Effect of Alcohol Ingestion on Carcinogenesis by Synthetic Estrogen and Progestin in the Rat Liver

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We examined the effect of alcohol ingestion on hepatocarcinogenesis induced by oral administration of synthetic female hormones, 0.075 mg of ethynylestradiol (EE) and 6.0 mg of norethindrone acetate (NA), every day for 12 months in female Wistar rats. Administration of 10% ethanol in drinking water for 5 days a week every week resulted in the development of hepatocellular carcinoma (HCC) in 38.4% of the hormone-treated rats at 12 months, which is approximately 5 times the incidence of HCC observed following EE and NA treatment alone. The number of hyperplastic nodules was significantly higher than the number observed in the case of EE and NA treatment alone after 4 months of the experimental period. The additional alcohol treatment also increased the value of unoccupied nuclear estrogen receptors (ERn) at months 6 and 8 of the experimental period, and increased the value of total ERn in the rat liver after 6 months of the experimental period. This indicates that additional alcohol treatment may increase occupied ERn (estrogen-ER complex) in the rat liver. A ^{32}P-postlabeling analysis of liver DNA revealed that the maximum number of extra spots consisting of modified nucleotides induced by EE and NA appeared earlier when the additional alcohol treatment was imposed. Consequently, alcohol affects the hepatocarcinogenesis by EE and NA, promoting not only the change in kinetics of ER, but also DNA adduct formation induced by EE and NA in the rat liver.

Key words: Female hormone — Hepatocarcinogenesis — Alcohol — Estrogen receptor — DNA adduct

Recently, an increased incidence of hepatocellular carcinoma (HCC)[2] and other alimentary tract cancers has been found in alcoholics.[1-3] Nakamura and Ohta reported that the incidence of HCC in hepatitis B (HB) sAg-positive alcoholics was extremely high, 65%.[1] Similarly, in a prospective atudy on cirrhotic patients carried out in West Germany, the highest incidence of HCC was observed in patients associated with both an HB virus (HBV) infection and excess alcohol consumption.[2] These epidemiological studies suggest that alcohol may act as a promoter of hepatocarcinogenesis induced by HBV. Furthermore, other epidemiological studies in areas where the prevalence of HBV is low have shown that alcohol consumption is associated with approximately a 4-fold increase in risk for HCC.[3] In experimental studies, alcohol has been reported to act as a cocarcinogen or promoter, activating the metabolism of carcinogens such as dimethylnitrosamine or nitrosopyrrolidine in the microsomes of the liver.[4, 5] Alcohol has also been reported to affect the microsomal metabolism of exogenous and endogenous steroids, and to enhance testosterone degradation and conversion to estrogens.[6] Nevertheless, the role of alcohol in carcinogenesis has not been elucidated.

We had, however, reported that the long-term oral administration of ethynylestradiol (EE) and norethindrone acetate (NA) developed HCC in female Wistar rats, indicating that EE and NA may act as complete carcinogens.[7] Recently, we have revealed through the use of a ^{32}P-postlabeling assay that EE and NA induce DNA adduct formation in the rat liver, as do other genotoxic chemical carcinogens. The present study was undertaken to elucidate the role of alcohol in hepatocarcinogenesis, using our experimental models of EE and NA administration to induce rat HCC, and paying special attention to the kinetics of cytosol and nuclear estrogen receptors (ER), as well as DNA adduct formation in the rat liver.

MATERIALS AND METHODS

Chemicals   EE and NA were purchased from Sigma Chemical Co. Ethanol came from Wako Chemical Co. and was of the purest grade available.

Treatment of animals   Female Wistar JCL rats, aged 4 weeks at the beginning of the experiment, were purchased from Shizuoka Laboratory Animal Center, Shizuoka. The rats were housed four or five per plastic

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[2] The abbreviations used are: HCC, hepatocellular carcinoma; EE, ethynylestradiol; NA, norethindrone acetate; ER, estrogen receptor; ERn, nuclear estrogen receptor; ERc, cytosol estrogen receptor; TED buffer, (10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM DTT, 10% glycerol, pH 7.4); TKED buffer, (10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM DTT, 0.6 M KCl); TLC, thin-layer chromatography.

