ASSESSMENT OF CARCINOGENIC RISKS OF CHEMICALS APPLICABLE FOR CANCER HIGH-RISK GROUPS

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Abstract
The present study was conducted to establish an assessment system for carcinogenic risks of chemicals existing in the human environment to be applicable for the situation of those who belong to cancer high-risk groups. Male Fischer 344 rats (6 weeks old, 12 per group) were fed a basal diet or a choline-deficient, L-amino acid-defined (CDAA) diet for 8 weeks, then administered varying doses of *N*-nirrosobis(hydroxypropyl)amine (BHP) or aminophenylnorharman (APNH) for 16 weeks under feeding the basal diet, and sacrificed to assess development of preneoplasms in major organs. BHP induced liver preneplasms only in rats given the CDAA-prefeeding, in which numbers of the preneoplasms increased in a dose-dependent manner. APNH induced liver preneoplasms only at the highest dose in rats not given the CDAA-prefeeding but at the middle and high doses in rats given such a treatment. Furthermore, APNH exerted a similar effect also in the colon. These results indicate that the CDAA-prefeeding introduces cancer-prone internal environment to enhance the carcinogenicity of chemicals. It is thus suggested that using the CDAA-prefeeding or other comparable treatments, carcinogenic risks of chemicals may be able to be assessed in a condition applicable for cancer high-risk groups.

Introduction
Cancer is one of the most horrible threats to humans. Because chemicals existing in the human environment are the most major cause of cancers, carcinogenic risk assessment and management of chemicals, including dioxins and other persistent organic pollutants, is crucially important to maintain and progress human health and welfare. It is well kwon that there are respective high-risk groups for frequent (if not all) cases of human cancers, and most of these are commonly characterized by the continuous tissue injury with a background signaling alteration complex, often by virtue of oxidative stress.[1-3] These situations sometimes induce cancers *per se*, but in most cases they provide a cancer-prone internal environment in which carcinogenic processes are to be enhanced, once risky chemicals are exposed.[4,5] This is because behaviour of chemicals under such an internal environment with altered metabolic, signaling, immunological and other circumstances must be different from that under the "normal" internal environment. While carcinogenic risks of chemicals are usually assessed using data obtained in animals (most frequently in rodents) through the extrapolation to the human situations, the data is basically obtained using "normal" animals to reflect the situation of general public. Recent efforts to use a variety of genetically modified animals in carcinogenic risk assessments are made mainly for the improvement of sensitivity and not necessarily baring cancer high-risk groups in mind. An assessment system for carcinogenic risks of chemicals, therefore, has not as yet been established to be applicable for the situation of those who belong to cancer high-risk groups. In this context, the present study was planned and conducted to try to establish a basic model of such assessment systems.

Chronic liver injury represented by repeating death and proliferation of hepatocytes and fibro/cirrhogenesis in association with a background signaling alteration complex, mainly by virtue of oxidative stress, is a major factor and a cancer-prone internal environment for human hepatocarcinogenesis, which is caused by a variety of reasons including chronic viral hepatitis, hemochromatosis, Wilson's disease, non-alcoholic steatohepatitis and others, and related to the situation of metabolic syndrome that is also cancer-prone.[6-8] Dietary choline deficiency in rodents well mimics most of phenomenological and mechanistic events occurring in the aforementioned human situation and develops hepatocellular carcinomas in the absence of exposure of any exogenous stimuli, and we ourselves have established one of the best rodent dietary choline deficiency models by producing a choline-deficient, L-amino acid-defined (CDAA) diet.[6,9,10] We have already shown that a combined treatment of a 1-week prefeeding and an 8-week postfeeding of the CDAA diet enhances hepatocarcinogenicity of not only hepatocarcinogens but also carcinogens not usually targetting the liver, and also enhances colon carcinogenicity of colon carcinogens.[6,11] The mechanism underlying this phenomenon has

been attributed to the introduction of a cancer-prone internal environment as described above.[6] The present study was planned on the basis of this data and tried to use the CDAA diet in a more sophisticated way. We featured 2 carcinogenic chemicals both present in the human environment as model test compounds. *N*-nitrosobis(hydroxypropyl)amine (BHP) is present in medical supplies, intermediate products of rubber, herbicides and surfactants, and it is a mutagenic carcinogen targetting the lung, liver, thyroid, kidney and urinary bladder.[12,13] Aminophenylnorharman (APNH) is a mutagenic compound internally synthesized from non-mutagenic norharman (present in cigarette smoke and cooked food) and aniline (present in cigarette smoke and some vegetables), and it is a carcinogen targetting mainly the liver and colon.[14-17]

