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Differential Effects of Nongenotoxic and Genotoxic Carcinogen on Cell Proliferation and c-Jun Expression in the Rat Liver Initiated with Diethylnitrosamine

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ABSTRACT : **Cell proliferation and c-Jun expression pattern in liver exposed by nongenotoxic carcinogens phenobarbital (PB) and clofibrate, and genotoxic carcinogen 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) were investigated to see whether differential effects of genotoxic and non-genotoxic carcinogens on the development of neoplastic foci may be related to differential effect on cell proliferation. Male F344 rats were initially given a single intraperitioneal injection of diethylnitrosamine (200 mg/kg body weight), and 2 weeks later, animals were fed diets containing 0.03% IQ or 0.5% CF or 0.05% PB or basal diet as a control for 6 weeks. All rats were subjected to the two-thirds partial hepatectomy (PH) at week 3. Sequential sacrifice of rats was performed until 8 weeks. Cell proliferation was examined by immunohistochemical staining of bromodeoxyuridine and c-Jun expression was determined by northern blotting. The increase of cell proliferation rate after PH was significant in the rats fed 0.05% IQ and continued until 8 weeks, while the increase was not significant in the rats fed phenobarbital and clofibrate compared to that in the rats fed control diet. mRNA level of c-Jun in the liver treated with IQ was about 7 fold higher than that of control and peak at 5 hours after PH. In the liver treated with CF, mRNA level of c-Jun was 3-4 fold higher than that of control and the highest level of mRNA of c-Jun was seen at 24 hours after PH. These results show that differential effects of genotoxic and non-genotoxic carcinogens on the development of neoplastic foci may be related to differential effect on cell proliferation pattern.**

Keywords: genotoxic, nongenotoxic carcinogen, cell proliferation, C-Jun expression

INTRODUCTION

Hepatic peroxisome proliferator clofibrate (CF) and phenobarbital (PB) have been found to be non-genotoxic hepatocarcinogens in rodents (Denda *et al.*, 1989; Huber *et al.*, 1996). 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ), a potent genotoxic carcinogen isolated from pyrolysates of amino acids and protein, has also been found to cause liver cancer in rodents and non-human primates (Ohgaki *et al.*, 1984;Takayama *et al.*, 1984). All three compounds have tumor promoting activities and IQ also has tumor initiating activity (Huber *et al.*, 1996; Hasegawa *et al.*, 1996).

It has been found that the incidence of preneoplastic foci such as γ-glutamyltranspeptidase (γ-GT) and glutathione S-transferase placental form (GST-P) after medium-term exposure of hepatocarcinogens using the two stage hepatocarcinogenesis models is correlated to their ability to induce tumors after the long-term exposure (Ito *et al.*, 1997). However, the expression of these markers is dependent upon the type and nature of tumor promoters. For example, phenobarbital enhanced γ-GT in the neoplastic foci initiated by diethylnitrosamine (DEN) whereas peroxisome proliferator CF did not, even though both chemicals have tumor promoting activity (Tatematsu *et al.*, 1988; Rao *et al.*, 1986). Previous our study showed that the expression of GST-P mRNA and development of GST-P positive foci were increased by IQ, but were decreased by clofibrate (Kim *et al.* 1998).

On the other hand, cell proliferation and expression of growth related genes have been known to be critical factors in the hepatocarcinogenesis. Chemicals which have tumor promoting activities give selective growth

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advantage for initiated cells to promote development of neoplastic foci in the multi-stage hepatocarcinogenesis. Therefore, it is possible that differential effects of genotoxic and non-genotoxic carcinogens on the development of neoplastic foci may be related to differential effect on cell proliferation in the initiated liver.

In the present study, we determined differential effects of non-genotoxic carcinogens PB and CF with genotoxic carcinogen IQ on the cell proliferation and c-Jun expression in the promotion stage of liver lesions initiated by DEN.