cage in an air-conditioned room at a temperature of $23 \pm 2°C$ with a daily 12-h light-dark cycle. The animals were divided into 4 experimental groups. In Group 1, 0.075 mg of EE and 6.0 mg of NA dissolved in 0.5 ml of olive oil was administered daily to the rats without anesthesia through a stomach tube for 12 months, and the rats had free access to water from plastic bottles. In Group 2, rats were administered the same doses of EE and NA by the same method as Group 1 for 12 months. Ethanol (10% w/v in the drinking water from plastic bottles) was given to the rats of Group 2 for 5 consecutive days a week *ad libitum*, during the period of synthetic female hormone treatment. In the remaining 2 days each week, pure water without alcohol was given from plastic bottles. In group 3, 0.5 ml of olive oil and 10% ethanol and water was given by the same method as Group 2 for 12 months. In Group 4, 0.5 ml of olive oil was administered to rats by the same method as Group 1, and water was given from plastic bottles *ad libitum* as a control. The amounts of ethanol intake in Groups 2 and 3 were monitored throughout the experimental period. Rats were killed at 2, 4, 6, 8, and 12 months after the initial treatment, and blood samples were drawn through the inferior vena cava during laparotomy under intraperitoneal Nembutal injection. These blood samples were taken for the measurement of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and γ-glutamyl-transpeptidase (GGT). The liver was immediately extirpated, and 2–3 g of the tissue was frozen at $-80°C$; the remnant tissue of the liver was placed in a 10% formalin solution for histological examination. The number of hyperplastic nodules greater than 0.2 mm in diameter in the liver was determined microscopically.

Determination of cytosol and nuclear ER in the liver: tissue extract preparation All procedures were carried out at 4°C unless otherwise specified. The tissue was kept at $-80°C$ for a maximum of 14 days until further handling. One g of the tissue was suspended in ice-cold TED buffer at a tissue:buffer ratio of 1:6 (w/v). After homogenization with a Polytron homogenizer, this sample was centrifuged at $800g$ for 10 min. The supernatant following this centrifugation was used for the determination of ERc. The pellet was resuspended using a Vortex mixer in the same volume of TED as that used for the first wash, and it was repelleted at $800g$ three times more. After the third wash, the crude nuclear pellet was resuspended in a TKED buffer (1:5, w/v) containing 0.6 M KCl. High salt extraction was performed by vortexing every 10 min for 1 h. The extract was centrifuged at $105,000g$ for 1 h, and the supernatant was used for the determination of ERn. Protein determination in cytosol and nuclear extracts were performed by using Lowry's method.

Cytosol and nuclear binding assay For the determination of levels of unoccupied ERc and ERn, duplicate samples

containing 50 μl of cytosol and nuclear extract, respectively, and 25 μl of ^{125}I-estradiol (7.5, 15.0, 30.0, 60.0, or 120.0 fmol/25 μl) were incubated for 16 h at 4°C with or without 2 μM diethylstilbesterol. For the determination of levels of total ERc and ERn, exchange techniques were used.[8, 9] Incubations with ^{125}I-estradiol were performed for 90 min at 30°C for the exchange reaction of the cytosol fraction, and for 180 min at 30°C for the nuclear fraction. Unbound ^{125}I-estradiol was absorbed by adding 500 μl aliquots of Dextran-coated charcoal, followed by incubation for 20 min at 4°C. Following centrifugation, 100 μl aliquots of the supernatant were removed for counting in a γ-counter. Specific binding was calculated as the difference in binding between the parallel incubations with and without diethylstilbesterol. Binding capacity and dissociation constants (K_d) were calculated by means of Scatchard analysis of the averages of these specific bindings.

The ^{32}P-postlabeling analysis of DNA adducts Prior to the ^{32}P-postlabeling assay, DNA was isolated from the frozen tissue (1 g) by a solvent extraction procedure involving digestion of protein and RNA with proteinase K and RNase, respectively, and extractions with phenol, phenol/Sevag (chloroform/isoamyl alcohol 24:1), and Sevag. DNA concentration was estimated spectrophotometrically at 260 nm. The solution was stored at $-80°C$ until use. For the detection of covalent DNA adducts, the ^{32}P-postlabeling assay reported by Gupta et al.[10] and Randerath et al.[11] was used. Briefly, DNA (2 μg) was digested to deoxyribonucleoside 3'-monophosphates, which were ^{32}P-labeled in the presence of $[\gamma-^{32}P]ATP$ and T4 polynucleotide kinase under ATP-deficient conditions. After removal of the normal nucleotides from the labeled digested matter by development with 1.0 M sodium phosphate on PEI-cellulose TLC, the adducts were separated by two-dimensional PEI-cellulose TLC with the following solvents: 3.5 M lithium formate, 7 M urea, pH 3.5 for the first dimension (D1); and 0.8 M lithium chloride, 0.5 M Tris-HCl, 7 M urea, pH 8.0 at right angles to D1 (D2). To remove nonspecific radioactive background, the chromatograms were given a final development with 3.5 M MgCl$_2$. Adducts were located by autoradiography on Kodak XAR-5 film with intensifying screens for 2–3 days at $-80°C$.

