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N-Nitroso-2-acetylaminofluorene: A Direct-acting Carcinogen Inducing Hepatocellular Carcinoma in Sprague-Dawley Rats

Yuan-Soon Ho and Jen-Kun Lin[1]

Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei, Taiwan, Republic of China

To compare the hepatotoxicity and hepatocarcinogenicity of N-nitroso-2-acetylaminofluorene (NO-AAF) and its parent compound, 2-acetylaminofluorene (AAF), male Sprague-Dawley rats were given intraperitoneal (i.p.) or subcutaneous (s.c.) injections of AAF or NO-AAF (60 mg/kg body weight/week) for ten months. In the AAF group, morphological changes were produced which involved gross distortions of the liver with multiple nodule formations. The rat livers in the NO-AAF group appeared to be smooth with a blunt-thick superior segment of the lateral lobe. The serum γ-glutamyl transpeptidase activity in both the AAF group and the NO-AAF group was significantly elevated ($P<0.0005$). The present study shows that i.p. and s.c. injections of NO-AAF resulted in a high incidence of well-differentiated hepatocellular carcinomas (HCC) (7/9 and 4/6, respectively), while poorly differentiated HCCs were induced by i.p. or s.c. administration of AAF (6/9 or 2/6, respectively). Subcutaneous lesions consisting of an inflammatory reaction and fibroadenoma formation were observed in the NO-AAF-treated rats, whereas no such skin lesions were detected in the AAF-treated animals. These results suggest that NO-AAF is a new direct-acting carcinogen which may be useful for investigating hepatocarcinogenesis.

Key words: NO-AAF — AAF — Hepatocarcinogenicity — Fibroadenoma — γ-Glutamyl transpeptidase

The well-known hepatocarcinogen AAF[2] is frequently used as a model compound for investigating the mechanism of hepatocarcinogenesis. The nitrosation of AAF in an acidic medium (pH 2.0) to form NO-AAF was described in our previous report.[1] NO-AAF is a direct-acting mutagen for all *Salmonella* tester strains, whereas AAF requires metabolic activation to a proximate mutagen. The mutagenicity of NO-AAF in the Ames test was greater than that of AAF, both in the presence and absence of rat liver microsomal enzymes.[1] In mammalian systems, the ability of NO-AAF to produce DNA single-strand breaks in C3H10T1/2 mouse fibroblasts and in Chinese hamster ovary cells has been demonstrated.[2] Without the metabolic activating system (rat liver S9 fraction), NO-AAF exhibits more direct and stronger damaging effects on DNA than AAF at equal concentrations in both cell lines. A relationship between DNA damage and ouabain-resistance mutation frequency in mammalian cell lines treated with NO-AAF has also been demonstrated.[3] Results from cell cycle-dependent transformation assays reveal that C3H10T1/2 cells in early S phase are more susceptible to induction of malignant transformation, chromosomal aberrations and sister chromatid exchanges by NO-AAF.[4] In our recent study, NO-AAF preferentially formed promutagenic lesions at exons 6–8 in the human p53 genomic DNA *in vitro* (Ho and Lin, in preparation). These findings provide evidence for the genotoxic and tumorigenic potential of NO-AAF. In the present study, we used 2 different routes (i.p. or s.c. injection) of administration to evaluate and compare the carcinogenicity of NO-AAF and its parent compound, AAF. The alterations of several enzymes in the serum and liver cells were also investigated.

MATERIALS AND METHODS

Chemicals AAF, glutathione, NADPH, glutathione reductase, cumene hydroperoxide and CDNB were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium azide and hydrogen peroxide were purchased from Merck Chemical Co. The reagent kits for serum GOT, GPT, and γ-GT assays were from BGH Co. (Taipei, Taiwan). NO-AAF was synthesized by nitrosation of AAF according to the procedure described earlier[1] and was purified by re-crystallization from 0.1% acetic acid in ethanol at −10 to −20°C.