MATERIALS AND METHODS

Chemicals

DEN and clofibrate (CF) were obtained from Tokyo Chemical Industry Co (Tokyo, Japan). Synthetic IQ was obtained from Nard Inst.(Osaka, Japan). PB was obtained from Iwaki Pharmaceutical Co. (Tokyo, Japan).

Animals and Treatments.

Five week old F344 rats (Charles River Japan Inc., Japan), housed five per plastic cage on wood chips for bedding, were maintained under constant conditions (12 h light/dark cycle, 50% humidity at 23±1°C) with Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. After 2 week of acclimation, the rats were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in 0.9% NaCl. Two weeks later, rats were placed on diets containing 0.03% IQ, 0.5% CF, 0.05% PB, or basal diets for 6 weeks. All animals were subjected to the two-thirds partial hepatectomy (PH) at week 3 to induce proliferation of hepatocytes as described in the previous study (Kim *et al.*, 1998). Rats were anesthetized under ether and killed. Each liver lobe was weighed separately, and then cut into 2~3 mm thick slices with a razor blade for immunostaining.

Immunohistochemical staining for BrdU

Cell proliferation was assessed by immunohistochemical staining of bromodeoxyuridine (BrdU) incorporated into liver. Two rats per experimental group at the each time point were given a single i.p. injection of BrdU (120 mg/kg b.w.) for 1 hour before sacrifice. The liver sections were fixed in ice-cold acetone, and routinely passed through xylene and a graded alcohol series, and then treated with 4 N HCl for 20 min. The liver section were then digested with 0.01% actinase E for 1 min after rinsing with boric acid-borated buffer (pH 7.6) for 5 min. After incubation with an 1:30 dilution of monoclonal mouse antibody to BrdU, liver sections were stained by the immunogold-silver staining method using Auro Probe LM and then Inten SEM kits (Jassen Biotech N.V., Olen, Belgium). Incorporation of BrdU into the nuclei was immunohistochemically visualized using the avidin-biotin-peroxidase complex with a monoclonal antibody against BrdU as described by Tatematsu *et al.* (1987). Sections were then counter-stained with hematoxylin for microscope examination. Cells labeled with BrdU were considered positive for proliferation. The results were expressed as a percentage of total cells.

RNA extraction and northern blotting

Total RNA was extracted by the method of Chomczynski and Sacchi (1987) using product specifications (Stratagene, La Jolla, CA). Liver tissues were washed with ice-cold PBS buffer, and then lysed with denaturating solution containing guanidine isothiocyanate and 2-mercaptoethanol followed by phenol-chloroform extraction.

For the northern blot, 10 μg of each RNA sample was dissolved in 10 μl denaturing buffer [5 μl formaldehyde, 2 μl formimide, 1 μl 10x MOPS buffer (MOPS; 0.1 M Sodium acetate, 10 (M EDTA, pH 7.0)] plus 1 μl ethidium bromide. The samples were electrophoresed through a 1.25% agarose gel. Fractionated RNA was transferred to a nylon membrane, and the membrane was dried and baked. A fragment of rat c-Jun cDNA was used for hybridization. The ^{32}P-labeled probe for c-Jun was generated with a random primed labeling kit (Ambion, Austin, TX, USA). The labeled c-Jun probe (1×10^6 dpm/ml) was denatured, and then added directly to prehybridization buffer (QuickHyb®, Ambion, Austin, TX, USA). The membrane was hybridized for 30 min at 68°C and washed three times in 2XSSC containing 0.5% SDS followed by one washing in 0.1XSSC with 0.1% SDS for 30 min. The final wash was done in 0.1XSSC with 0.1% SDS for 1 h at 56°C. The membrane was dried and then autoradiographed using Kodak MR film at −80°C overnight. Autoradiographs of northern blots were quantitated by using a laser densitometer. The mRNA level was expressed by the ratio of c-Jun level/β-actin as an internal positive.