Statistical analysis was carried out by using the paired Student's *t* test. The difference between the incidence of HCC in Groups 1 and 2 was analyzed by using the chi-square test. Values of $P < 0.05$ were considered to be significant.

RESULTS

The amount of ethanol intake in Groups 2 and 3 Ethanol intake (g) per body weight of rats (kg) in one day in

Table I. Incidence of Hepatocellular Carcinoma and Hyperplastic Nodules Induced by Ethynylestradiol (EE), Norethindrone Acetate (NA) and Alcohol (AL) in Rat Liver

Group	Duration of administration (months)	No. of affected rats	Hepatocellular carcinoma	Hyperplastic nodules
1	2	5	0	0
(EE+NA)	4	5	0	5 (100%)
	6	5	0	5 (100%)
	8	5	0	5 (100%)
	12	12	1 (8.3%)	12 (100%)
2	2	5	0	0
(EE+NA+Al)	4	4	0	4 (100%)
	6	5	0	5 (100%)
	8	5	0	5 (100%)
	12	21	8[a](38.4%)	21 (100%)
3	2	5	0	0
(Al)	4	5	0	0
	6	5	0	0
	8	5	0	0
	12	10	0	0
4 (Control)		25[b]	0[c]	0[c]

a) Multiple tumors of hepatocellular carcinoma were found in two rats (9.5%). $P<0.05$ vs. the incidence of hepatocellular carcinoma at 12 months in Group 1.
b) The number at 2, 4, 6, 8 and 12 months was five.
c) No hepatocellular carcinomas or hyperplastic nodules were found during the experimental period.

Group 2 was 9.6 ± 2.6 g/kg at the beginning of the experiment, and 11.3 ± 3.7 g/kg at 12 months. In Group 3, the intake was 9.9 ± 2.5 g/kg at the beginning of the experiment, and 11.7 ± 4.1 g/kg at 12 months. These amounts of ethanol intake were not significantly different among the rats in the two groups.

Analysis of blood samples: GOT, GPT, and GGT Serum levels of GOT in Groups 1 and 2 increased similarly and peaked at 138 ± 22 U/liter and 152 ± 27 U/liter, respectively, at 4 months, being significantly higher than those in control Group 4 (101 ± 21 U/liter) at 4 months ($P<0.05$). At 8 and 12 months in Group 1, these levels were not significantly different from those of the control, but in Group 2, the levels were significantly higher, reaching 115 ± 15 U/liter ($P<0.05$) at 8 months and 143 ± 52 U/liter ($P<0.05$) at 12 months, compared to the Group 4 levels of 67 ± 18 U/liter, 75 ± 11 U/liter, respectively. In Group 3, serum levels were significantly higher, at 120 ± 18 U/liter, than those of the control at 8 months only ($P<0.05$). Serum levels of GPT were not significantly different among the four groups except for the levels of Group 2 at 12 months, which were markedly higher, at 103 ± 47 U/liter ($P<0.05$). Serum levels of GGT in Group 2 increased to 2.1 ± 0.7 U/liter, 2.7 ± 0.5 U/liter,

and 2.5 ± 1.0 U/liter at 4, 8 and 12 months, respectively, showing significant differences from the levels of the other three groups ($P<0.05$, 0.01). In Group 1, GGT levels at 4 months only were significantly higher than those of the control. In Group 3, the levels were not significantly different from those of the control.