Animals and experimental design Forty-five male Sprague-Dawley rats (five weeks old) were used in this study. They were purchased from the animal center of the National Taiwan University Hospital (Taipei). The

[1] To whom correspondence should be addressed.

[2] Abbreviations: AAF, 2-acetylaminofluorene; HCC, hepatocellular carcinomas; NO-AAF, N-nitroso-2-acetylaminofluorene; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; γ-GT, γ-glutamyl transpeptidase; GST, glutathione S-transferase; GSHPX, glutathione peroxidase; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; CDNB, 1-chloro-2,4-dinitrobenzene.

weights of the rats at the beginning of the study ranged from 70 to 100 g. After one week of acclimatization, the rats were treated with AAF or NO-AAF (60 mg/kg body weight/week) by either i.p. (9 rats in each group) or s.c. (6 rats in each group) injection into the lower abdomen. The experiments were terminated after 10 months, and the rats were killed by ether anesthetization. Blood was collected from the jugular vein, serum was separated for the measurement of GOT, GPT, and γ-GT, and the livers were quickly excised. Part of each liver was frozen in liquid nitrogen immediately and the remainder was fixed and stained with hematoxylin and eosin, and examined under a light microscope. Tumors were classified as well, moderately, or poorly differentiated HCC, according to standard histologic criteria.[5,6]

Serum enzyme assays Serum GOT and GPT activities were determined according to the method of Reitman and Frankel.[7] Serum was added to a buffer solution of α-ketoglutamic acid and aspartic acid or alanine, and the amount/concentration of the resulting oxaloacetic acid or pyruvic acid formed after incubation was measured colorimetrically at 546 nm by reaction with dinitrophenylhydrazine. The activity of serum γ-GT was measured according to the method of Persijin and van der Slik.[8] Serum was incubated with L-γ-glutamyl-3-carboxyl-4-nitroanilide and glycylglycine, and the amount/concentration of the resulting 5-amino-2-nitro-benzoate was determined spectrophotometrically at 405 nm.

Liver GST and GSHPX assay The GST and GSHPX activities were estimated according to the methods of Habig *et al.*[9] and Paglia and Valentine,[10] respectively. A small portion of the rat liver (1 g) was homogenized in 4 volumes (w/v) of 50 m*M* Tris buffer (pH 7.5) containing 0.25 *M* sucrose. Homogenates were centrifuged at 105,000*g*, and the supernatant fractions were used for the estimation of GST (CDNB as the substrate) and GSHPX (H_2O_2 as the substrate) activity. Protein concentration was determined[11] by using a standard commercial kit (Bio-Rad Laboratories Ltd., Watford, UK) with bovine serum albumin as the standard.

Statistical analysis All enzyme assays were performed in duplicate. Data are expressed as mean ± SEM and statistical significance was evaluated by using Student's *t* test. The chi-square test was used to evaluate the statistical significance of the liver cancer induction by either i.p. or s.c. injection of NO-AAF and AAF.

RESULTS

Morphological characteristics of HCC induced by NO-AAF and AAF The mortality, liver/body weight ratio, and activities of the liver and serum enzymes of Sprague-Dawley rats treated with AAF or NO-AAF for ten months are summarized in Table I. Macroscopic observation of the livers in the NO-AAF group revealed different characteristics from those seen in the AAF or control group. The NO-AAF livers were smooth, lacked nodules, and showed a blunt-thick superior segment of the lateral lobe (Fig 1A, arrow). The enlarged rat livers in the AAF group showed gross distortions with multiple tumor nodule formations (Fig. 1B, arrow) and the liver/body weight ratio was elevated when compared with that of either the NO-AAF or control group (Table I).

