Preventive Effect of FK143, a 5α-Reductase Inhibitor, on Chemical Hepatocarcinogenesis in Rats

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ABSTRACT
The incidence of hepatocellular carcinoma (HCC) is more prevalent in males than in females. 5α-Dihydrotestosterone is the most potent form of androgen and is converted from testosterone by 5α-reductase. The antitumor effect of a 5α-reductase inhibitor (FK143) was evaluated in a rat chemical hepatocarcinogenesis model (Solt-Farber). Male Fischer 344 rats were used in three groups: (a) control group; (b) low-dose FK143 (FKL) group (20 ppm FK143); and (c) high-dose FK143 (FKH) group (200 ppm FK143). The numbers of both glutathione S-transferase placental form-positive foci (P < 0.05) and hyperplastic nodules (HNs; P < 0.01) in the liver were significantly lower in the FKH group than in the control group. The numbers (P < 0.05) and tumor volume (P < 0.01) of HCCs per liver were significantly lower in the FKL group when compared with the control group. All HCCs were well differentiated in the FKL group, whereas 38% and 36% of HCCs were moderate to poorly differentiated in the control group and the FKH group, respectively. The proliferating cell nuclear antigen labeling index:apoptotic index ratios of enzyme-altered foci, HNs, and HCCs were significantly lower in the FKL group than in the control group. Serum 5α-dihydrotestosterone was significantly lower in both the FKL and FKH groups. However, a high dose of FK143 (200 ppm) provided no protection against hepatocarcinogenesis, and the level of serum testosterone was elevated in this group when compared with that in the control group. The low dose of FK143 significantly suppressed the formation of enzyme-altered foci, HNs, and HCCs in rat hepatocarcinogenesis. This may indicate that 5α-dihydrotestosterone enhances hepatocarcinogenesis. We conclude that an optimal dose of FK143 may have a suppressive effect on hepatocarcinogenesis.

INTRODUCTION
Effective chemoprevention is warranted for a population at high risk for development of HCC.2 The number of annual deaths from liver cancer in Japan exceeds 33,000 (1). However, there are few reports of chemoprevention studies in this relentless tumor (2–5). Accordingly, the development of effective chemoprevention is desirable.

The incidence of HCC is more prevalent in males than in females (6). Alcohol abuse, smoking, and chronic hepatitis virus infection may account in part for male predominance. The sex differences are much discussed and have been proved in experimental animal models for spontaneous mouse hepatoma (7, 8), chemical-induced hepatocarcinogenesis in rats (9–13), and carcinogenesis in transgenic mice with the HBx gene (14) and hepatitis C virus core gene (15). In addition, it was reported that elimination of androgens by castration decreased the incidences of HCCs and preneoplastic lesions in rodents (10–12), and these tumors reappeared upon replacement of androgens (11, 12).

The significance of AR expression in human HCC and the surrounding liver has been studied extensively (16–21). The presence of the AR in human HCC was first demonstrated by Iqbal et al. (16) in 1983. We reported that the AR levels are significantly higher in HCC than in the surrounding liver (17). Experimentally, the growth of Morris hepatoma 7787, an AR-expressing rat cell line, was shown to be well modulated by androgens (22). Similarly, we have shown that the growth of an AR-positive human HCC cell line can be modulated by androgen manipulation (23, 24). In addition, the elevation of hepatic AR content has also been noted in chemical-induced hepatocarcinogenesis in the rat (25, 26).