Gross and histological findings of the liver The results are summarized in Table I. HCC developed in 8 out of 21 rats (38.4%) at 12 months in Group 2, and in only one out of 12 rats (8.3%) at 12 months in Group 1; multiple tumors (two and three HCC) were found in 2 out of 21 rats (9.5%) in Group 2. No hepatocellular carcinomas in Groups 3 and 4 developed during the experimental period. Alcohol ingestion markedly increased the incidence of HCC induced by EE and NA at 12 months following these hormone administrations ($P<0.05$). Figure 1 shows three tumors developing in the liver of a rat of Group 2 treated with EE, NA and alcohol for 12 months. As shown in Fig. 2, these tumors were histologically well-differentiated HCC, composed of neoplastic cells with basophilic cytoplasm, and exhibiting variation in size and shape. The nucleus-cytoplasmic ratios were large, and mitotic figures were occasionally recognized. Similar histological characteristics were ob-
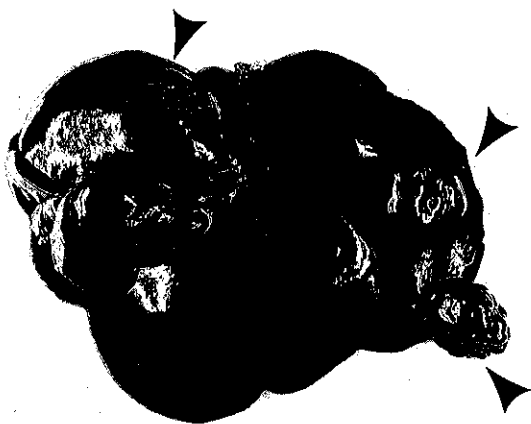
Fig. 1. Photograph of multiple hepatocellular carcinoma induced by EE and NA with additional 10% ethanol administration in drinking water at 12 months. Three tumors are indicated with arrows.
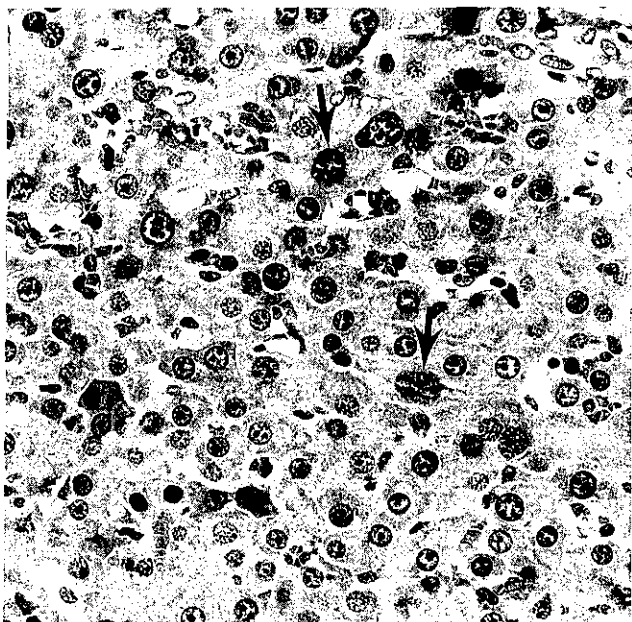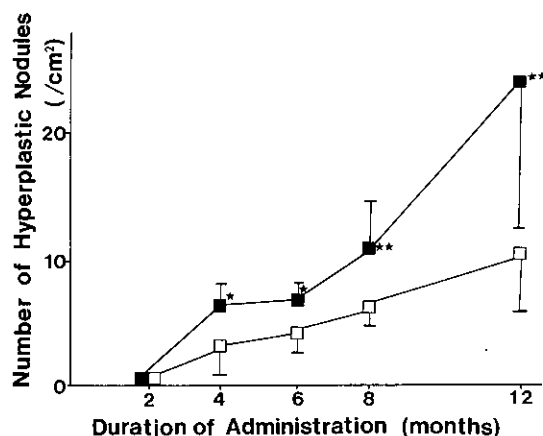

Fig. 2. Microscopic findings of hepatocellular carcinoma in Fig. 1. The tumor is histologically well differentiated, composed of neoplastic cells with basophilic cytoplasm, exhibiting variation in size and shape. The nucleus-cytoplasmic ratios are large. Mitotic figures are indicated with arrows.

