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Anti-Carcinogenic Effects of a Serine Protease Inhibitor (FOY-305) through the Suppression of Neutral Serine Protease Activity During Chemical Hepatocarcinogenesis in Rats

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ABSTRACT

Anti-carcinogenic effect of a serine protease inhibitor, [N, N-dimethylcarbamoylmethyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate (FOY-305), was studied in rats with neutral serine protease during hepatocarcinogenesis induced by a single intraperitoneal administration of diethylnitrosamine (DEN) and following feeding of a diet containing 2-N-fluorenylacetamide (FAA) for 32 weeks. Oral administration of FOY-305 significantly suppressed development of γ-glutamyl transpeptidase (γ-GTP)-positive hyperplastic nodules, preneoplastic lesion, at the 8th week of DEN injection, and that of hepatocellular carcinoma (HCC) formation at the 32nd week. Neutral protease activity increased in the preneoplastic and neoplastic livers. The activities in the preneoplastic and tumor-bearing livers were much lower in FOY-305-treated group compared with those in control group. Neutral protease partially purified from neoplastic liver at the 32nd week was inhibited by FOY-305 *in vitro*. The data suggest that neutral protease plays a crucial role in the process of chemical hepatocarcinogenesis.

Involvement of proteases in the malignant transformation of cells has been proposed[1,2]. Thiol and/or serine protease inhibitors suppress the expression of mutation in bacteria or neoplastic transformation of cells *in vitro*[6,8]. Anticarcinogenic effects of thiol and/or serine protease inhibitors have also been reported in skin and mammary carcinogenesis of mouse and rat[15,20]. However, there are no reports on efficient inhibitory effects of protease inhibitor for hepatocarcinogenesis.

In recent years, increased activity of neutral serine protease was reported in rat hepatocarcinogenesis, in malignant cells and in human hepatoma[4,16,17], emphasizing the role of neutral serine protease on carcinogenesis. The plasma membranes from human liver and hepatoma tissues are known to contain diisopropylfluorophosphate (DFP) sensitive neutral proteolytic activity[4]. These reports led us to investigate the effect of serine protease inhibitor on hepatocarcinogenesis.

In the present study, we examined the anticarcinogenic effects of [N, N-Dimethylcarbamoyl-

Abbreviations: FOY-305. [N,N-dimethylcarbamoylmethyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate; DEN, diethylnitrosamine; FAA, 2-N-fluorenylacetamide; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; BAPA, N-α-benzoyl-DL-arginine-p-nitroanilide; PMSF, phenylmethylsulfonylfluoride; DFP, diisopropylfluorophosphate; EDTA, ethylenediamine-tetraacetic acid.

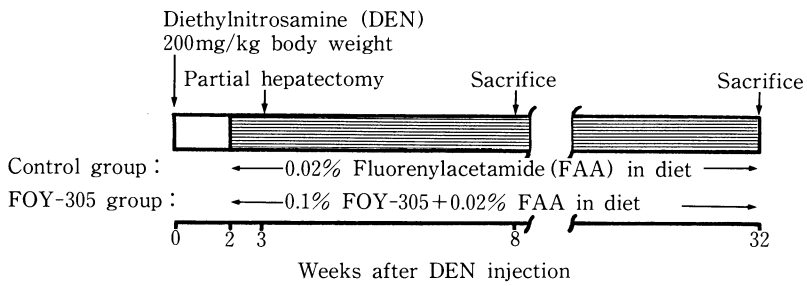

Fig. 1. Experimental schedule. DEN was injected to rats intraperitoneally. Rats were maintained with the basal diet for 2 weeks (open bar), and then fed up for 32 weeks with a 0.02% FAA containing diet (hatched bar) in control group and with a 0.1% FOY-305 and 0.02% FAA containing diet in FOY-305 group. Partial hepatectomy was performed at the end of the 3rd week in both groups.

methyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate (FOY-305) on chemical hepatocarcinogenesis in rats induced by diethylnitrosamine (DEN) and N-2-fluorenylacetamide (FAA). Neutral serine protease activity was measured in preneoplastic and neoplastic livers, and the effect of FOY-305 on the partially purified enzyme was also studied *in vitro*.