Statistics

Statistical comparisons of data were performed using the one way analysis of variance followed by Scheffe's test as a post hoc test of significance.

RESULTS

First, we compared the rate of cell proliferation by the genotoxic carcinogen IQ, and by the clofibrate and phenobarbital. As seen in Fig. 1, the rate of cell proliferation was significantly increased by partial hepatotectomy (PH) in the all of the rats (50-70 fold increase). The highest rate of cell proliferation was seen 24 after PH. The increase of cell proliferation rate after PH was significant in the rats fed 0.05% IQ, while the increase was not significant in the rats fed phenobarbital and clofibrate compared to that in the rats fed control diet. The higher rate of cell proliferation was continued until 8 weeks in the IQ treatment. No significant regional difference (Left and right lateral and median) of the cell proliferation was found. Slightly higher rate of cell proliferation was seen in the right lateral cranial compared to other regions (Table 1).

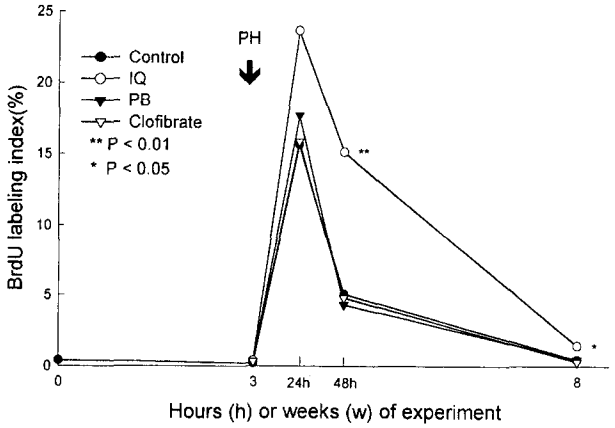

Fig. 1. Sequential changes in BrdU labeling indices of hepatocytes in rats initiated with diethylnitrosoamine followed by IQ, phenobarbital and clofibrate. Results are expressed as % of labeled cells. All animals were treated with a single i.p. injection of DEN 200 mg/kg body weight, and then given partial hepatectomy (PH) at the third week, and treated for 8 weeks with each chemical. Labeling indicies were detected at 0, 2, 3, and 8 weeks, and 24 and 48 hours after PH. IQ; 2-amino-3-methylimidazo (4,5-f)quinoline, PB; phenobarbital * and **; Significantly different from control group values at P<0.05 or 0.01, respectively.

In agreement with the cell proliferation pattern, the expression of c-Jun mRNA after PH in the liver was significantly increased. However, mRNA level of c-Jun in the liver treated with IQ was peak at 5 hours after PH. At 24 hours after PH, mRNA level in the liver treated with IQ was only two times higher than that in the control liver. At 48 hours after PH, the level was only 50% higher than that of control. In the liver treated with clofibrate, mRNA level of c-Jun was three to four folds higher than that of control until 24 hours after PH, and about 2 fold at 48 hours after PH. The highest level of mRNA of c-Jun was seen at 24 hours after PH (Fig. 2 and Table 2).

DISCUSSION

In the present study, we compared the effect of three hepatic carcinogens on the cell proliferation and growth regulatory oncogene expression (c-Jun) at the liver in-

Table 1. BrdU labelling index (%) in rat liver initiated with DEN followed by IQ, PB and CF

Agents	0	3 W	3 W+24 h	3 W+48 h	8 W
Control					
Total	0.41±0.01	0.21±0.04	15.65±2.71	5.08±0.05	0.45±0.06
A+B	0.43±0.03	0.21±0.07			
C	0.44±0.01	0.20±0.04	14.57±5.13	5.02±0.18	0.45±0.04
C'	0.30±0.01	0.25±0.02	16.95±2.34	5.16±0.11	0.45±0.06
0.03% IQ					
Total		0.40±0.03*	23.70±0.16	15.14±0.62**	1.45±0.17*
A+B		0.43±0.06			
C		0.32±0.01	25.19±2.96	16.13±0.63	1.59±0.11
C'		0.38±0.05	21.22±5.85	13.71±0.98	1.06±0.11

All animals were treated with a single i.p. injection of DEN 200 mg/kg body weight , and then given PH at the third week, and treated for 8 weeks with each chemical.