Fig. 3. Sequential changes in the number of hyperplastic nodules of Group 1 (EE and NA treatment) and Group 2 (EE and NA with additional administration of 10% ethanol in drinking water). Those in Group 2 were significantly different from those in Group 1 ($P<0.05$; at 4, 6 months, $P<0.01$; at 8, 12 months). Open squares: Group 1. Closed squares: Group 2. $\star P<0.05$, $\star\star$ $P<0.01$.
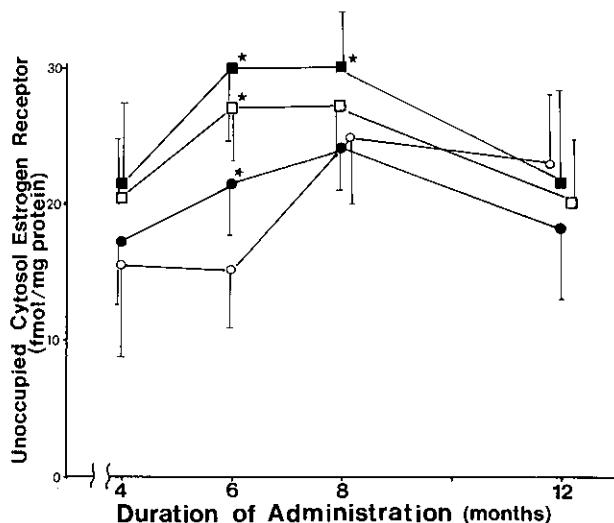

Fig. 4. Sequential changes in the value of unoccupied ERc in the liver determined by ^{125}I-estradiol binding assay during the experimental period. Open squares: Group 1 (EE+NA). Closed squares: Group 2 (EE+NA+Al). Closed circles: Group 3 (Al). Open circles: Group 4 (none). $\star$ $P<0.05$ vs. Group 4.

served in the other HCC developing in Groups 1 and 2. Hyperplastic nodules were found in all affected rats in Groups 1 and 2 from 4 months through to the end of the experimental period. The number of hyperplastic nodules

in Group 2 was $5.5\pm1.9/cm^2$, $6.1\pm1.6/cm^2$, $11.8\pm3.7/cm^2$, and $24.2\pm12.5/cm^2$, at 4, 6, 8, and 12 months, respectively, as shown in Fig. 3. These were significantly higher than those in Group 1 at each point during the
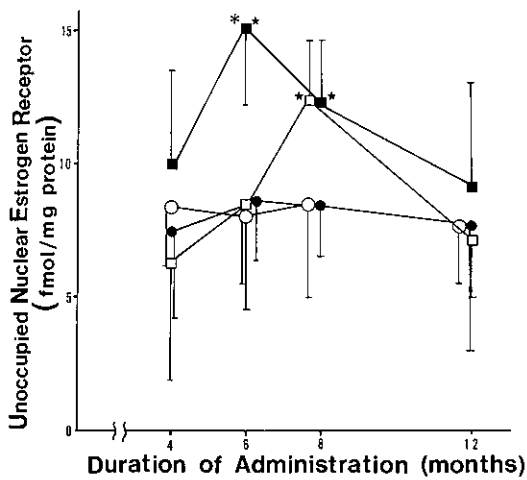
Fig. 5. Sequential changes in the value of unoccupied ERn in the liver determined by ^{125}I-estradiol binding assay during the experimental period. Open squares: Group 1 (EE+NA). Closed squares: Group 2 (EE+NA+Al). Closed circles: Group 3 (Al). Open circles: Group 4 (none). ★P<0.05 vs. Group 4. * P<0.05 vs. Group 1.

Fig. 7. Sequential changes in the value of total ERn in the liver determined by ^{125}I-estradiol binding exchange assay. Open squares: Group 1 (EE+NA). Closed squares: Group 2 (EE+ NA+Al). Closed circles: Group 3 (Al). Open circles: Group 4 (none). ★ P<0.05 vs. Group 4. * P<0.05 vs. Group 1.
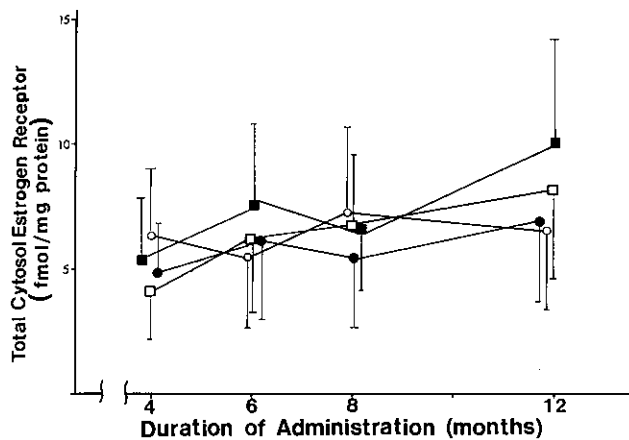

Fig. 6. Sequential changes in the value of total ERc in the liver determined by ^{125}I-estradiol binding exchange assay during the experimental period. Open squares: Group 1 (EE+NA). Closed squares: Group 2 (EE+NA+Al). Closed circles: Group 3 (Al). Open circles: Group 4 (none).

