Tumor Res. **20**, 61–65 (1985)

Phalloidin Uptake by Preneoplastic Rat Hepatocytes

Toshihiro MITAKA
Department of Pathology, Cancer Research Institute, Sapporo Medical College
South 1, West 17, Chuo-ku, Sapporo 060, Japan

SUMMARY

Preneoplastic hepatocytes were isolated by a collagenase perfusion method from F344 male rats fed a diet containing 0.02% 2-acetylaminofluorene for 14 weeks. Most of these hepatocytes were putatively preneoplastic cells. Phalloidin sensitivity of the preneoplastic cells was considerably lower than that of normal hepatocytes. The uptake of phalloidin was much lower in the preneoplastic cells than in normal cells. The low sensitivity of the preneoplastic cells is considered to depend on the low consumption of the toxin by the cells.

Key words: Phalloidin sensitivity, Phalloidin uptake, Preneoplastic hepatocytes

INTRODUCTION

Phalloidin is a bicyclic toxic heptapeptide isolated from a poisonous mushroom called *Amanita phalloides*. It is specifically taken up by hepatocytes. In *in vitro* experiments using isolated hepatocytes, multiple cytoplasmic blebs are induced by the toxin over the cell surface (18, 19).

A lesser sensitivity to phalloidin was observed in isolated hepatocytes obtained from baby or young rat liver (20), regenerating liver (20), liver-tumor promoter treated liver (15, 17) and carcinogen treated liver (13, 14, 21). In addition, AS-30D ascites hepatoma cells were reported to be completely insensitive to the toxin(10). As for the mechanism of the decrease in the sensitivity, It was reported that the uptake of the toxin decreased in hepatocytes treated with various liver-tumor promoters (17) and that ascites hepatoma cells completely did not take up the toxin (4). Furthermore, it was reported that ascites hepatoma cells took up negligible amounts of ^{3}H-demethylphalloin, a cyclopeptide similar to phalloidin in its molecular structure (8).

In the present experiments, we examined whether a decrease in the sensitivity

of preneoplastic cells induced by 2-acetylaminofluorene (2-AAF)* is also due to a decrease in cellular uptake of the toxin.

MATERIALS AND METHODS

Adult male F344 rats (Charles River Co., Atugi) weighing about 180 g were fed a diet (M, Oriental Yeast Co., Tokyo) containing 0.02% 2-AAF (Tokyo Kasei Indust. Co., Tokyo) for 14 weeks and then placed on a basal diet for one week. The animals were maintained at 23±1℃ on a standard 12-h light-dark cycle.

Isolation of preneoplastic hepatocytes was carried out according to the two step liver perfusion method of Seglen (16) with some modifications. The liver was perfused *in situ* through the portal vein for 2.5 min at a flow rate of 55 m*l*/min with Ca^{2+}-free Hanks' balanced salt solution (CF-HBSS) supplemented with 0.5 mM ethylene glycol-bis-(β-amino ethyl ether) N, N'-tetraacetic acid (EGTA), 0.5 μg/m*l* insulin, 0.04 mg/m*l* streptomycin and 40 IU/m*l* penicillin-G. After washout-perfusion, Hanks' solution containing 130 units per m*l* collagenase (Wako Pure Chem. Indust. Co., Osaka) was perfused for 15 min at a flow rate of 25 m*l*/min. The liver was then minced by scissors in the enzyme solution and teased by the aid of 12 m*l*-injection syringe equipped with 20-gauge needle. The cells were further bubbled with a gas mixture ($95\%O_2-5\%CO_2$) at 37℃ for 10 min. The pellet was resuspended in CF-HBSS containing 1.5% bovine serum albumin (Fraction V, Seikagaku Kogyo Co., Tokyo) and centrifuged at 50×g for 1 min. This procedure was repeated 3 times. After the last centrifugation, the pellet was resuspended in Williams' medium E (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% calf serum (GIBCO, Grand Island, N. Y.), 0.5 μg/m*l* insulin, 0.04 mg/m*l* streptomycin and 40 IU/m*l* penicillin-G, and centrifuged. The pellet was resuspended in the culture medium and filtered through 250 μm nylon mesh and further through 40 μm mesh. After viability of the cells was examined by trypan blue exclusion test (viability in this experiments was 85−90%), the cells were inoculated in a 35-mm plastic dish at a concentration of 3×10^5 viable cells/1.5 m*l* of the medium for determination of the sensitivity, and in a 60-mm plastic dish at a concentration of 3×10^6 viable cells/3 m*l* of the medium for estimation of the consumption, respectively. EGTA had no effect on the phalloidin sensitivity.

