Co-Carcinogenic Effect of Cyclohexanol on the Development of Preneoplastic Lesions in a Rat Hepatocarcinogenesis Model

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Cyclohexanol is a basic industrial chemical widely used because of its versatility as an industrial solvent. No studies have been conducted to evaluate the carcinogenic/co-carcinogenic hazards associated with cyclohexanol exposure. In male Fisher 344 rats liver preneoplastic lesions were induced by N-nitrosodiethylamine (150 mg/Kg) i.p., followed by the tumor promoter 2-acetylaminofluorene (2-AAF: 20 mg/kg) orally administered on three consecutive days before partial hepatectomy. The cyclohexanol administration in this hepatocarcinogenesis assay revealed that it has a strong tumor co-promoter potential. There is clear evidence that oxidative stress and the CYP2E1 are components of carcinogenesis. Although no changes in the lipid peroxidation levels were observed between treated and untreated animals, a significant increase in CYP2E1 expression was observed when cyclohexanol was administered 24 h after the last 2-AAF dose. On the other hand, levels of the proliferation markers PCNA and Ki-67 were not increased after treatment with cyclohexanol, but a marked downregulation of the Bax proapoptotic protein was found exclusively in mitochondrial extracts of animals treated with cyclohexanol. This study represents the first report of the ability of cyclohexanol-induced lesions, when administered simultaneously with 2-AAF, to potentiate the development of preneoplastic liver. © 2006 Wiley-Liss, Inc.

Key words: cyclohexanol; hepatocarcinogenesis; co-carcinogenic effect

INTRODUCTION

Most occupational carcinogens known were discovered circumstantially [1]. They were first suspected on the basis of case reports by clinicians [2]. Although a small fraction of occupational agents have been adequately investigated with epidemiologic data, it is reasonable to suspect that there may be some, perhaps many, as yet undiscovered occupational carcinogens.

Cyclohexanol is a basic industrial chemical used in the production of nylon, lacquers, paints, varnishes, degreasers, plastics and plasticizers, soaps and detergents, textiles, and insecticides [3]. The 1983 National Occupational Exposure Survey estimated 68 715 U.S. workers have the potential for exposure to cyclohexanol [4]. Occupational exposure may occur through inhalation or via dermal contact with cyclohexanol-containing solutions. It is also possible for the general population to be exposed to cyclohexanol, most probably via ingestion of contaminated drinking water or food, inhalation of contaminated air, or dermal contact with contaminated water. Reported environmental releases of cyclohexanol for the U.S. totaled 3892943 pounds in 1999 [5].

Despite the wide use of cyclohexanol, resulting from its versatility as an industrial solvent, few studies are available on the toxicological effects of cyclohexanol. However, the little information available on cyclohexanol shows it to be irritating to the skin and mucous membranes, and that it affects the

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Abbreviations: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; GGT, γ -glutamyltranspeptidase; GST-P, glutathione Stransferase placental form; PH, partial hepatectomy; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; ENU, N-ethyl-Nnitrosourea.

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central nervous system [4]. Additionally, exposure to cyclohexanol has been associated with the induction of hepatic xenobiotic metabolism [4]. A previous report showed that the S9 fraction obtained from rat livers, which was orally pretreated for 5 d with cyclohexanol, was able to activate several nitrosamines into highly mutagenic metabolites detected in the TA-100 strain of Salmonella typhimurium [6]. Several studies with environmental substances have shown the importance of the modulation of CYP450 isozymes for liver carcinogenicity through formation of highly reactive electrophilic intermediates [7]. Therefore, our hypothesis has resulted from the issue that cyclohexanol increases CYP2E1 and then this could increase reactive oxygen species (ROS). We considered that cyclohexanol would increase the preneoplastic lesions in this way, because there are clear examples of the participation of ROS in hepatocarcinogenesis in rats [8].

In an extensive literature survey, we did not find any reports regarding experimental carcinogenesis following exposure with cyclohexanol. Therefore, this study was aimed to determine whether cyclohexanol favors the development of hepatic preneoplastic lesions induced by DEN, and promoted by 2-AAF and partial hepatectomy (PH).

MATERIALS AND METHODS

Animals and Experimental Protocol

Male Fisher 344 rats (180–200 g) were obtained from Laboratory Animal Facility (UPEAL, CINVES-TAV-IPN). The animals were housed in groups of five animals in polycarbonate cages with wood-chip bedding and were maintained, prior to and during experiments, on a 12 h light: 12 h dark cycle at $22\pm1^{\circ}$ C and 50 ± 10 relative humidity. Rodent chow (Purina, St. Louis, MO) and water were available ad libitum. The study was approved by the Institutional Animal Care and Use Committee of our institution, and conducted in accordance with established guidelines of the care and use of laboratory animals of the National Research Council, USA.

We divided the animals into six groups (Table 1). The basic model used in this study was the Semple– Roberts hepatocarcinogenesis model (Group 2) [9]. This protocol includes the administration of one dose 150 mg/kg diethylnitrosamine (DEN, Sigma Chemical Co., St. Louis, MO) i.p. One week later, the animals received 2-acetylaminofluorene (2-AAF, Sigma Chemical Co., St. Louis, MO) orally, at doses of 20 mg/kg/d during three consecutive days before PH, which was performed 10 d after initiation. PH was performed under light ether anesthesia by midventral laparotomy with 70% liver resection, and the median and left lateral lobes of the liver were ligated and excised. Then, the incision was closed by using a continuous two-layer suture technique. The animals were sacrificed under ether anesthesia at 25 d postexperiment initiation.