*,** : Significantly different from the value of control group at P<0.05 and 0.01.

IQ; 2-amino-3-methylimidazo(4,5-f)quinoline, PB; phenobarbital, CF; clofibrate

A; left lateral, B; median, C; right cranial part, C'; right lateral caudate part

Table 2. Level of c-Jun mRNA in the rat liver initiated DEN followed by IQ or CF

Time	IQ	CF
0	0.61±0.66	0.61±0.66
2 weeks	0.18±0.15	1.18±0.15
3 weeks	4.28±1.95	2.14±1.08
3 week+5h[a]	5.00±0.60	2.06±0.27
3 weeks+24h	1.38±0.75	3.10±1.24
3 weeks+48h	0.91±0.22	1.17±0.53
8 weeks	0.00±0.00	0.03±0.03

All animals were treated with a single i.p. injection of DEN 200 mg/kg body weight, and then given partial hepatectomy ([a]) at the third week, and treated for 8 weeks with each chemicals. Autoradiographs of northern blots were quantitated by scanning densitometry, using a laser densitometer. The mRNA level was expressed by the ratio of c-Jun level / β-actin as internal positive.
Data were expressed as mean±SD (n=5 rats)
IQ; 2-amino-3-methylimidazo(4,5-f)quinoline

itiated with DEN followed by genotoxic carcinogen IQ, and/or nongenotoxic carcinogens, clofibrate and phenobarbital. The increase of cell proliferation rate after PH was significant in the rats fed 0.05% IQ and continued untill 8 weeks, while the increase was not significant in the rats fed phenobarbital and clofibrate compared to that in the rats fed control diet. mRNA level of c-Jun in the liver treated with IQ was about 7 fold higher than that of control and peak at 5 hours after PH. In the liver treated with clofibrate, mRNA level of c-Jun was three to four folds higher than that of control and the highest level of mRNA of c-Jun was seen at 24 hours after PH.

All three chemicals have been found to have tumor promoting and carcinogenic activities (Hurber *et al.*, 1996; Hosogkawa *et al.*, 1989). However, our previous and other data have shown that preneoplastic liver lesions (identified by GST-P) was not induced by the clofibrate (Kim *et al.*, 1998; Hasegawa *et al.*, 1994), while the genotoxic carcinogen IQ enhanced the development of the DEN-initiated GST-P positive preneoplastic lesions. It has been thought that cell proliferation is a critical factor to expand initiated cells in the two stage hepatocarcinogenesis. Therefore, we determined whether the differential effect of nongenotoxic carcinogen CF and genotoxic carcinogen IQ on the development of the preneplastic foci is related to differential effect of two compounds on the cell proliferation. The differential effect of non-genotoxic and genotoxic carcinogen on the cell proliferation in this study is agreed with the previous