experimental period (P<0.05, at 4 and 6 months, P< 0.01, at 8 and 12 months, respectively). Alcohol ingestion markedly increased the number of hyperplastic nodules induced by EE and NA. No hyperplastic nodules were found in Groups 3 and 4 throughout the experimental period. Specific lesions induced by alcohol, such as necrosis, fibrosis, fatty change, ballooning of liver cells and
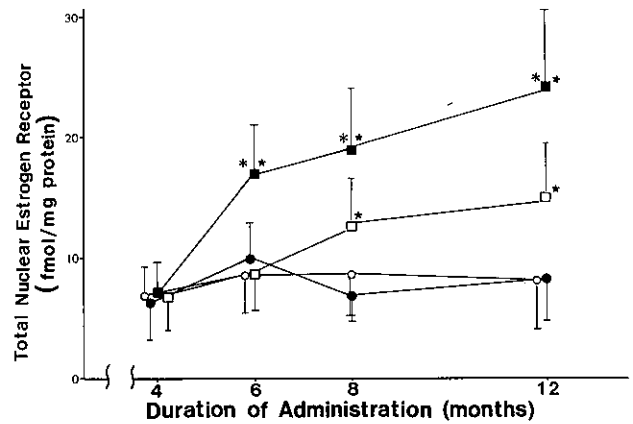
alcoholic hyaline bodies, were not found in the livers of Groups 2 and 3.

Analysis of ERc and ERn in the liver The values of unoccupied ERc in Groups 1 and 3 at 6 months were 26.7±4.9 fmol/mg protein, and 21.4±3.8 fmol/mg protein, respectively, which were significantly higher than those in Group 4 (P<0.05); but at the other points, the unoccupied ERc values in Groups 1 and 3 were not significantly different from those in Group 4. The values in Group 2 at 6 and 8 months were 29.8±5.6 fmol/mg protein and 30.1±4.3 fmol/mg protein, respectively, significantly higher than those in Group 4 (P<0.05), but not significantly different from those in Group 1 (Fig. 4). The values of unoccupied ERn in Group 1 were 12.5± 2.0 fmol/mg protein at 8 months, significantly higher than those in Group 4 (P<0.05); but at the other points they were not significantly different from those in Group 4. However, in Group 2, the values of ERn at 6 and 8 months were 15.2±2.7 fmol/mg protein and 