MATERIALS AND METHODS

Chemicals. DEN and FAA were obtained from Wako Pure Chemical Industries, Ltd. and Nakarai Chemical Co., respectively. FOY-305 was kindly provided by Ono Pharmaceutical Co. Ltd. N-α-benzoyl-DL-arginine-p-nitroanilide (BAPA), phenylmethylsulfonylfluoride (PMSF), diisopropylfluorophosphate (DFP) and soybean trypsin inhibitor were obtained from Sigma Chemical Co. Leupeptin, antipain and chymostatin were supplied by Peptide Institute, Inc.

Animals. Male Sprague-Dawley rats, weighing approximately 250 g, obtained from Clea Japan, Inc. were divided into 2 groups of each 26 rats. These rats were kept for a week on a basal diet (CE-2, Clea Japan, Inc.) prior to the experiment. The room temperature was kept constant at 24°C with an alternating 12 hr light-dark cycle.

Experimental schedule. After a single intraperitoneal administration of DEN at a dose of 200 mg/kg body weight, rats were fed with the basal diet for 2 weeks (Fig. 1)[13]. From the beginning of the 3rd week, FOY-305 group was given a diet containing 0.02% FAA and 0.1% FOY-305, and control group was fed with a diet containing 0.02% FAA alone. Partial hepatectomy was done in both groups at the end of the 3rd week of the experiment. Six rats in each group were sacrificed at the end of the 8th week, when hyperplastic nodules were already formed. Eight rats in control group and 7 in FOY-305 group were sacrificed at the 32nd week, when hepatocellular carcinoma (HCC) developed. Paired feeding was done in control and FOY-305 groups, and experimental diet was changed to the basal diet 3 days before sacrifice. Body weights were measured biweekly for the first 8 weeks, and 4 week intervals thereafter.

Preparation of enzyme solution. Liver was perfused with cold saline, cut up with scissors, and homogenized with 4 volumes of 250 mM sucrose. The homogenate was centrifuged at 27,000 × *g* for 15 min at 4°C. The supernatant fraction was centrifuged at 105,000 × *g* for 90 min at 4°C to obtain the cytosol fraction.

Assay of neutral protease and other enzyme activities. Neutral protease activity was measured using BAPA as substrate according to Wada et al's method[17] with minor modification. Three tenth ml of enzyme solution was incubated with 2 mM BAPA in 1 ml of 50 mM potassium phosphate buffer (pH 7.3). After incubation at 37°C for 3 hr, the reaction was terminated by adding 0.5 ml of ethanol, and a mixture was centrifuged at 16,000 × *g* for 5 min. The supernatant was then filtered through a membrane filter (Millipore HA, 0.45 μm) and absorbance of the filtrate was measured using Hitachi Digital Spectrophotometer 102 at 410 nm. Control tubes were run in the same way except that substrate was added after the termination of reaction. One unit of enzyme activity is defined as μmol of p-nitroaniline liberated per

hr and the specific activity as units per mg protein. The protein concentration of tissue extracts were determined by Lowry et al's method[7].

Serum glutamate pyruvate transaminase (GPT) and γ-glutamyl transpeptidase (γ-GTP) activity were routinely determined using a GPT-UV Test and γ-GTP C Test (Wako Pure Chemical Co.), respectively.

Partial purification of neutral protease from rat liver. Prior to chromatography, the supernatant fraction was adjusted to pH 5.0 with 1 N acetic acid and centrifuged at 27,000 × *g* for 15 min to obtain the pH 5.0 supernatant fraction. The pH of supernatant fraction was then re-adjusted to 7.0 with careful addition of 1N ammonium solution[17]. Thirty to 80% saturated-ammonium sulfate precipitation was done, and the precipitate was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol. The dialyzed solution was applied to a DEAE-cellulose chromatography column (Whatman DE-52; 1.4 × 35 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 250 mM sucrose and 1 mM 2-mercaptoethanol. A linear gradient to a final concentration of 500 mM potassium chloride was applied. Eluted peak fractions were used for an experiment of protease inhibitors on this enzyme activity. DFP, PMSF, FOY-305, soybean trypsin inhibitor, antipain, chymostatin and ethylenediamine-tetraacetic acid (EDTA) were also added to the above incubation mixture.