Culture and measurement of the sensitivity of the cells to phalloidin (Sigma Chemical Co., St. Louis, Mo.) were made according to the method previously described in detail (13, 14). The cells in the 35-mm dishes were fixed in ice-cold

* The abbreviations used are: 2-AAF, 2-acetylaminofluorene; CF-HBSS, Ca^{2+}-free Hanks' balanced salt solution; EGTA, ethylene glycol-bis-(β-amino ethyl ether) N, N'-tetraacetic acid; GGT, γ-glutamyltransferase.

ethanol for 10 min and stained for γ-glutamyltransferase (GGT) activity (11). The sensitivity was expressed as the percentage of the number of the cells that had formed multiple cytoplasmic blebs over the cell surface to the number of the observed cells.

Phalloidin consumption experiments were done according to the method previously described (17).

RESULTS AND DISCUSSION

It was reported that 2-AAF induced numerous preneoplastic nodules in rat livers after a long period of dietary administration (6, 14). Most of the hepatocytes isolated from the liver of rats fed the carcinogen for 14 weeks appeared to be derived from the preneoplastic nodules.

As shown in Table 1,the phalloidin sensitivity of both GGT-negative and GGT-positive cells of 2-AAF treated rats was evidently decreased in each concentration of phalloidin. The sensitivity of GGT-positive cells was much lower than that of GGT-negative cells in both carcinogen treated rats and control rats. The percentage of GGT-positive cells was remarkably increased. These results coincided with those of our previous experiments (13, 14).

In examinations of phalloidin uptake, as shown in Figure 1, the decreases in phalloidin sensitivity started after the first incubation with the toxin and drew a linear regression line. The regression equation of 2-AAF treated hepatocytes was $Y=62.7-0.152X$ ($r=-.989$), whereas, that of control was $Y=65.7-0.389X$ ($r=-.993$). The slope of the regression line was far more gentle in the cells of the

Table 1 *Sensitivity of GGT-negative and GGT-positive cells to phalloidin.*

GGT-negative cells					
Treatment	No. of rats	Phalloidin sensitivity			
		2.5[a]	5	10	20
CONTROL	5	68.7[b]±5.2	87.4±1.7	91.9±1.9	94.1±1.5
2-AAF	4	21.8±3.9	41.6±4.4	61.9±4.3	78.0±2.5
GGT-positive cells					
Treatment	No. of rats	Percentage of GGT(+) cells	Phalloidin sensitivity		
			5[a]	10	20
CONTROL	5	0.2[b]±0.1	34.6±2.2	52.1±4.1	62.3±2.5
2-AAF	4	24.6±6.8	7.8±3.8	15.8±4.2	27.5±3.6

a) Concentration of Phalloidin (μg/ml)
b) Mean±SD

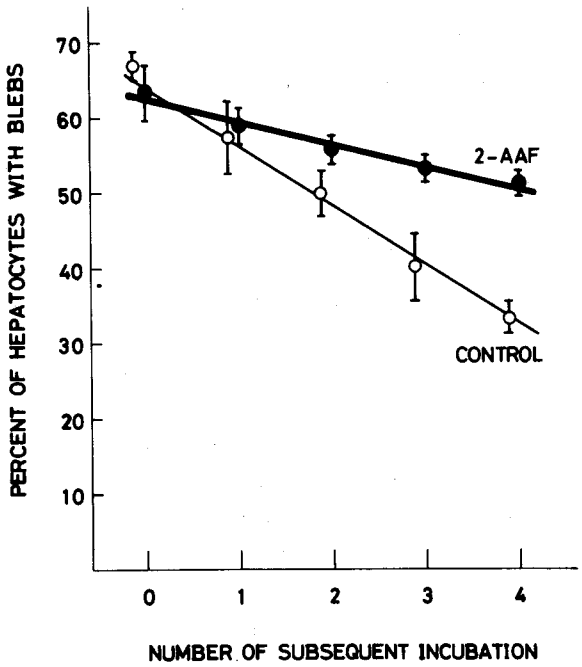

Fig. 1 Consumption of phalloidin of preneoplastic rat hepatocytes induced by 2-AAF.

carcinogen treated rats than in control. It is likely that the decrease in the sensitivity of the preneoplastic cells to the toxin is related to the decrease in the cellular uptake of the toxin. Comparison of ascites hepatoma cells (4), preneoplastic hepatocytes and hepatocytes of liver-tumor promoter treated rats (17) suggests that a close correlation is present between the degree of the sensitivity to and the degree of the uptake of the toxin.