Materials and Methods

Our in-house committees evaluated the experimental protocol beforehand and monitored the actual experiment to obey domestic and international laws, regulations, guidelines and rules for animal welfare. A total of 192 male Fischer 344 rats (Charles River Japan, Inc., Japan) were obtained at their 5 weeks of age, acclimatized for 1 week on the CE-2 basal diet (Clea Japan, Inc., Japan) and used for the experimentation at 6 weeks of age by randomly divided into 16 groups each consisting of 12 animals. Animals were maintained in an air-conditioned room under constant conditions of 24 ± 2ºC and 55 ± 10% humidity with a 12-hour light/dark cycle, allowed free access to food and drinking water and regularly monitored general condition, body weight, water intake and food consumption. The CDAA diet, BHP and APNH were obtained from Dyets Inc. (USA), Nakalai Tesque Co., Ltd. (Japan) and Nard Institute (Japan), respectively.

In the BHP experiment, groups 1, 2, 3 and 4 received the CE-2 diet and the plain drinking water for 8 weeks and then the CE-2 diet and the drinking water containing BHP at concentrations of 0, 10 100 and 1000 ppm, respectively, for 16 weeks. Groups 5, 6, 7 and 8 received the CDAA diet and the plain drinking water for 8 weeks and then the CE-2 diet and the drinking water containing BHP at concentrations of 0, 10 100 and 1000 ppm, respectively, for 16 weeks. At the end of week 24, all animals were sacrificed by exanguination under light ether anesthesia, and after macroscopic observation major organs were removed. From the organs, formalin-fixed, paraffin-embedded sections were prepared, stained by a standard hematoxylin and eosin staining procedure, and used for histopathological examination. In addition, development of preneolasm was quantitatively assessed in the liver and colon by using surrogate biomakers of glutathione *S*-transferase placental form (GST-P)-positive foci of cellular alteration[11] and aberrant crypt foci,[18,19] respectively. In the APNH experiment, groups 1-8 were treated principally identical to the BHP experiment with the exception that APNH at concentrations of 0, 0.4, 4 and 40 ppm were administered by admixing into the CE-2 diet instead of BHP.

Numerical data was statistically assessed for the significance of intergroup difference using a one-way analysis of variance and the Student-Newman-Keuls *post-hoc* test, where the difference with p value less than 0.05 was considered significant.

Table 1. Effect of the CDAA-prefeeding on the development of preneoplasms in the liver induced by BHP (12 animals per group)

Group	Treatment(s)	Liver preneoplasm	
		Number/cm^2	Average area (mm^2)
1	CE-2 BHP 0 ppm	0	-
2	CE-2 BHP 10 ppm	0	-
3	CE-2 BHP 100 ppm	0	-
4	CE-2 BHP 1000 ppm	0	-
5	CDAA BHP 0 ppm	0.0035 ± 0.0040[a,c]	0.0024 ± 0.0030
6	CDAA BHP 10 ppm	0.0060 ± 0.0100	0.0017 ± 0.0024
7	CDAA BHP 100 ppm	0.0181 ± 0.0100[b,c]	0.0156 ± 0.0189[b]
8	CDAA BHP 1000 ppm	0.1139 ± 0.0790[b,c]	0.0007 ± 0.0006

[a]Mean ± standard deviation.
Significantly different from [b]group 5 value or [c]BHP-same-dose group value ($p < 0.05$).

Results and Discussion

In the BHP experiment, GST-P-positive, liver preneoplasms were developed only in groups 5-8 given the CDAA-prefeeding (table 1). Among groups 5-8, the lesion numbers increased in a dose-dependent

manner, and values of groups 7 and 8 (given the middle and high doses of BHP, respectively) became significantly higher than that of group 5 (not given BHP) (table 1). The lesions were very small in groups 5, 6 and 8, whereas the lesion size was significantly larger in group 7 than in group 5 (table 1). It is thus apparent that the CDAA-prefeeding clearly enhances hepatocarcinogenicity of BHP. In the lung, proliferative lesions were developed only in groups 4 and 8 (both given the high dose of BHP) at the 100% incidence. They were mostly atypical alveolar hyperplasias, but adenomas were observed in 1 rat of group 4 and 1 rat of group 8. No difference was noted either for the incidence or the multiplicity of lung proliferative lesions between these 2 groups, indicating no influence of the CDAA-prefeeding. In the other organs including the colon, no apparent preneoplams were detected in any groups.