Histochemical study. Liver tissues were fixed with cold acetone and staining of γ-GTP was performed according to the modified methods of Rutenburg et al[9]. The area (mm^2) of γ-GTP-positive hyperplastic liver nodules and foci per cm^2 of liver section were quantitatively determined on enlarged photomicrographs using a Medical Graphics Analyzer (Good Man Co.). The liver tissues adjacent to those used for biochemical and histochemical studies were fixed in 10% buffered neutral formaldehyde solution and stained with hematoxylin and eosin.

Statistical analysis. All the data were expressed as the mean ± SD. Statistical differences between the mean values were determined by Student's t-test, and for categorical variables with Chi-square test.

RESULTS

No significant difference of body weights through the experiment in FOY-305 and control groups (Fig. 2) was present. There was also no significant difference in the ratio of liver weight to body weight and serum GPT and γ-GTP activities between the two groups (Table 1). The liver at the 8th week of the experiment showed granular surface in control group (Fig. 3A) and increased γ-GTP-positive foci and nodules (Fig. 3B). In contrast, less granular surface (Fig. 3C) and fewer γ-GTP-positive foci (Fig. 3D) were observed in FOY-305 group. Areas of γ-GTP-positive foci and nodules in FOY-305 group were smaller than that of control group (Table 2).

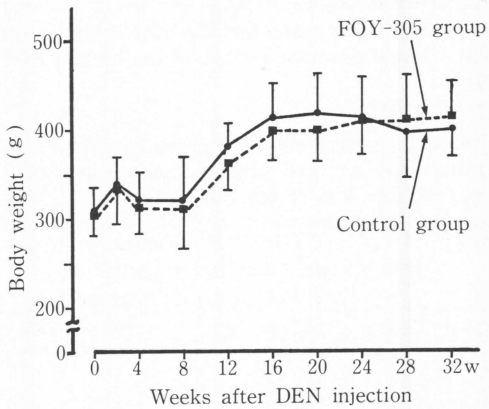

Fig. 2. Changes in body weight during the experiment. (●) Growth curves of control groups. (■) FOY-305 groups. Vertical lines indicate standard deviation of the mean.

Table 1. Ratio of liver weight to body weight, serum GPT and γ-GTP activity in rats determined at the end of 8th and 32nd week

Group	Week	No. of rats	Liver weight/Body weight(%)	Serum GPT (IU)	Serum γ-GTP (U)
Control	8	6	4.0 ± 0.9	29 ± 7	19 ± 4
FOY-305	8	6	4.8 ± 0.8	31 ± 10	17 ± 6
Control	32	8	7.1 ± 1.9	83 ± 56	151 ± 83
FOY-305	32	7	7.0 ± 1.3	76 ± 30	102 ± 43

Mean ± SD.

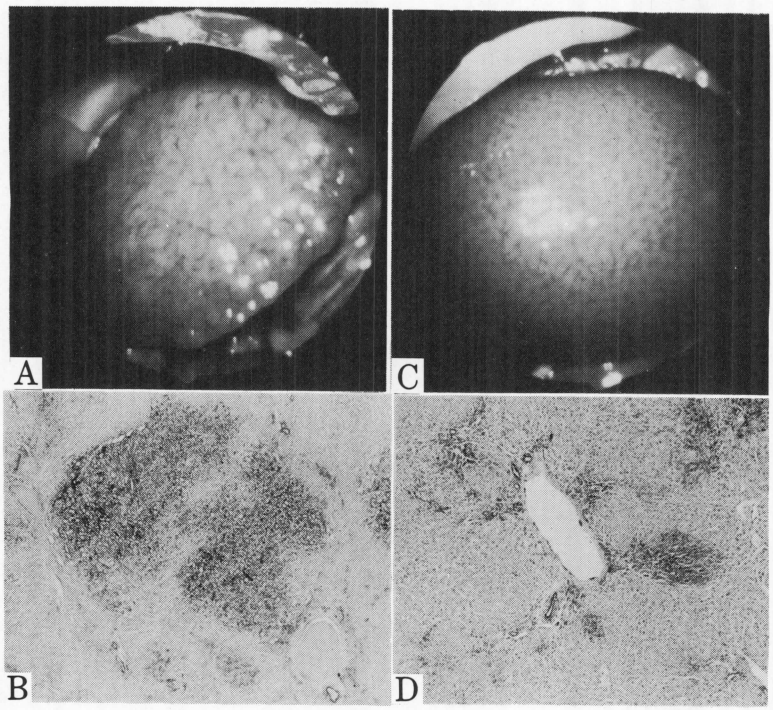

Fig. 3. Peritoneoscopic findings and γ-GTP staining-positive liver nodules and foci in the 8th week of the experiment. (A) Granular surface of the liver in control group. (B) Large γ-GTP-positive liver nodules in control group. (×25) (C) Less granular surface of the liver in FOY-305 group. (D) Small γ-GTP-positive foci in FOY-305 group. (×25)