Microscopic observations of the hepatic lesions induced by NO-AAF and AAF Results of microscopic observation of the hepatic lesions induced by AAF or NO-AAF are summarized in Table II. It was found that NO-AAF, introduced either by i.p. or s.c. injection, produced a high incidence (7/9 or 4/6) of well-differentiated HCCs (Fig. 2, A and C). The carcinoma cells exhibited trabecular

Table I. Mortality, Liver/Body Weight Ratio and Marker Enzymes of Sprague-Dawley Rats Treated with AAF or NO-AAF

Group[a]		Survival rate	Body weight[c] (g)	Liver weight[c] (g)	Liver/body weight (%)	GOT[b] (IU/dl)	GPT[b] (IU/dl)	γ-GT[b] (IU/dl)	GST[b] (nmol/min/mg protein)	GSHPX[b] (nmol/min/mg protein)
AAF	i.p.	8/9	554±23.2	22.3±4.3	4.0	135.2±19.3	91.3±16.2	1.29±0.154	0.112±0.018	0.025±0.0032
	s.c.	5/6	563±18.8	27.1±3.1	4.8	171±18.3	104±17.8	4.6[d]±0.312	0.117±0.028	0.022±0.0021
NO-AAF	i.p.	9/9	560±28.5	16.2±1.3	2.9	93.5±23.2	80.2±19.5	1.71±0.117	0.0904±0.012	0.020±0.0011
	s.c.	6/6	553±20.6	15.5±2.1	2.8	99±21.1	76±20.1	5.26[d]±0.334	0.112±0.011	0.022±0.0014
Control	i.p.	9/9	587±33.3	16.8±0.9	2.8	69.7±16.7	64.6±11.7	0.73±0.105	0.07±0.018	0.016±0.002
	s.c.	5/6	574±21.2	16.5±1.2	2.8	81±19.8	58±22.2	0.84±0.168	0.078±0.014	0.015±0.0009

a) The experimental rats were treated with AAF or NO-AAF (60 mg/kg body weight, weekly) for 10 months either by intraperitoneal (i.p.) or by subcutaneous (s.c.) injection.
b) The serum enzymes (GOT, GPT, and γ-GT) and the hepatic enzymes (GST and GSHPX) were measured as described in the text. Data are expressed as mean ± SEM; all measurements were performed in duplicate.
c) The average weight of rat liver in the AAF group treated either by i.p. or s.c. injection was significantly higher than that of the NO-AAF or control group.
d) Significantly different ($P<0.0005$) from corresponding control values.