Enzyme 5α-reductase catalyzes the conversion of testosterone into DHT with NADPH as the cofactor (27, 28). DHT exhibits the most potent biological activity among various androgens (27). 5α-Reductase has two major isoforms of type I and type II (28). The former is predominant in the liver, and the latter is predominant in the reproductive tissues in the male (28). Therefore, considering the androgen-responsive nature of HCC, it should be made clear whether or not inhibition of 5α-reductase could give rise to the rational blockage of this tumor. FK143 is a nonsteroidal and noncompetitive inhibitor of...
5α-reductase of both human and rat origin (29). In the present study, the preventive effect of FK143 was examined in detail with respect to the incidence of enzyme-altered foci, HNs, and HCCs during chemical-induced hepatocarcinogenesis in the rat. For elucidation of the antitumor mechanism of FK143, incidences of cellular proliferation and apoptosis were examined in enzyme-altered foci, HNs, and HCCs.

MATERIALS AND METHODS

Chemicals and Diets. FK143, 4-[3-[3-[bis(4-isobutylphenyl)methyl-amino]-benzoyl]-1H-indol-1-yl]-butyric acid, was kindly provided by the Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). DEN was purchased from Wako Pure Chemical Co., Ltd. (Kyoto, Japan), and 2-AAF was from Sigma Chemical Co. (St. Louis, MO). Basal diet (CRF-1) was obtained from Oriental Food Corp. (Tokyo, Japan), and all of the mixed diets fed in the experiments were based on it.

Animals and Treatments. Male 6-week-old Fischer 344 rats (Charles River Japan, Inc., Kanagawa, Japan) were used. The animals were housed in an air-conditioned room at 25°C with a 12-h light/dark cycle. Diets and water were given ad libitum during the experiments. All experiments were performed after a 1-week acclimatization period on a basal diet. A modified protocol of chemical hepatocarcinogenesis originally described by Solt and Farber (30) was used (Fig. 1). According to this protocol, rats were given DEN at a single i.p. dose of 200 mg/kg. Two weeks after DEN administration, rats were fed a 0.01% 2-AAF-containing diet for 2 weeks. A two-thirds partial hepatectomy was performed after the completion of 1 week of 2-AAF consumption.

At appropriate time intervals, animals were sacrificed to investigate the effect of FK143 on the stepwise development of enzyme-altered foci (experiment 1), HNs (experiment 2), and HCCs (experiment 3). Each experimental setting comprised three groups of animals: (a) control group; (b) FKL group (20 ppm FK143); and (c) FKH group (200 ppm FK143). FK143 treatment was started 1 week before DEN injection in experiment 1 and after completion of 2-AAF treatment in experiments 2 and 3. Rats were sacrificed at 5 weeks (control group, n = 10; FKL group, n = 7; FKH group, n = 9), 16 weeks (control group, n = 7; FKL group, n = 7; FKH group, n = 7), and 40 weeks (control group, n = 8; FKL group, n = 9; FKH group, n = 11) after DEN injection in experiment 1, 2, and 3, respectively. After sacrifice, livers were removed, weighed, and cut into 2-mm-thick sections for 10% formalin fixation. Residual samples were frozen in liquid nitrogen and stored at −80°C until use. All experimental methods were conducted according to the instructions of the Institute of Animal and Experimental Research Center, Shimane Medical University (Izumo, Japan).

Histopathological Assessment. For histopathological examination, formalin-fixed paraffin-embedded sections were stained with H&E and randomly examined in a blind manner. The morphology of HCC was diagnosed by one of the authors (K. O.).

Immunohistochemistry of GST-P and PCNA. Immunohistochemical staining was performed according to the ABC method. A polyclonal rabbit antibody to GST-P (31) and a
mouse monoclonal antibody against PCNA (PC-10; Dako, Glostrup, Denmark) were used as the primary antibodies. The ABC assay was done by using commercial kits [Vectastain-ABC kits (Vector Laboratories, Burlingame, CA) and Histofine kit (Nichirei, Tokyo, Japan)]. The peroxidase reaction was visualized with diaminobenzidine. For PCNA staining, slides were counterstained with hematoxylin.

