Tumor Res. **23**, 59—64 (1988)

Effects of Cholesteryl Hemisuccinate on the Phalloidin Sensitivity of Normal and Preneoplastic Rat Hepatocytes

Toshihiro MITAKA, Kazunori FURUKAWA and Hideyuki TSUKADA
Department of Pathology, Cancer Research Institute, Sapporo Medical College, Minami-1-jo, Nishi-17-chome, Chuo-ku, Sapporo 060, Japan

SUMMARY

Normal and preneoplastic hepatocytes were isolated from the untreated liver and carcinogen-induced hyperplastic liver nodules of rats, respectively. Phalloidin sensitivity of the 1-hour cultured cells decreased in the presence of cholesteryl hemisuccinate (CH). This was seen more markedly in the order of peneoplastic hepatocytes positive in gamma-glutamyltransferase (GGT), the preneoplastic cells negative in GGT, GGT-positive normal hepatocytes, and GGT-negative normal hepatocytes. Coexistence of phosphatidylcholine and CH inhibited the CH-inducible decrease completely in GGT-negative normal hepatocytes and partially in the normal cells positive in GGT, but failed to recover the decrease in both GGT-negative and positive preneoplastic hepatocytes. These phenomena are suggested to be ascribed to the differences of the cell membrane fluidity among these cells.

Key words: Cholesteryl hemisuccinate, Phosphatidylcholine, Phalloidin sensitivity, Preneoplastic hepatocytes

INTRODUCTION

Sensitivity of hepatocytes to phalloidin, a mushroom toxin, is suggested to depend primarily on the efficiency of the inward transport of the toxin into the cells (4, 6, 7, 10, 16). The transport was reported to be mediated by carrier proteins in the cell membranes(18). The changes in the sensitivity are suggested to be ascribable to the amount of carrier proteins. On the other hand, functional capacity of carrier proteins in the cell membranes is known to be influenced by the fluidity of the cell membranes(5, 13). Phalloidin sensitivity of the cells may also be influenced by

* Abbreviations:
CH, cholesteryl hemisuccinate;
WME, Williams' Medium E;
PC, phosphatidylcholine;
GGT, gamma-glutamyltransferase.

the state of the cell membrane fluidity.

Since an addition of cholesterol or cholesteryl hemisuccinate (CH*) was reported to decrease the cell membrane fluidity(2, 3, 14, 17), the present experiments were made in order to examine whether an addition of CH was effective to alter the sensitivity to phalloidin of normal rat hapatocytes and also of carcinogen-induced preneoplastic rat hepatocytes, the sensitivity of which had already been decreased (6, 11, 12).

MATERIALS AND METHODS

Male inbred F344 Du/Crj rats (Charles River Co., Atsugi) were used. The age of rats was 12-14 weeks and 6 weeks for yielding normal hepatocytes and preneoplastic hepatocytes, respectively. They were fed a diet (MF, Oriental Yeast Co., Tokyo) and received drinking water *ad libitum*. They were maintained at 22±1℃ on a standard 12-hour light-dark cycle. Hyperplastic liver nodules were induced according to Solt-Farber protocol(15), and examinations were made on the 20th week after an intraperitoneal injection with 200 mg/kg body weight diethylnitrosamine (Tokyo Kasei Indust., Tokyo).

Nomal hepatocytes were isolated by the collagenase perfusion method as previously described(12). The isolated cells were finally suspended in the culture medium which consisted of Williams' Medium E (WME) (Flow Labs. Inc., Irvine, UK) supplemented with 1.0% bovine serum albumin (Fraction V, Seikagaku Kogyo Co., Tokyo), 0.04 mg/m*l* streptomycin sulfate and 40 IU/m*l* penicillin G. After viability of the cells was determined by the trypan blue exclusion test (90-95% in these experiments), the cells were centrifuged and resuspended in the culrure medium containing CH (Sigma Chemical Co., St. Louis, USA), phosphatidylcholine (PC) (from egg yolk, Sigma), or containing both compounds in 1.0% ethanol, in a concentration of 3×10^5 viable cells/1.5 m*l*. One and half milliliters of the cell suspension were inoculated into 35-mm plastic dishes, which had been coated with bovine serum by incubation for 2 days before use in WME supplemented with 10% bovine serum (Hyclone Labs. Inc., Logan, USA) and antibiotics. As for the isolation of preneoplastic hepatocytes, hyperplastic nodules were carefully enucleated from the livers after collagenase perfusion. The nodules were minced with scissors in the enzyme solution, and then teased with the aid of a syringe equipped with a 20-gauge needle. The specimens were further dissociated by bubbling with a gas mixture (95% O_2+5% CO_2) at 37℃ for 10 min. After viability of the isolated cells was determined (80-90%), 3×10^5 viable cells in 1.5 m*l* of the culture medium containing CH, PC, or both were inoculated into the serum-coated dishes.