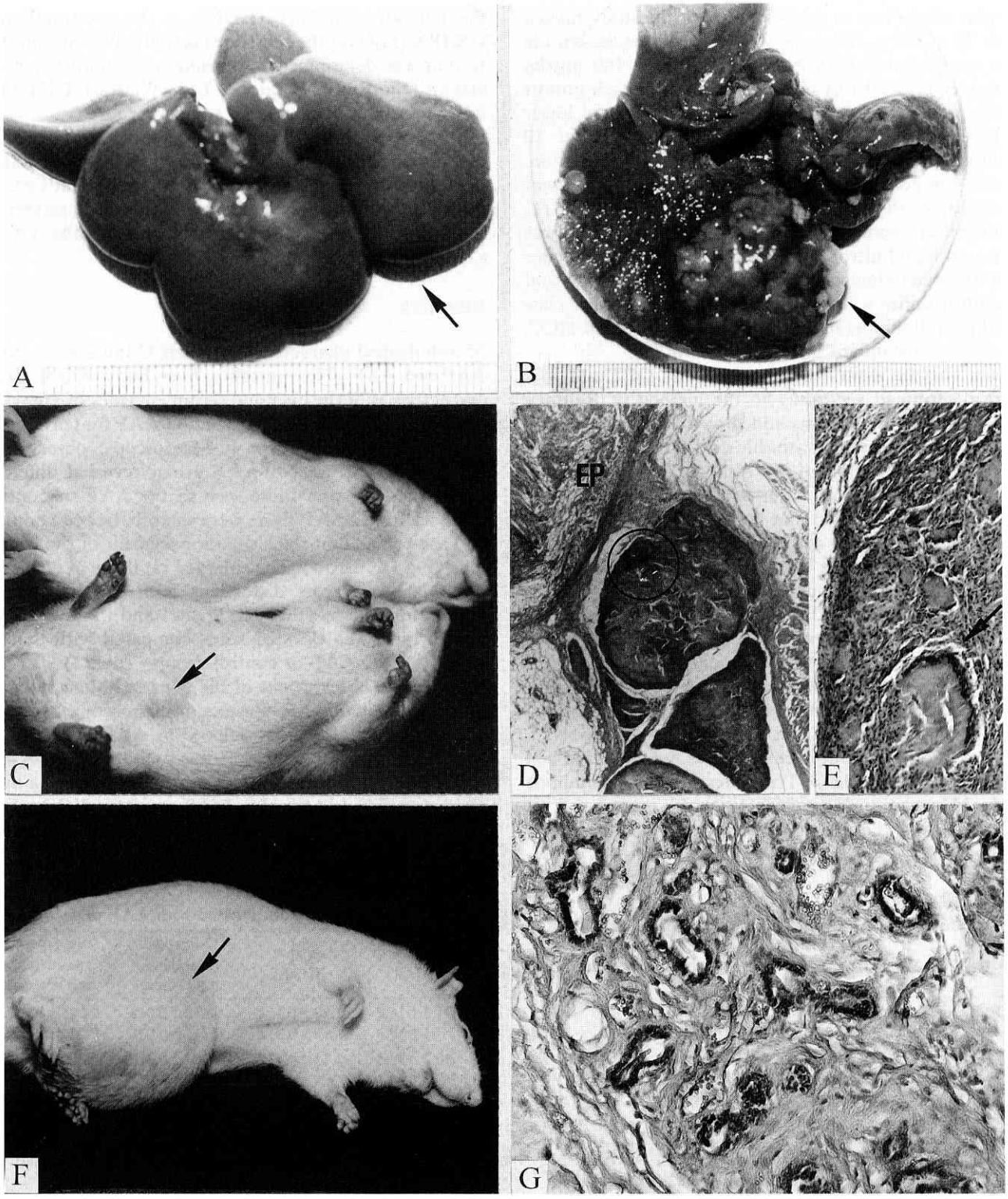

Fig. 1. Comparison of NO-AAF- and AAF-induced hepatocarcinogenesis by macroscopic observation. A: NO-AAF-induced HCC. B: AAF-induced-HCC. C: Subcutaneous lesions (arrow) induced by s.c. injection of NO-AAF. D: Microscopic view of inflammatory lesions under the epidermis (EP). H-E, ×40. E: High magnification of Fig. 1D. H-E, ×100. F: NO-AAF-induced subcutaneous fibroadenoma. G: Microscopic view of Fig. 1F. H-E, ×250.

patterns (plates of hepatocytes of multiple-cell thickness; Fig. 2A, arrowhead) with some fat vacuole depositions (Fig. 2A, arrow). These cells were varied in size and shape, and the nuclei were vesicular with a prominent nucleus (Fig. 2C, arrow). A high incidence of cholangiocarcinoma (4/9) was induced by AAF (Table II). The carcinoma cells formed a glandular structure and abundant fibrous stroma in the cords of tumor tissues (Fig. 2B, arrowhead). Some liver parenchyma cells can also be seen in the transitional (or bridging) area (Fig. 2B, arrow). In addition, poorly differentiated HCCs were found to be induced by i.p. or s.c. administration of AAF (6/9 or 2/6, respectively) (Table II, Fig. 2D). Cancer cells grew in sheets and attempted to form ducts in some parts. The nuclei were varied in size and shape with hyperchromatic patterns. Moderately differentiated HCCs were also induced by both AAF and NO-AAF (Fig. 2, E and F). A giant multinucleated tumor cell (centre) contrasting with the smaller cells (above) was induced by NO-AAF (Fig. 2E, arrow). The hepatotoxic features (including necrosis, cirrhosis, etc.) induced by AAF and NO-AAF are listed in Table II. From these data we can conclude that NO-AAF is more potent than AAF in the production of rat liver inflammatory reactions.