**In Situ Labeling of Apoptosis.** The TUNEL method was adopted to detect apoptosis and was performed according to the protocol provided by the manufacturer (DeadEnd colorimetric apoptotic detection system; Promega, Madison, WI). Briefly, tissue sections were deparaffinized, rehydrated in graded alcohols, and permeabilized with proteinase K treatment. After equilibration with a buffer solution, the slides were treated with terminal deoxynucleotidyl transferase reaction mix (equilibration buffer, biotinylated nucleotide mix, and terminal deoxynucleotidyl transferase enzyme) for 1 h at 37°C. Endogenous peroxidase was blocked with 0.3% H₂O₂. Subsequently, slides were treated with horseradish peroxidase-labeled streptavidin, and color was developed with diaminobenzidine. Slides were counterstained with hematoxylin. Positive and negative controls were incorporated in each run of the staining procedure.

**Evaluation of Enzyme-altered Foci, PCNA LI, and AI.**

The numbers of GST-P-positive enzyme-altered foci were counted on images captured by a CCD camera on a Macintosh computer. All GST-P-positive enzyme-altered foci over 0.2 mm in diameter were counted. The number of enzyme-altered foci per unit volume was estimated on the stereological study according to the method of Campbell et al. (32). The PCNA LI and AI were represented as the percentage of positively stained nuclei by counting 1000–2000 cells. Areas of the lesion showing the highest density of cells with positively stained nuclei were chosen.

**Western Blot Analysis of Hepatic AR Proteins.** Protein extraction was done with ice-cold TEDG buffer [10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol (pH 7.2)]. Amounts of proteins were measured using the Coomassie Brilliant Blue protein assay kit (Nakarai, Kyoto, Japan). SDS-PAGE was carried out with 8% SDS-polyacrylamide gel. The nitrocellulose membrane-transferred proteins were immunostained using an anti-AR monoclonal antibody (AN-15; Ref. 33) and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The band intensity was estimated by a densitometer, laser scanner model. Results were corrected against a standard positive control sample of rat testis.

**RIA for Serum Testosterone and DHT.** Serum concentrations of testosterone and DHT were quantitated in eight animals from each of the control, FKL, and FKH groups (experiment 3) with a commercial RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA) as described previously (34). In brief, the serum steroids were extracted with ethyl acetate:hexane (3:2) and evaporated, followed by application of the extracts to a microcolumn of celite that had been equilibrated with isooctane. Testosterone was eluted with 15% ethyl acetate/iso-octane and concentrated with evaporation. DHT was eluted from the column with 5% ethyl acetate/iso-octane. The steroids were assayed with specific antisera and the ³H-labeled standards, and the charcoal-unbound hormones were counted by a liquid scintillation counter as reported previously (34).
**Tumor Volumetry.** Volumetry of HCCs was done on a liver cross-section stained with H&E. Tumor volumes were estimated by the following equation (35): 
\[ \text{volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times \frac{1}{2}. \]

**Statistical Analysis.** Values are represented as mean ± SD unless noted otherwise. Statistical comparisons for significance were evaluated by using Wilcoxon's rank-sum test. \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**Effects of FK143 on the Formation of GST-P-positive Enzyme-altered Foci, HNs, and HCCs.** The estimated number of enzyme-altered foci per unit volume in the liver was significantly lower in the FKL group (\( P < 0.05 \)) than in the control group (Fig. 2). The numbers of HNs as seen on the liver surface were significantly lower in the FKL (\( P = 0.01 \)) and FKH (\( P < 0.01 \)) groups than in the control group (Fig. 3A). The numbers of HNs in the liver were significantly lower in the FKL group (\( P < 0.01 \)) than in the control group (Fig. 3B). Microscopic HCCs were present in all animals in both the control and treatment groups. However, the incidences of macroscopic lesions on the liver surface were 75% (6 of 8), 33% (3 of 9), and 91% (10 of 11) in the control group, FKL group, and FKH group, respectively (Table 1). The macroscopic appearance of liver tumors in the control group, FKL group, and FKH group is shown in Fig. 4, A–C, respectively. The number of microscopic HCCs/liver was significantly lower in the FKL group than in the control group (\( P < 0.05 \); Fig. 5A). The average estimated tumor volume in the FKL group was significantly lower than that in the control group (\( P < 0.01 \); Fig. 5B). On histological examination (Fig. 6A), all tumors in the FKL group were well differentiated, whereas 38% and 36% of tumors were moderate to poorly differentiated in the control group and FKH group, respectively (Table 1).