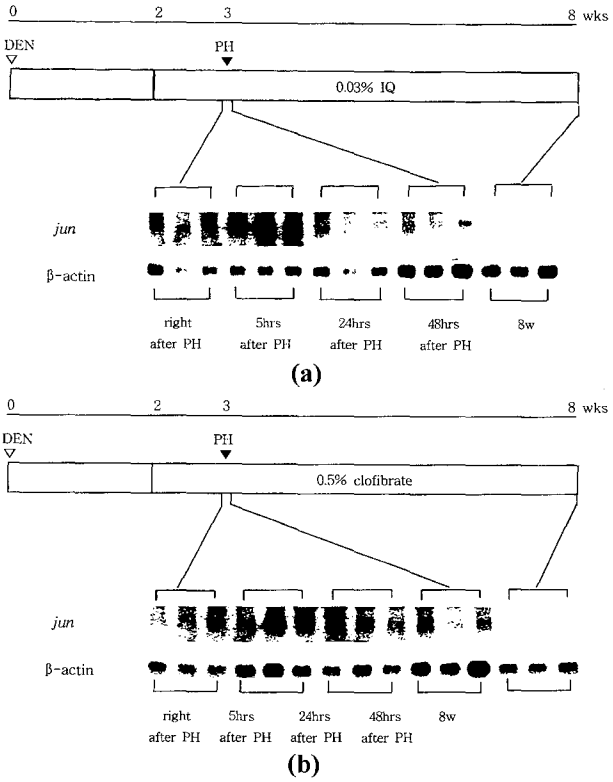

Fig. 2. Sequential changes of c-Jun expression in the rats treated rats initiated with DEN followed by IQ (a) and CF (b). All animals were treated with a single i.p. injection of DEN 200 mg/kg body weight, and then given partial hepatectomy (PH) at the third week, and treated for 8 weeks with each chemical. c-Jun exprssion were detected at 0, 2, 3 and 8 weeks, and 5, 24, and 48 hours after PH. IQ; 2-amino-3-methylimidazo(4,5-f)quinoline.

data showing differential effects of two chemicals on the development of preneoplastic foci, and suggests that cell proliferation maybe partially contributes to increase the development neoplastic foci. However, It is not clear that increase of cell proliferation in the liver treated with IQ result from increase of only initiated cell proliferation. The expression of c-Jun was somewhat different with the cell proliferation pattern. The expression of c-Jun was peak soon after PH to 5 hours after PH in the liver treated with IQ and the increase was about seven folds compared to that of control liver. However, c-Jun expression in the liver treated with clofibrate was peak at 24 hours after PH and the increase was three to four folds. IQ treatment injection resulted in two times higher expression of c-Jun mRNA than that induced by CF treatment soon after PH. The higher expression (seven folds increase) continued for only 5 hours (the time point detected) in the

liver treated with IQ, whereas, the expression in the liver treated with clofibrate continued for 48 hours with three to four folds. It can not be definitely answered about this difference, but it can be assumed that even short term period time after treatment with different promoting agents could change certain environments bringing up growth signals or growth factors themselves.

It is noteworthy that the expression of GST-P mRNA in putative initiated hepatocytes was controlled by the transcription factor AP-1 which is the complex of c-fos/c-jun or c-jun/c-jun (Okuta *et al.*, 1989). Therefore, it is possible that IQ may provide initiated cells with favor environments (for examles, increase of production of growth factors or comitogenic factor such as eicosanoids) for growth, which may not be induced or decreased by clofibrate. In fact, growth response to hepatocyte growth factors was decreased in the cultured hepatocytes isolated from rats exposed with a peroxisome proliferator ciprofibrate, a derivate of CF compared to control untreated hepatocytes (Lindroos and Michalopoulos, 1993). It has also been suggested that the difference of eicosanoids metabolism may be related to the tumor promoting activity (Hong *et al.*, 1995; Hong and Glauert, 1996). Furthermore, prostaglandins stimulated peroxisome proliferator ciprofibrate-induced AP-1 activity in cultured hepatocytes (Hong and Glauert, 1998). It was also found that indomethacin, a cyclooxygenase inhibitor inhibited clofibrate-induced Jun B expression in the immortalized liver cell lines, BNL-CL2 and ML-457 (Ledwith *et al.*, 1996).

Although the genotoxic carcinogen IQ and nongenotoxic carcinogen clofibrate have tumor promoting activity, the ability of increase of cell proliferation was different and there was different pattern in c-Jun expression in the liver initiated with DEN followed by IQ and clofibrate. Further studies to determine what is (are) the factor(s) which can cause this different effect on the cell proliferation and expression of c-Jun are required.

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