Subcutaneous lesions induced by NO-AAF NO-AAF was a strong irritating agent. Macroscopic and microscopic observation of subcutaneous lesions induced by NO-AAF revealed certain characteristic changes, including an inflammatory reaction and fibroadenoma formations under the epidermis (EP). These characteristics are shown in Table II and Fig. 1, C and D. The inflammatory process did not subside, presumably since the agent responsible for inducing the inflammatory process remained. The granulation tissue also continued to form, and dense connective tissue was found. In addition, lymphocytes and polymorphonuclear cells were found to accumulate in the tissues where necrosis and calcification lesions were also found (Fig. 1E, arrow). One encapsulated fibroadenoma weighing 130 g developed rapidly after subcutaneous injection of NO-AAF (Fig. 1F). Fibrous tissues with pseudoglandular formation were found in the fibroadenoma by microscopic observation (Fig. 1G). There was no difference in the inflammatory lesions found in the rats given either i.p. or s.c. injection of NO-AAF.

Elevations of the levels of serum and liver cytosolic enzymes The serum GOT, GPT and γ-GT activities were estimated in order to evaluate the level of hepatic damage (Table I). γ-GT activity in the rats treated with AAF or NO-AAF by s.c. administration was significantly elevated when compared to the control rats ($P<0.0005$). The serum GOT, GTP, GSHPX, and GST levels in

Table II. Incidence of Liver and Subcutaneous Lesions in Sprague-Dawley Rats Treated with AAF or NO-AAF

Group	AAF		NO-AAF		Control	
Injection[a)]	i.p.	s.c.	i.p.	s.c.	i.p.	s.c.
Survival rate	8/9	5/6	9/9	6/6	9/9	5/6
HCC						
Well differentiated	0	1	7	4	0	0
Moderately differentiated	1	1	1	1	0	0
Poorly differentiated	6	2	0	0	0	0
Cholangiocarcinoma	4	0	0	0	0	0
Metastasis	1	0	0	0	0	0
Hepatotoxicity						
Necrosis	4	3	6	4	1	1
Cirrhosis	2	1	4	2	0	1
Cholestasis	3	1	3	1	1	2
Infarct	2	0	3	1	0	0
Ballooning degeneration	1	0	3	1	0	0
Acidophilic body	1	2	1	3	2	2
Hyperplasia						
Kupffer cell	3	1	2	4	0	0
Oval cell	2	3	7	4	0	1
Subcutaneous lesion						
Inflammatory reaction	0	0	2	5	0	0
Fibroadenoma[b)]	0	0	0	1	0	0

a) The experimental rats were treated with AAF or NO-AAF (60 mg/kg, weekly) for up to 10 months either by intraperitoneal (i.p.) or subcutaneous (s.c.) injection.
b) Fibroadenoma = 130 g.