**Proliferation and Apoptotic Activities of Enzyme-altered Foci, HNs, and HCCs.** PCNA-positive nuclei and TUNEL-positive nuclei were stained brown and could be clearly distinguished from other nuclei under the light microscope, respectively. Typical appearance of PCNA-positive and TUNEL-positive HCCs is shown in Fig. 6, B and C, respectively. The PCNA LI of enzyme-altered foci were 52.7 ± 5.2, 27.5 ± 1.4, and 42.5 ± 3.5 in the control group, FKL group, and FKH group, respectively. The PCNA LI of HNs were 21.9 ± 2.5, 8.3 ± 2.1, and 14.9 ± 1.5 in the control group, FKL group, and FKH group, respectively. The PCNA LI of HCCs were 26.1 ± 2.7, 12.5 ± 0.8, and 37.4 ± 9.6 in the control group, FKL group, and FKH group, respectively. The AI of enzyme-altered foci (\( P < 0.01 \)), HNs (\( P < 0.01 \)), and HCCs (\( P < 0.05 \)) in the FKL group was significantly lower than that in the control group. The PCNA LI of enzyme-altered foci (\( P < 0.01 \)) and HNs (\( P < 0.01 \)) and HCCs (\( P < 0.05 \)) in the FKH group was significantly lower than that in the control group. The number of tumors in the FKL group (A) was remarkably reduced compared with that in the control group. The number of tumors in the FKH group (C) did not differ from that in the control group.

![Fig. 4 Macroscopic appearance of liver tumors in experiment 3. Multiple tumors were seen in the control group (A), whereas the number of tumors in the FKL group (B) was remarkably reduced compared with that in the control group. The number of tumors in the FKH group (C) did not differ from that in the control group.](image-url)
than that in the control group. The PCNA LI:AI ratio was used for the adjusted estimation of the net growth potential of enzyme-altered foci, HNs, and HCCs. The PCNA LI:AI ratios of enzyme-altered foci (P < 0.01), HNs (P < 0.01), and HCCs (P < 0.05) in the FKL group were significantly lower than those in the control group (Fig. 7). The PCNA LI:AI ratios of enzyme-altered foci (P < 0.01) and HNs (P < 0.01) were significantly lower in the FKH group than in the control group (Fig. 7).

**Serum Androgen Concentrations and Hepatic AR Proteins.** The serum androgen concentrations showed that there was a significant decrease in serum DHT level in the FKL (P < 0.01) and FKH (P < 0.01) groups compared with that in the control group (Table 2). In contrast, the level of serum testosterone was significantly increased only in FKH group when compared with the control group (P < 0.01; Table 2). The level of AR expression in HCCs was significantly higher than that of the surrounding liver in the control group (P < 0.05; Fig. 8). The AR levels of HCCs in the FKL (P < 0.05) and FKH (P < 0.05) groups were significantly reduced compared with that in the control group (Fig. 8).

**Total Administration of FK143 and General Findings.** The total administered doses of FK143 in the FKL group in experiments 1, 2, and 3 were 9.3, 34.2, and 98.9 mg/rat, respectively. The amounts of FK143 consumed in the FKL group in experiments 1, 2, and 3 were 91.3, 344.6, and 979.9 mg/rat, respectively. There was no clear evidence of toxicity in the rats fed the FK143 diets. The relative liver weights and body weights in all experiments showed no significant difference between the control group and the FK143-treated groups.