Table 2. Effect of the CDAA-prefeeding on the development of preneoplasms in the liver and colon induced by APNH (12 animals per group)

Group	Treatment(s)	Liver preneoplasm		Colon preneoplasm	
		Number/cm^2	Average area (mm^2)	Number/animal	Number of crypts/lesion
1	CE-2 APNH 0 ppm	0	-	0	-
2	CE-2 APNH 0.4 ppm	0.0003 ± 0.0009[a]	0.0006 ± 0.0020	0	-
3	CE-2 APNH 4 ppm	0.0010 ± 0.0020	0.0026 ± 0.0054	0	-
4	CE-2 APNH 40 ppm	0.4583 ± 0.1318[b]	0.1092 ± 0.0209	1.33 ± 0.89[b]	1.88 ± 0.72
5	CDAA APNH 0 ppm	0.0158 ± 0.0069[d]	0.2920 ± 0.1780[d]	0	-
6	CDAA APNH 0.4 ppm	0.0210 ± 0.0105[d]	0.1861 ± 0.0002[d]	0	-
7	CDAA APNH 4 ppm	0.0429 ± 0.0124[c,d]	0.1640 ± 0.0900[d]	0.08 ± 0.29	0.17 ± 0.58
8	CDAA APNH 40 ppm	0.5500 ± 0.1294[c,d]	0.1248 ± 0.0401[d]	2.67 ± 1.67[c,d]	3.53 ± 1.08[d]

[a]Mean ± standard deviation.
Significantly different from [b]group 1 value, [c]group 5 value or [d]APNH-same-dose group value ($p < 0.05$).

Among groups 1-4 not given the CDAA-prefeeding in the APNH experiment, GST-P-positive, liver preneoplasms were significantly developed only in group 4 (given the high dose of APNH) (table 2). Among groups 5-8 given the CDAA-prefeeding, the lesion numbers were significantly higher in groups 7 and 8 (given the middle and high doses of APNH, respectively) than in group 5 (not given APNH) (table 2). The lesion sizes were not altered by the administration of any doses of APNH. Values of numbers and sizes of the lesions were significantly higher in groups given the CDAA-prefeeding than in groups not given such a treatment. While preneoplastic, aberrant crypt foci of the colon were significantly developed only in groups 4 and 8 (both given the high dose of APNH), values of the lesion number and size were both significantly higher in group 8 (given the CDAA-prefeeding) than in group 4 (not given the CDAA-prefeeding) (table 2). It is thus apparent that the CDAA-prefeeding clearly enhanced not only hepatocarcinogenesis but also colon carcinogenesis of APNH. Furthermore, whereas APNH has been shown to develop carcinomas in the liver and colon at 20 and 40 ppm, the significant increase of their incidences has been obtained only at 40 ppm but not at 20 ppm.[14] The present results thus indicate that the CDAA-prefeeding makes carcinogenicity of low dose APNH significantly revealed at least in the liver. In the other organs no apparent preneoplams were detected in any groups.

A 8-week feeding of the CDAA diet causes chronic liver injury represented by repeating death and proliferation of hepatocytes and scant fibrosis in association with a background signaling alteration complex, mainly by virtue of oxidative stress.[6,9,10] This is a typical cancer-prone internal environment for the liver as aforementioned, corresponding to the situation of human high-risk group for liver cancer. The CDAA diet *per*

se induces liver preneoplasms by feeding for 8 weeks, but these are incapable of progressing to hepatocellular adenomas or carcinomas in the absence of further feeding of the CDAA diet.[6,9,10] Dietary choline deficiency exerts strong liver cancer promoting activity,[6,9,10] but in the present study the CDAA diet was not administered after starting of the carcinogen exposure. It may thus be likely that the present enhancing effect of the CDAA-prefeeding on the liver carcinogenicity of BHP and APNH as well as the colon carcinogenicity of APNH is due to the introduction of cancer-prone internal environment as preliminarily shown previously.[6] The fact that the CDAA-prefeeding enhanced the colon carcinogenicity of APNH is noteworthy, because even a long-term administration of the CDAA diet for up to 2 years does not cause any apparent morphological changes in the colon.[20] This suggests that cancer-prone internal environment in one organ (in this case the liver) can infect other organ(s) (in this case the colon). It is reasonable, because while the CDAA diet causes morphological changes mostly in the liver, its causing oxidative stress and related signaling alterations are easily expected to affect extra-liver organs.[6,9,10] This may be analogous to human metabolic syndrome situation.[7,8]

In conclusion, the CDAA-prefeeding introduces cancer-prone internal environment to enhance the carcinogenicity of chemicals. It is thus suggested that using the CDAA-prefeeding or other comparable treatments, carcinogenic risks of chemicals may be able to be assessed in a condition applicable for cancer high-risk groups.

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