It is well known that the preneoplastic cells acquire a relative resistance to hepatotoxins (3, 5, 7, 12). The mechanisms of the resistance have been suggested to involve a decrease in uptake (3), a decrease in activation (2) and an increase in detoxication of the toxins (1). Phalloidin was reported neither to be metabolized (9) nor to bind to ligandin (22) within hepatocytes.

In this context, the results of the present experiments suggest that the first possibility of the above-mentioned mechanisms, in other words, alteration (s) of the cell membranes is worthy of note as a mechanism of the resistance.

ACKNOWLEDGMENT

I am indebted to Dr. H. Tsukada for his encouragement and critical review of

the manuscript, and to Mrs. M. Kuwano, Miss Y. Takahashi and Mrs. T. Toriyabe for their skillful technical assistance. This work has been supported in part by Grant-in-aid for Cancer Research 59010076 and 60010076 from the Ministory of Education, Science and Culture, Japan.

REFERENCE

1. BOCK, K. W., LILIENBLUM, W., PFEIL, H. and ERIKSSON, L. C.: Cancer Res. **42,** 3747-3752 (1982).
2. CAMERON, R., SWEENEY, G. D., JONES, K., LEE, G. and FARBER, E.: Cancer Res. **36,** 3888-3893 (1976).
3. FARBER, E., PARKER, S. and GRUENSTEIN, M.: Cancer Res. **36,** 3879-3887 (1976).
4. GRUNDMANN, E., PETZINGER, E., FRIMMER, M. and BOSCHEK, C. B.: Naunyn-Schmiedeberg's Arch. Pharmacol. **305,** 253-259 (1978).
5. JUDAH, D. J., LEGG, R. F. and NEAL, G. E.: Nature **265,** 343-345 (1977).
6. KITAGAWA, T. and SUGANO, H.: Cancer Res. **33,** 2993-3001 (1973).
7. LAISHES, B. A., ROBERTS, E. and FARBER, E.: Int. J. Cancer **21,** 186-193 (1978).
8. PETZINGER, E., ZIEGLER, K. and FRIMMER, M.: Naunyn-Schmiedeberg's Arch. Pharmacol. **307,** 275-281 (1979).
9. PUCHINGER, H. and WIELAND Th.: Eur. J. Biochem. **11,** 1-6 (1969).
10. RUFEGER, U. and GRUNDMANN, E.: Naunyn-Schmiedeberg's Arch. Pharmacol. **297,** R17 (1977).
11. RUTENBURG, A. M., KIM, H., FISCHBEIN, J. W., HANKER, J. S., WASSERKRUG, H. L. and SELIGMAN, A. M.: J. Histochem. Cytochem. **17,** 517-526 (1969).
12. SAWADA, N., FURUKAWA, K., GOTOH, M., MOCHIZUKI, Y. and TSUKADA, H.: Gann **72,** 318-321 (1981).
13. SAWADA, N., FURUKAWA, K. and TSUKADA, H.: J. Natl. Cancer Inst. **69,** 683-685 (1982).
14. SAWADA, N. and TSUKADA, H.: Tumor Res. **17,** 39-50 (1982).
15. SAWADA, N. and TSUKADA, H.: Gann **74,** 35-40 (1983).
16. SEGLEN, P. O.: Exp. Cell Res. **82,** 391-398 (1973).
17. TSUKADA, H., SAWADA, N., MITAKA, T. and GOTOH, M.: Carcinogenesis **7,** in press (1986).
18. WEISS, E., STERZ, I., FRIMMER, M. and KROKER, R.: Beitr. Pathol. **150,** 345-356 (1973).
19. WIELAND, Th. and FAULSTICH, H.: CRC Crit. Rev. Biochem. **5,** 185-260 (1978).
20. ZIEGLER, K., PETZINGER, E., GRUNDMANN, E. and FRIMMER, M.: Naunyn-Schmiedeberg's Arch. Pharmacol. **306,** 295-300 (1979).
21. ZIEGLER, K., PETZINGER, E. and FRIMMER, M.: Naunyn-Schmiedeberg's Arch. Pharmacol. **310,** 245-247 (1980).
22. ZIEGLER, K., GRUNDMANN, E., VEIL, L. B. and FRIMMER, M.: Naunyn-Schmiedeberg's Arch. Pharmacol. **317,** 364-367 (1981).