**DISCUSSION**

5α-Reductase type I is abundant in the liver, and this enzyme plays an essential role in the production of DHT in vivo. DHT exhibits strong androgenic activities on at least two points: (a) its greater affinity to AR than testosterone (27); and (b) lack of aromatization (27). In a recent study, it was stated that DHT enhanced cell proliferation and 3[H]thyidine uptake in an AR-positive human HCC cell line (23). The proliferative effect of DHT was partially inhibited by antiandrogen treatment using CPA (23). Similarly, it was shown in the clinical setting that an antiandrogen treatment regimen using CPA produced regression of advanced HCCs, which was associated with a decrease of serum levels of DHT (36). Iqbal et al. (18) found that both DHT levels and DHT:testosterone ratios increased in HCC patients compared with those in patients with cirrhotic liver, and they pointed out a plausible relationship between development of HCC and 5α-reductase activity. Thus, DHT is thought to be one of the important accelerators in the development and growth of HCC.

The results of the current study clearly demonstrate that a low dose of FK143 significantly suppressed the formation of enzyme-altered foci, HNs, and HCCs in rat chemical hepatocarcinogenesis. FK143 treatment not only reduced the hepatic tumor burden but also modulated the differentiation status of tumor in some animals. The most significant antitumor effect of FK143 was evident in the HN phase of hepatocarcinogenesis. Obviously, complete prevention of development of HCC by FK143 treatment was not possible in this model of chemical-induced hepatocarcinogenesis. Although macroscopic HCCs were seen in only three of nine rats, HCCs remained microscopic in all animals in the FKL group. However, it is worth mentioning that all of the HCCs in these rats were well differentiated, in contrast to the 38% moderate to poorly differentiated HCCs seen in the control group. Also, the number and estimated volume of HCCs were significantly lower in the FKL group than in the control group. The data from serum androgen measurement showed that there was a significant decrease in the serum DHT level in the FKL group as compared with that in the control group (Table 2). Accordingly, the antitumor effects of FKL might indicate that blockage of DHT production by 5α-reductase inhibitor would work in treating hepatocarcinogenesis (Fig. 9). Alternatively, this may indicate that DHT is one of the responsible factors for hepatocarcinogenesis in male rats.

In contrast to the antitumor effect of FKL, the effect of FKH was controversial. In the early phases of hepatocarcinogenesis, especially at the HN phase, FKH had a significant preventive effect (Fig. 3) on tumor development. However, the long-term effect of FKH on HCC development was not different from that in the control group. This discordant result could be explained by a significant elevation of the serum testosterone level in the FKH group compared with the control group (Table 2). It was recently reported that testosterone at high concentrations interacts with the AR in a manner similar to that of DHT (37). Similarly, in a prostatic cancer
model in rats, Homma et al. (38) reported a preventive effect in the FKL group (20 ppm FK143) but reported a high incidence of tumor-bearing rats in the FKH group (200 ppm FK143). They finally concluded that there was a lack of dose dependency on the effect of the FK143 treatment in the rat prostatic carcinogenesis. Therefore, the optimal dose of

### Table 2  Serum androgen concentrations in experiment 3

<table>
<thead>
<tr>
<th>Group</th>
<th>DHT (ng/ml)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.059 ± 0.012</td>
<td>0.494 ± 0.119</td>
</tr>
<tr>
<td>FKL group</td>
<td>0.032 ± 0.010*</td>
<td>0.507 ± 0.122</td>
</tr>
<tr>
<td>FKH group</td>
<td>0.036 ± 0.008*</td>
<td>1.392 ± 0.238*</td>
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* Versus control group (P < 0.01).

![Fig. 6](image1.png) Representative sections of HCC stained by H&E (A), anti-PCNA (B), and TUNEL (C) are shown (original magnification: A, ×200; B, ×200; C, ×200; inset, ×400).