12.3±2.2 fmol/mg protein, respectively, significantly higher than those in Group 4; furthermore, those at 6 months were significantly higher than those in Group 1. In Group 3, values of ERn were not significantly different from those in Group 4 at any point during the experimental period (Fig. 5). The values of total ERc in all of the groups were not different from each other at any point during the experimental period (Fig. 6). However, the values of total ERn in Groups 1 and 2 increased during the experimental period. Those in Group 2 were 16.7±4.1 fmol/ mg protein, 18.7±5.2 fmol/mg protein, and 24.2±6.5 fmol/mg protein at 6, 8, and 12 months, respectively, significantly higher than those in the other groups (P<
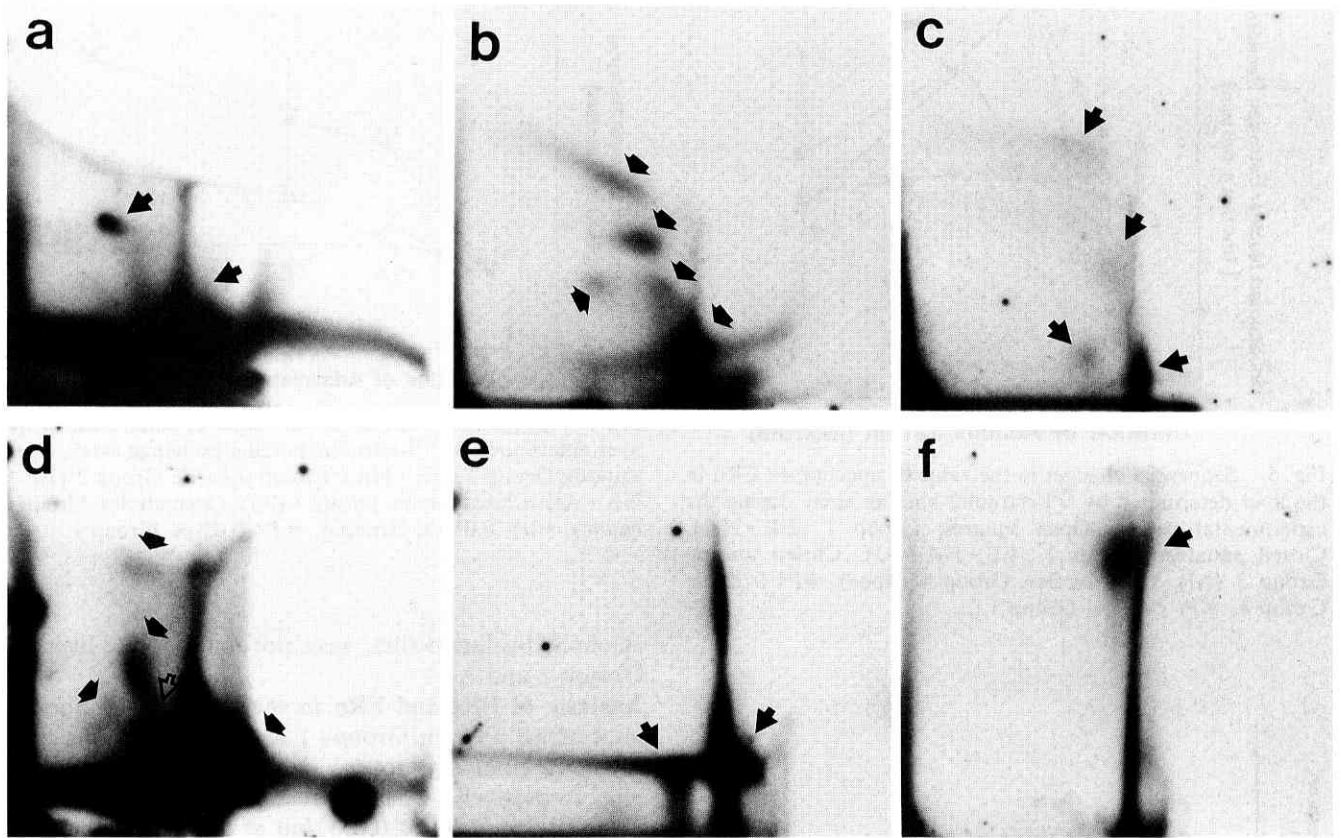
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Fig. 8. Autoradiograms of PEI cellulose TLC of ^{32}P-labeled digests of rat liver DNA obtained from rats in Groups 1 and 2 during the experimental period. The panels of a, b and c are from Group 1 (EE + NA) at 2, 6 and 8 months, respectively. The panels of d, e and f are from Group 2 (EE + NA + Al) at 2, 6 and 8 months, respectively. Extra spots, indicated with arrows, are apparent. The number of extra spots in Group 1 peaked at 6 months (b), but that in Group 2 peaked at 2 months (d). The characteristic localization of extra spots in Group 2 corresponded to that in Group 1.