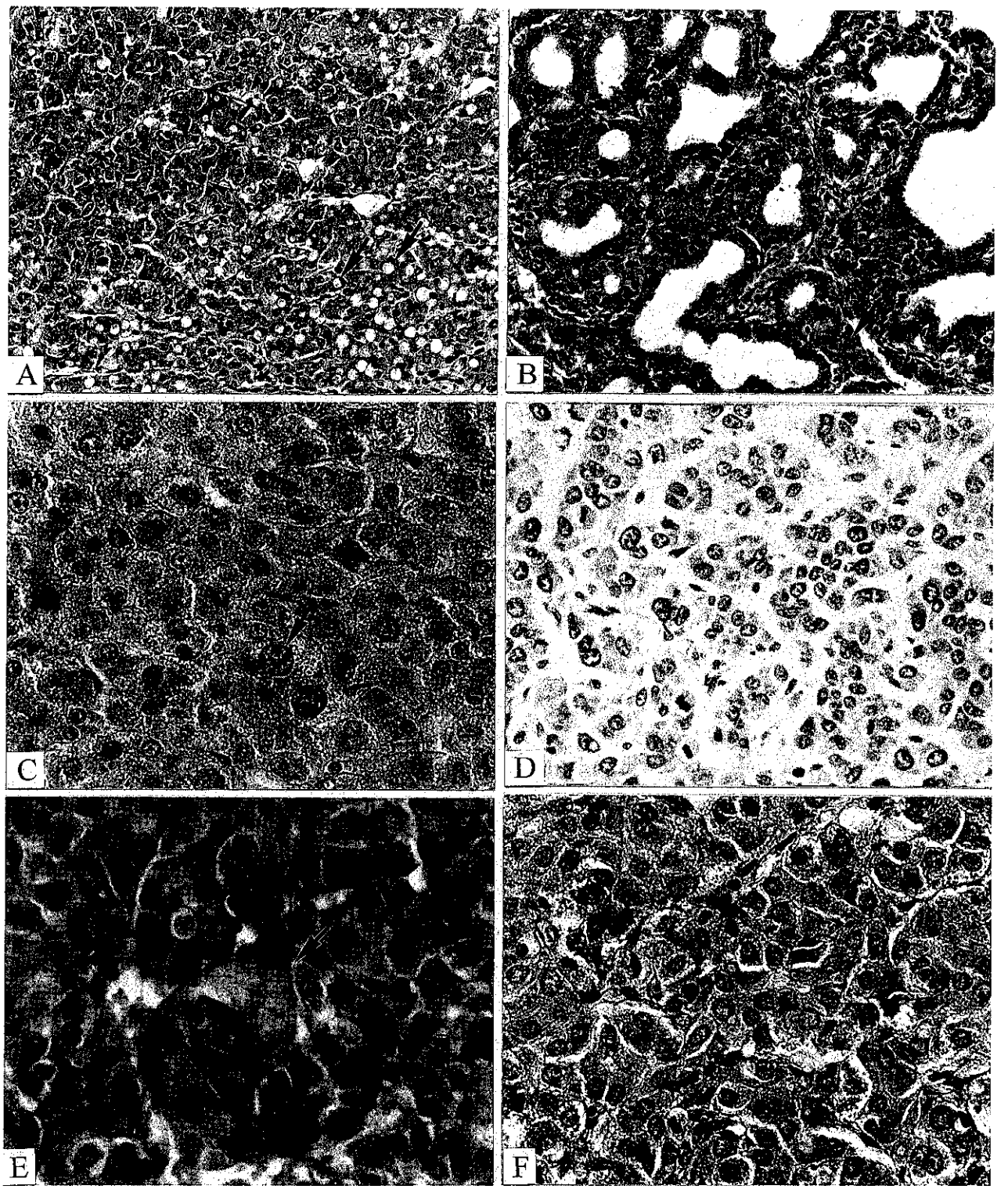

Fig. 2. Microscopic view of different types of HCC induced by NO-AAF or AAF. A, C: Well-differentiated HCC induced by NO-AAF (A: H-E, ×100; C: H-E, ×400). B: Multicystic cholangiocarcinoma induced by AAF. H-E, ×100. D: Poorly differentiated HCC induced by AAF. H-E, ×250. E, F: Moderately differentiated HCC induced by NO-AAF (E) and AAF (F). H-E, ×400.

hepatocytes resulting from AAF or NO-AAF administration were in the following order: AAF > NO-AAF > control (Table I).