After a one-hour cultivation in a CO_2-incubator at 37℃, the cells were washed

once with the culture medium without CH, PC, or both, and they were incubated in 1.5 m*l* of WME containing phalloidin (Sigma) and antibiotics at 37℃ for 20 min. Evaluation of the phalloidin sensitivity was done according to the previously described method(16) using the cytochemistry of gamma-glutamyltransferase (GGT).

RESULTS AND DISCUSSION

As shown in Table 1, phalloidin sensitivity of normal hepatocytes decreased by addition of CH in a dose-dependent manner, more markedly in the GGT-positive cells than in the negative cells. Since the cell membrane fluidity was reported to decrease by incorporation of cholesterol or CH(2, 3, 14, 17), the decrease in phalloidin sensitivity of the CH-treated cells is suggested to be ascribed to a decrease in the cell membrane fluidity. This assumption might be supported by the observations that 1) a decrease in the cell membrane fluidity of aged rat hepatocytes or of hepatocytes of rats treated with ethynylestradiol resulted in a decrease in bile salt uptake by these cells(1, 8), 2) in aged rat hepatocytes and hepatocytes of rats treated with ethynylestradiol, phalloidin sensitivity and the uptake of the toxin were

Table 1 *Effects of cholesteryl hemisuccinate on phalloidin sensitivity of normal and preneoplastic rat hepatocytes*

Cholesteryl hemisuccinate (μg/m*l*)	Phalloidin sensitivity			
	GGT[a]-negative cells		GGT-positive cells	
	20[b]	10	20	10
Normal				
Control	93.9±1.1[c]	91.9±1.0	68.5±2.2	57.3±1.9
12.5	92.8±1.9	89.3±3.3	68.2±2.9	51.3±3.5[d]
25	89.5±2.3[e]	84.5±3.4[f]	57.9±3.2[e]	38.2±1.4[f]
50	87.0±2.7[e]	77.8±1.5[f]	43.5±0.6[f]	21.8±1.9[f]
Preneoplastic cells				
Control	58.6±2.4	42.5±4.0	10.1±1.3	ND
12.5	40.1±4.3[h]	29.5±2.2[g]	2.0±0.5[h]	ND
25	37.3±3.8[h]	27.8±2.8[g]	2.3±0.6[h]	ND
50	34.2±3.6[h]	24.1±2.3[g]	1.8±0.4[h]	ND

[a] GGT, gamma-glutamyltransferase.
[b] Concentration of phalloidin; μg/m*l*.
[c] Means±SD of 5 examinations.
[d,e,f] Significantly different from control of normal: *d*, $P<0.05$; *e*, $P<0.01$; *f*, $P<0.001$.
[g,h] Significantly different from control of preneoplastic cells: *g*, $P<0.01$; *h*, $P<0.001$.
ND: Not determined.

decreased (7 and our unpublished observations), and 3) identical carrier proteins in the cell membranes were involved in the uptake of bile acids and phalloidin(9, 18, 19). Table 1 also shows that preneoplastic hepatocytes had lower sensitivity to phalloidin than normal hepatocytes, and that the degree of decrease in the sensitivity caused by CH treatment was far more outstanding in GGT-negative preneoplastic cells than in the negative normal cells. Furthermore, CH-treated GGT-positive preneoplastic cells were practically completely resistant to phalloidin, irrespective of the dose of CH used. The response of the cells to CH treatment, as evaluated by the decrease in phalloidin sensitivity, might be greater in the order of GGT-positive preneoplastic cells, GGT-negative preneoplastic cells, GGT-positive normal cells, and GGT-negative normal cells. Although the cell membrane fluidity of these cells and the degree of incorporation of CH into the cells remained to be studied, it is assumed that fluidity is the lowest in GGT-positive preneoplastic cells and the highest in GGT-negative normal hepatocytes.

Effects on phalloidin sensitivity of PC, at the two fold concentration of CH,

Table 2 *Effects of phosphatidylcholine and cholesteryl hemisuccinate on phalloidin sensitivity of normal and preneoplastic rat hepatocytes*

Treatment	Phalloidin sensitivity					
	GGT[a]-negative cells			GGT-positive cells		
	20[b]	10	5	20	10	5
Normal						
Control	ND	92.3±1.5[c]	85.1±2.0	ND	58.6±0.9	36.1±1.4
CH	ND	86.6±2.6[d]	71.2±2.6[e]	ND	38.1±1.1[e]	19.8±0.9[e]
CH+PC	ND	91.9±1.6	81.2±4.2	ND	64.2±3.6[e]	28.9±0.6[e]
PC	ND	93.6±2.3	85.1±3.5	ND	58.6±0.9	35.9±1.8
Preneoplastic cells						
Control	59.2±2.9	41.1±5.9	ND	10.8±2.4	ND	ND
CH	33.8±2.7[g]	22.5±0.4[f]	ND	2.0±0.5[f]	ND	ND
CH+PC	36.7±0.7[g]	22.6±2.8[f]	ND	1.9±0.4[f]	ND	ND
PC	59.2±2.0	40.4±5.7	ND	10.2±1.8	ND	ND

[a] GGT, gamma-glutamyltransferase.