Table 2. Area of γ-GTP-positive hyperplastic nodule and foci and neutral protease activity in liver (the end of the 8th week)

Group	No. of rats	γ-GTP-positive nodule and foci (mm^2/cm^2 liver)	Neutral protease activity (μU/mg protein)
Control	6	41 ± 9 *	1980 ± 486 *
FOY-305	6	26 ± 14 *	1520 ± 172 *

Mean ± SD. *: $p<0.05$.

Furthermore, the neutral protease activity in FOY-305 group was significantly lower than control. In the 32nd week, HCC developed from 7 out of 8 rats in control group (Table 3). However, HCC developed from only 2 out of 9 rats in FOY-305 group. The neutral protease activity in HCC was much higher than that in tumor-bearing liver. The activity in tumor-bearing liver was lower in FOY-305 group than that in control group. The activity in HCC ap-

Table 3. The number of tumor-bearing rats and neutral protease activity in liver (the end of the 32nd week)

Group	No. of rats (Total/Carcinoma)	Tissue	Neutral protease activity (μU/mg protein)
Control	8/7	Tumor-tissue	2730 ± 1620 (5) *
		Surroundings	980 ± 410 (8) *
FOY-305	9/2 #	Tumor-tissue	1060 (2) *
		Surroundings	640 ± 160 (7) *

#: $p<0.05$, Statistical analysis with Chi square-test vs control. ( ): Number of samples in which the activity was measured. The neutral protease activity in untreated rats were 520 ± 100 (No. of rats = 6).
Mean ± SD. *: $p<0.05$ by Student's t-test.

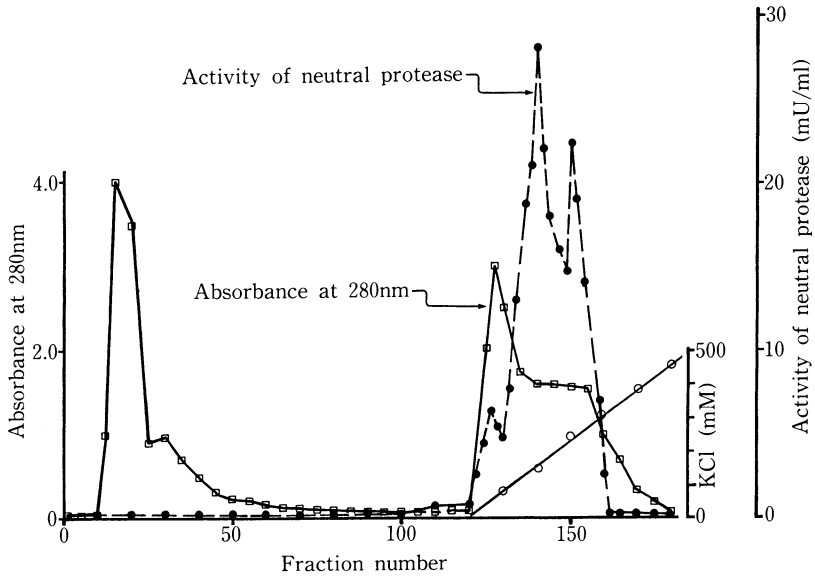

Fig. 4. DEAE-cellulose column chromatography of the neutral protease from the liver treated with hepatocarcinogen for 32 weeks. DEAE-cellulose column (1.4 × 35 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 250 mM sucrose and 1 mM 2-mercaptoethanol. Neutral protease was eluted with a linear gradient up to 500 mM KCl at a flow rate of 18.4 ml/hr. Eluate was collected 2.9 ml per tube. □ Absorbance at 280 nm. ● Activity of neutral protease. ○ KCl concentration.

