs coexisted. Most HCAs were less than 10 mm in diameter and the majority of HCCs were more than 5 mm in diameter. HCC nodules occasionally contained a component of HCA, and a differential diagnosis between HCA and HCC was very difficult in some cases. These observations imply that a considerable number of HCCs developed from HCAs.

EGCG and lycopene did not exhibit obvious tumor-suppressive effects for any XPA gen-

Table 1. Liver Tumors of the Mice at the Age of 18 Months

Control group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	16	18	18
tumor-bearing mice (%)	14 (88%)	16 (89%)	12 (67%)
HCA-bearing mice (%)	12 (75%)	11 (61%)	12 (67%)
HCC-bearing mice (%)	8 (50%)	11 (61%)	4 (22%)
tumors per mouse (average ± S.D.)	1.8 ± 1.2	2.2 ± 1.9	1.3 ± 1.3
HCAs per mouse (average ± S.D.)	1.1 ± 0.8	1.4 ± 1.9	1.1 ± 1.1
HCCs per mouse (average ± S.D.)	0.7 ± 0.9	0.8 ± 0.7	0.3 ± 0.6[b]
diameter of tumors (mm, average ± S.D.)	8.1 ± 8.0	8.2 ± 6.8	5.7 ± 5.0[c]
HCAs (mm, average ± S.D.)	3.3 ± 2.4	3.8 ± 2.0	3.5 ± 2.2
HCCs (mm, average ± S.D.)	15.6 ± 8.0	16.2 ± 4.6	13.8 ± 4.1

EGCG group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	15	17	17
tumor-bearing mice (%)	13 (87%)	11 (65%)	15 (88%)
HCA-bearing mice (%)	11 (73%)	10 (59%)	13 (76%)
HCC-bearing mice (%)	10 (67%)	4 (24%)	7 (41%)
tumors per mouse (average ± S.D.)	2.9 ± 2.0	1.2 ± 1.3	2.0 ± 1.5
HCAs per mouse (average ± S.D.)	2.0 ± 1.8	0.9 ± 0.9	1.4 ± 1.3
HCCs per mouse (average ± S.D.)	0.9 ± 0.9	0.4 ± 0.7	0.6 ± 0.9
diameter of tumors (mm, average ± S.D.)	7.6 ± 7.0	6.7 ± 6.7	7.4 ± 6.7
HCAs (mm, average ± S.D.)	3.9 ± 2.5	3.3 ± 2.4	3.7 ± 2.1
HCCs (mm, average ± S.D.)	15.6 ± 6.7	15.3 ± 6.2	16.3 ± 5.3

Lycopene group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	14	15	16
tumor-bearing mice (%)	11 (79%)	11 (73%)	14 (88%)
HCA-bearing mice (%)	10 (71%)	11 (73%)	11 (69%)
HCC-bearing mice (%)	6 (43%)	6 (40%)	9 (56%)
tumors per mouse (average ± S.D.)	1.6 ± 1.3	2.3 ± 2.0	2.0 ± 1.3
HCAs per mouse (average ± S.D.)	1.1 ± 0.9	1.7 ± 1.6	1.1 ± 0.9
HCCs per mouse (average ± S.D.)	0.5 ± 0.7	0.6 ± 0.9	0.9 ± 1.0[b]
diameter of tumors (mm, average ± S.D.)	6.9 ± 7.1	5.7 ± 5.2[a]	9.7 ± 8.1[a,c]
HCAs (mm, average ± S.D.)	3.0 ± 2.4	3.1 ± 1.7	3.2 ± 2.0
HCCs (mm, average ± S.D.)	15.3 ± 6.4	13.3 ± 4.6	17.1 ± 5.8

a, b, c Significantly different ($p<0.05$). HCA, hepatocellular adenoma ; HCC, hepatocellular carcinoma.