CH: Cholesteryl hemisuccinate, 25 μg/m*l*; PC: Phoshatidylcholine, 50 μg/m*l*; ND: Not determined.

[b] Concentration of phalloidin; μg/m*l*.

[c] Means±SD of 4 examinations.

[d,e] Singnificantly different from control of normal: *d*, $P<0.01$; *e*, $P<0.001$.

[f,g] significantly different from control of preneoplastic cells: *f*, $P<0.01$; *g*, $P<0.001$.

were examined with the results shown in Table 2. The presence of PC in the culture medium without CH did not affect the sensitivity of both normal and preneoplastic hepatocytes. However, coexistence of PC and CH was found to prevent the CH-inducible decrease in the sensitivity almost completely in GGT-negative normal hepatocytes and partially in the positive normal cells. In contrast, the presence of PC with CH did not bring about the recovery of the CH-inducible decrease in the sensitivity of preneoplastic hepatocytes, regardless of their GGT positiveness. Two possibilities to explain this phenomenon may exist; one is that PC sufficient to alter the cholesterol/PC ratio in the cell membrane does not enter the preneoplastic cells, and the other is that the cell membranes of the preneoplastic cells are too rigid to be affected by the dose of PC used in the present experiments, in contrast to the normal cell membranes.

ACKNOWLEDGMENTS

We are indebted to Mrs. M. Kuwano, Miss Y. Takahashi, and Mrs. T. Toriyabe for their skillful technical assistance. This work has been supported in part by a Grant-in-Aid for Cancer Research (62010075) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. BERR, F., SIMON, F. R. and REICHEN, J.: **Am. J. Physiol. 247**, G437-443 (1984).
2. BOROCHOV, H., ABBOTT, R. E., SCHACHTER, D. and SHINITZKY, M.: **Biochemistry 18**, 251-255 (1979).
3. BOROCHOV, H. and SHINISITZKY, M.: **Proc. Natl. Acad. Sci. USA 73**, 4526-4530 (1976).
4. FRIMMER, M., PETZINGER, E., ZIEGLER, K. and VEIL, L. B.: **Naunyn-Schmiedeberg's Arch. Pharmacol. 311**, 91-94 (1980).
5. MILLS, P. R., MEIER, P. J., SMITH, D. J., BALLATORI, N., BOYER, J. L. and GORDON, E. R.: **Hepatology 7**, 61-66 (1987).
6. MITAKA, T.: **Tumor Res. 20**, 61-65 (1985).
7. MITAKA, T. and TSUKADA, H.: **Jpn. J. Cancer Res. (Gann) 78**, 1193-1197 (1987).
8. OHTA, M. and KITANI, K.: **Biomed. Gerontol. 11**, 75-76 (1987).
9. PETZINGER, E.: **Naunyn-Schmiedeberg's Arch. Pharmacol. 316**, 345-349 (1981).
10. PETZINGER, E., ZIEGLER, K. and ERIMMER, M.: **Naunyn-Schmiedeberg's Arch. Pharmacol. 307**, 275-281 (1979).
11. SAWADA, N., FURUKAWA, K. and TSUKADA, H.: **J. Natl. Cancer Inst. 69**, 683-686 (1982).
12. SAWADA, N. and TSUKADA, H.: **Tumor Res. 17**, 39-50 (1982).
13. SCHACHTER, D.: **Hepatology 4**, 140-151 (1984).
14. SHINITZKY, M., SKORNICK, Y. and HARAN-GHERA, N.: **Proc. Natl. Acad. Sci. USA 76**, 5313-5316 (1979).
15. SOLT, D. B. and FARBER, E.: **Nature (Lond.) 263**, 702-703 (1976).

16. TSUKADA, H., SAWADA, N., MITAKA, T. and GOTOH, M.: **Carcinogenesis 7**, 335-337 (1986).
17. WHETTON, A. D., GORDON, L. M. and HOUSLAY, M. D.: **Biochem. J. 210**, 437-449 (1983).
18. WIELAND, T. H., NASSAL, M., KRAMER, W., FRICKER, G., BICKEL, U. and KURZ, G.: **Proc. Natl. Acad. Sci. USA 81**, 5232-5236 (1984).
19. ZIEGLER, K., FRIMMER, M., MOLLER, W. and FASOLD, H.: **Naunyn-Schmiedeberg's Arch. Pharmacol. 319**, 249-253 (1982).