0.05). Those in Group 1 were 12.7 ± 3.8 fmol/mg protein and 15.0 ± 4.6 fmol/mg protein at 8 and 12 months, significantly higher than those in Group 4 ($P < 0.05$). Those in Group 3 were not significantly different from those in Group 4 (Fig. 7).

The ^{32}P-postlabeling analysis of DNA adducts When the ^{32}P-postlabeling assay was applied to liver DNA obtained from rats in Groups 1 and 2, the results shown in Fig. 8 were obtained. In Group 1, one extra spot and one cluster, indicated with arrows, were detected at 2 months (Fig. 8a). The number of extra spots increased to five at 6 months (Fig. 8b), and then decreased to four at 8 months (Fig. 8c). In Group 2, four extra spots and one cluster, indicated with arrows, were detected as early as 2 months (Fig. 8d). The number of extra spots decreased to two at 6 months (Fig. 8e), and one at 8 months (Fig. 8f). These extra spots were not detected in the liver DNA

of Groups 3 and 4 (data not shown). Extra spots detected in the liver DNA of Group 2 were considered to be adducts formed by EE and NA, not by alcohol, since the characteristic localizations corresponded to those of Group 1.

DISCUSSION

Since Caroli et al.[12] first reported the development of HCC associated with anabolic androgenic steroids in 1953, and Baum et al.[13] reported 7 cases of liver cell adenomas in young women who had received oral contraceptives in 1973, the association between liver tumors and the use of contraceptives and anabolics in women and men, respectively, had drawn attention to hepatocarcinogenesis by sex hormones. We have established an experimental animal model for synthetic estrogen and

progestin-induced HCC.[7] In our model, EE and/or NA induced HCC in 8–74.5% of female Wistar rats, depending on the daily doses and the duration of hormone administration, within 8 to 18 months after the initial oral administration.[14] In our experimental series, tamoxifen, an antiestrogen, inhibited the development of hyperplastic nodules and HCC induced by EE.[15] Further, Ishida[16] revealed that the values of occupied ERn (estrogen-ER complex) increased during the course of the hepatocarcinogenesis by EE and NA; he indicated that the estrogen-ER complex might play an important role in the mechanisms of the hepatocarcinogenesis by EE and NA.

The present study demonstrates that alcohol ingestion increases the incidence of both tumor development and the number of hyperplastic nodules in the course of hepatocarcinogenesis by EE and NA in rats. It is indicated that alcohol ingestion has a promoting effect on the hepatocarcinogenesis by EE and NA. It was also found that the additional alcohol treatment with EE and NA increased the values of unoccupied ERn in the rat liver only at 6 months after the initial administration, but did not increase the values of unoccupied ERc during the experimental period. Recent studies provided evidence that the estrogen receptors are located in cell nuclei, and that cytosolic localization of unoccupied receptors is the result of leaching of the receptor subsequent to cellular disruption.[17, 18] These studies also showed that additional alcohol treatment increased the value of unoccupied ER in liver cells at 6 months in the course of the hepatocarcinogenesis by EE and NA. On the other hand, the present study demonstrates that the additional alcohol treatment with EE and NA increased the values of total ERn in the rat liver after 6 months during the experimental period, but did not increase the total ERc. This data may indicate that additional alcohol ingestion increases the values of occupied ERn (estrogen-ER complex) in the rat liver. Cells containing unoccupied ERn may maintain the capacity to respond to hormonal stimuli, whereas cells containing occupied ERn that are associated with chromatin may be growing under the influence of hormones.[19] Thus, we consider that additional alcohol treatment enhances that capacity of hepatocytes to respond to estrogen by increasing ER in the pre-stage of cancer development in the experimental period, and may promote hepatocyte or probably neoplastic clonal growth under the influence of the estrogenic activity of EE and NA by increasing estrogen-ER complex. Such a mechanism remains to be firmly established.

Until recently, EE and NA had been reported to be non-mutagens. However, the present study revealed by ^{32}P-postlabeling assay that these synthetic female hormones are complete carcinogens, inducing covalent DNA adduct formation in the liver. Furthermore, the present study demonstrated that additional alcohol administration accelerated the formation of DNA adducts induced by EE and NA. Alcohol is negative in both the Ames mutagenicity test and the sister chromatid exchange test, which is the other test widely used for in vitro screening for carcinogens.[20, 21] We did not detect DNA adduct formation in the liver of alcohol-treated rats in this study. Liehr et al.[22] first demonstrated that several synthetic and natural estrogens induced DNA adduct formation in the kidney of the Syrian hamster, and postulated that endogenous DNA adducts generated during microsomal cytochrome P-450-mediated catecholquinone oxidation processes play a role in hormonal carcinogenesis.[23] It has been found that chronic alcohol consumption has an effect on the hepatocarcinogenesis by nitrosamine compounds such as diethylnitrosamine and N-nitrosomorpholine, increasing the induction of preneoplastic foci as a co-carcinogen or promoter in an animal experimental model.[24] Lieber et al.[4, 6, 25] concluded that these effects of alcohol on carcinogenesis were due to an enhancement of the induction of cytochrome P-450 microsomal enzyme systems, especially a microsomal ethanol-oxidizing system, which might activate the metabolism of carcinogens. Therefore we consider that alcohol enhances the capacity for microsomal activation of synthetic female hormones through the cytochrome P-450 enzyme system, and promotes the formation of DNA adducts induced by these compounds.

The effect of alcohol as a co-carcinogen and promoter may be associated with its well-known toxic action, which leads to single liver cell necrosis and regeneration.[26] The present study revealed that the combined administration of synthetic female hormones with alcohol enhanced the toxic action of these compounds, increasing serum levels of GOT and GPT in the late stages of the experimental period, although no evidence of liver injury was observed histologically.

In conclusion, alcohol may have a promoting effect on the hepatic carcinogenesis of EE and NA, not only by promoting the kinetics of ER, but also by promoting DNA adduct formation induced by EE and NA by activating the metabolism of these compounds in the rat liver.

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