![Fig. 7](image2.png) Analysis of PCNA LI:AI ratio in enzyme-altered foci, HNs, and HCCs. The PCNA LI:AI ratio of enzyme-altered foci (P < 0.01), HNs (P < 0.01), and HCCs (P < 0.05) in the FKL group was significantly lower than that in the control group. The PCNA LI:AI ratio of enzyme-altered foci (P < 0.01) and HNs (P < 0.01) was significantly lower in the FKH group than in the control group. The data are shown as the mean ± SD.

![Fig. 8](image3.png) AR concentrations in HNs, HCCs, and the surrounding liver tissues. The level of AR expression in the HCCs was significantly higher than that in the surrounding liver in the control group (P < 0.05). The AR levels of HCCs in the FKL (P < 0.05) and FKH groups (P < 0.05) were significantly reduced compared with that in the control group. The data are shown as the mean ± SD.
FK143 is crucial for prevention of carcinogenesis. The antitumor effect of FK143 lies primarily with the prevention of conversion of less potent testosterone to more potent DHT. DHT is responsible for enhanced tumor cell proliferation and acts as a survival factor during carcinogenesis (39, 40). Therefore, treatment with a low dose of FK143 decreased the PCNA LI and increased the AI in enzyme-altered foci and HNs. The PCNA LI:AI ratio was used for the adjusted estimation of the net growth potential of enzyme-altered foci, HNs, and HCCs. The PCNA LI:AI ratios of enzyme-altered foci, HNs, and HCCs were significantly lower in the FKL group than in the control group (Fig. 7). This is supported by the fact that androgen ablation represses cell proliferation and accelerates apoptosis in prostatic cancers (39, 40). We showed previously that apoptosis induced by CPA treatment in an AR-positive HCC cell line was associated with overexpression of transforming growth factor β1, a proapoptotic signal (24). Also, there is a unique molecular mechanism in androgen-dependent cells by which deprivation of androgen up-regulates the expression of testosterone-repressed prostatic message-2 (41). Testosterone-repressed prostatic message-2 is also an important signal necessary for apoptosis (41). Accordingly, the antitumor mechanism of 5α-reductase inhibitor is thought to be suppression of cell proliferation and enhancement of apoptosis in the hepatic lesions during the carcinogenic process.

It was elucidated that AR expression in HCC was positive in 74% of male patients and in only 37% of female patients (42). It was reported that AR expression in human liver diseases increases in the order of cirrhosis, dysplastic nodule, and HCC (43). Such a gradual elevation of hepatic AR has been noted during rat chemical hepatocarcinogenesis (25, 26), including the current study. In fact, FK143 treatment significantly repressed the expression of AR in the HCCs in both treatment groups as compared with the control group (Fig. 8). In a study using the testicular feminization mutant mice having mosaic for the AR mutation, Kemp et al. (44) recently proved that tumor promotion by testosterone requires a functional AR; however, this was not cell autonomous. It was also shown in a series of works on mice strains that androgens are closely related to susceptibility to hepatocarcinogenesis by interacting with the hepatocarcinogen sensitivity (Hcs) gene (45, 46). Thus, androgen and its receptor appear to play an important role in the development and growth of HCC. This is the first experimental study in which 5α-reductase inhibitor was used as a chemopreventive agent in HCC. This is a new concept in terms of inhibition of DHT production for the prevention of hepatocarcinogenesis. In conclusion, a low dose of FK143 significantly suppressed the formation of enzyme-altered foci, HNs, and HCCs in the rat hepatocarcinogenesis. FKHl could be preventive in the early phase of hepatocarcinogenesis but becomes detrimental in long-term use due to reciprocal testosterone elevation. We conclude that an optimal dose of FK143 is crucial and effective in prevention of hepatocarcinogenesis. The data from our study may indicate the possible application of FK143 and other steroid 5α-reductase inhibitors for clinical chemopreventive trials of human HCC.

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