Table 4. Effects of protease inhibitors on the activity of neutral protease partially purified from DEN-FAA treated liver

Inhibitor	Concentration	Activity remained (%)	
		Peak 1	Peak 2
PMSF	0.5mM	21	18
DFP	5mM	0	0
FOY-305	1mM	21	20
Leupeptin	20mM	24	40
Antipain	10μg/ml	27	57
Chymostatin	10μg/ml	144	133
Soybean trypsin inhibitor	50μg/ml	113	130
EDTA	5mM	169	200

peared to be lower in FOY-305 group compared with that in control group.

DE-52 column chromatography of neutral protease from neoplastic liver at the 32nd week of the experiment was shown in Fig. 4. Two peaks of neutral protease activity were eluated at the concentration of 150 mM and 220 mM KCl. The first and second peak fractions were pooled separately and tested for inhibition study. DFP and PMSF, the specific inhibitors of serine protease, inhibited the activity of both fractions (Table 4). FOY-305 also inhibited both, whereas the activities were not inhibited by soybean trypsin inhibitor.

DISCUSSION

In vivo administration of FOY-305, a potent inhibitor of serine proteases such as trypsin, plasmin, kallikrein and thrombin, diminished the appearance of γ-GTP-positive hyperplastic liver nodules, which have been considered to be preneoplastic lesion. Tumor formation was also suppressed by FOY-305. Neutral serine protease activity in preneoplastic and tumor-bearing livers were lower in FOY-305 group compared with that in control group. Furthermore, activity of neutral serine protease, partially purified from the liver at the 32nd week was inhibited in vitro by FOY-305 as well as DFP and PMSF. Therefore, the results suggest that FOY-305 suppressed carcinogenesis in rat liver through inhibiting the neutral serine protease activity.

Process of chemical carcinogenesis is considered to be at least three distinct stages, i.e., initiation, promotion and progression[11]. Protease inhibitors are reported to act more intensively at the promotion step rather than at the initiation step[6]. Actually, protease inhibitors were administered at the promotion step in most of the previous investigations[6,15,20]. In the

present experiment, FOY-305 was treated at the promotion step and effectively suppressed the carcinogenesis, indicating that FOY-305 acts probably at the promotion step.

Reactive oxygen species, which are induced by various tumor promoters, have recently been paid attention because of their tumor promoting effect[11]. Phorbol myristate acetate, a potent epidermal tumor promoter, induces not only superoxide radical formation in cultured polymorphonuclear leukocyte but also protease production such as plasminogen activator in normal chick embryo fibroblasts and Rous sarcoma virus-transformed chick embryo fibroblasts[14]. In cultured polymorphonuclear leukocytes, addition of protease inhibitors such as DFP, leupeptin and soybean trypsin inhibitor antagonizes the activation of polymorphonuclear leukocyte oxygen consumption[5]. Therefore, in consideration of these data together with the results of the present study, it is likely that proteases are induced in the promotion step, and thus accelerate the formation of reactive oxygen species directly or indirectly, resulting in DNA-damage[3].

It is interesting that neutral protease activity increases not only in HCC but in fetal liver and carbon tetrachloride (CCl_4)-injured liver (Yamauchi, Y., et al, to be published elsewhere). Oral CCl_4 administration of similar dose used in the present study with dietary FAA exposure has a strong promoting effect on hepatocarcinogenesis[10,12]. Thus, increased neutral serine protease activity may participate promotional effect of CCl_4 on hepatocarcinogenesis. Alpha-fetoprotein (AFP) increased in fetal liver and in serum of CCl_4-injured rat[18,19]. The elevation of serum AFP concentrations following CCl_4 administration reached a maximum in 4 days[19], being later than that of neutral protease activity (data not shown). The increase of neutral protease activity after partial hepatectomy was very limited as similarly observed in AFP[19]. Increased activity of neutral protease and concentration of AFP, an onco-fetal protein, in similar situations are very interesting and noteworthy.

In the present study, we demonstrated FOY-305-induced suppression of hepatocarcinogenesis through inhibiting neutral serine protease activity, though the role of neutral serine protease on chemical hepatocarcinogenesis is still unclear at present. Further investigations are needed to clarify the exact role of neutral serine protease during hepatocarcinogenesis.

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