DISCUSSION

When the chemical carcinogen AAF binds to DNA *in vivo*, two major DNA adducts, namely N-(deoxyguanosine-8-yl)-AAF and N-(deoxyguanosine-8-yl)-AF, are formed. These products trigger important structural changes in the DNA secondary structure.[12] The correlation between the carcinogen-induced conformational changes of DNA structure and the corresponding mutation specificity is well documented.[13] More than 90% of the mutations induced by N-(deoxyguanosine-8-yl)-AAF adducts are frame-shift mutations, while the N-(deoxyguanosine-8-yl)-AF adducts induce primarily base substitution mutations (85%), mainly of the G to T transversion type. In our recent study,[14] the four nucleosides, namely adenosine, guanosine, cytidine and thymidine, were used as substrates to test the deamination ability of NO-AAF. Xanthine, hypoxanthine, uracil and some unusual bases such as 8-OH-dG and 8-OH-dA were found. Furthermore, our recent studies revealed that NO-AAF and Aco-AAF, which is the active form of AAF, are highly electrophilic and can react extensively with an isolated plasmid (php53B) which contains the human p53 gene to form corresponding adducts *in vitro*. DNA polymerase footprinting analysis[15] was employed for identifying the substituted bases in the DNA carcinogen adducts. The following results were obtained: G and A are the two hot bases that are attacked, and there are two "hot regions" through exons 6–7 and 7–8 of the p53 gene that are more easily attacked by the hepatocarcinogen NO-AAF. In another of our studies (unpublished data), the p53 gene mutations in rat HCCs induced by NO-AAF and AAF were detected by PCR-SSCP and direct sequencing. Noteworthy features of the mutation spectrum of the p53 gene in HCCs were as follows: (a) the mutation frequencies of the p53 gene in rat HCCs induced by NO-AAF and AAF were 19.23% and 31.1%, respectively, and (b) the incidence and sites of the p53 gene mutations were highly associated with the degree of differentiation of cancer cells. In poorly differentiated HCCs, p53 mutations were clustered on exon 7–8, whereas in well-differentiated HCCs mutations were found on exon 5. Therefore, we hypothesize that formation of NO-AAF-DNA and AAF-DNA adducts in the "hot regions" largely destroys the tumor-suppressing activity of the p53 gene, thus providing the molecular basis for the initiation stage of hepatocarcinogenesis.

An excellent study by Oda *et al.* with a human hepatocarcinogenesis model has led to the hypothesis that the different sites and types of mutations in the p53 gene are significantly associated with cancer cell differentiation.[16] They found that in poorly differentiated HCCs, p53 mutations were frequent (54%) and clustered on domains IV and V (corresponding to exons 7 and 8). In well or moderately differentiated HCCs, the mutations were less frequent (21%) and equally distributed on domains II to V (corresponding to exons 5 to 8). HCC may develop in a stepwise manner, through the phases of initiation, promotion and progression. The DNA-binding of hepatocarcinogens, such as NO-AAF and AAF, may play an important role in the initiation step of HCC development. It is likely that different sites of DNA adducts attacked by hepatocarcinogens may lead to the initiation of different types of HCCs. More experimental results on the molecular structures of these DNA-adducts are needed to prove or disprove this contention.

NO-AAF was a strong irritating agent, which caused subcutaneous inflammatory reactions and more potent hepatic parenchyma cell necrosis than AAF (Table I). These results imply that NO-AAF has potent hepatotoxicity and causes extensive elevation of the cytosolic enzymes in liver cells. Liver GSHPX and GST are involved in cellular detoxification,[17] and because these enzymes are sensitive to environmental pollutants and drugs,[18, 19] they have been used as indicators of the toxic effects of ingestion or inhalation of various xenobiotics. It has been suggested that γ-GT could be a sensitive oncofetal marker of hepatocarcinogenesis.[20, 21] The elevation of γ-GT levels in the liver carcinomas induced by AAF[22, 23] and its active metabolite, N-hydroxy-2-acetylaminofluorene,[24] suggest the involvement of γ-GT in hepatocarcinogenesis.

Our results in this study indicate that the major type of neoplasm induced by NO-AAF was well-differentiated HCC, whereas mixed cell carcinomas, including poorly differentiated HCC and pseudo-glandular cholangiocarcinoma, were induced by AAF. This confirmed previous findings[25, 26] that administration of 0.05% AAF to rats induced cholangiocarcinoma, and cholangiofibrosis was a common feature. Further experiments on the carcinogenicity of NO-AAF with or without active metabolites of AFF might be interesting. The results obtained here indicate that NO-AAF is a new model compound for investigating hepatocarcinogenesis and may help us to understand the molecular etiology of different types of hepatocellular carcinoma.

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