Histochemical GGT Staining

For histochemical GGT staining, liver sections of 15 µrere obtained in a cryostat (Slee cryostat MTC, <u>sectimany</u>^{Q2}) fixed in absolute ethanol for 5 min at 4°C and stained according to Rutenburg [10]. Briefly, γ-glutamyl-4-methoxy-2-naphthylamine, glycyl-glycine and 4-benzoylamino-2.5-diethoxybenzene-diazonium chloride hemi (zinc chloride) salt (Sigma Chemical Co., St. Louis, MO) were added to the sections and the reaction was kept for 30 min, then cupric sulfate was added for 2 min. Finally, GGT-positive nodules were quantitatively analyzed with images of histological sections taken with a digital competence (Color-View 12, Soft Imaging System GmbH, Color-View 12, Soft Imaging System 12, Soft Imaging System GmbH, Color-View 12, Soft Imaging System 1 softwar alysis kit (AnalySIS, Soft Imaging System GmbH, <u>ermany</u>^{Q4}). Focal GGT-positive areas greater than 0.01 mm² were registered to avoid small bile-duct cell detection (arbitrary intensity values) and were classified according to GGT stain intensity. The number of preneoplastic lesions per cu cm was estimated with the formula that $N = [(1/r_1) + (1/r_1)]$ r_2) + (1/ r_3)... + (1/ r_n)]/ πA . The number of foci per liver was calculated by multiplying the number of foci per cu cm by the weight of the liver in g [11]. The data were analyzed by a two-tailed Student's t-test and *P*-values of ≤ 0.05 were considered statistically significant.

Histological Analysis

For histopathological evaluation, two slices from each lobe were fixed in 10% buffered formalin and embedded in paraffin blocks. Then, 5 μ m thick sections were cut and stained with hematoxylin and eosin (H&E).

Immunohistochemical Analysis for PCNA

We cut sections 5 µm thick from paraffinembedded liver tissues and sequentially deparaffinized and rehydrated through xylene and graded alcohol solutions for immunohistochemical analysis. Antigen retrieval was performed by immersion of tissue sections in preheated citric acid buffer (10 mM, pH 6) with constant heat in a water bath (95–99°C) for 30 min. The slides were then treated with 3% hydrogen peroxide in PBS to block endogenous peroxidase. After rinsing the sections with PBS for 30 min, they were incubated for 1 h with the primary antibody anti-PCNA (Zymed, San Francisco, CA) at 1:50 dilution. The labeled Streptavidin-Biotin method with the LSAB Plus-kit (DAKO Corporation, Carpinteria, CA) was applied according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin.

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DEN, 150 mg/kg; at days 7, 8, 9, 1 2-AF was administered at doses of 20 mg/kg/d and PH was performed on day 10; A cylohexanol doses, 100 mg/kg/d. The animals were sacrificed 25 d after DEN administration. Groups without = DEN or 12-AF were performed and PBS was instead administered. Asterisks indicate a significant increase in comparison with Group 2. $*P \le 0.05$. $**P \le 0.001$.

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Western Blot Analysis

Nuclear, mitochondrial, and microsomal liver fractions were prepared as previously described [12–14]. The cytosolic fraction was obtained from a supernatant at high speed (100 000 g for 90 min). The soluble protein was quantified by Bradford analysis [15] and then separated on SDS-PAGE. The protein was then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA), and then blocked with 5% nonfat dry milk; incubated with either of the following: anti-GST-P (1:1000 dilution; DAKO Corporation); anti-PCNA (1:1000 dilution; Zymed); anti-ki67 (1:1000; DAKO Corporation); anti-Bcl-2, Bcl-X_L, or Bax (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-CYP2E1 (1:2000; Oxford Biomedical Research, Oxford, MI). The secondary antibody used was horseradish peroxidase-conjugated (Zymed); and detection was performed by chemiluminescence with ECL detection reagents (Amersham Biosciences, Piscataway, NJ) and developed with Konica Film (Tokyo, Japan). These membranes were then re-probed with goat polyclonal anti-lamin B (Santa Cruz Biotechnology), anti-cytochrome oxidase and anti-acting noclonal antibodies (Cinvestav, <u>Mexico City</u>, as loading controls of nuclear, mitochondrial and cytoplasmic extracts, respectively. Protein levels were determined by scanning densitometry of the autoradiography with a Gel Analysis Software (SIGMA GEL.LNK).

Assay of Lipid Peroxidation

Liver samples of 200 mg of tissue were homogenized in four volumes 225 mM saccharose, 110 mM Tris-HCl, 0.3 mM EGTA, pH 7.4, in a Potter-Elvehjem homogenizer with a Teflon pestle at 4°C. The homogenates were collected and used for the measurement of thiobarbituric acid reactive substances (TBARS). The amounts of aldehydic products generated by lipid peroxidation were assayed in liver homogenates with TBARS [16] and modified as previously reported [17]. Results were listed as nanomoles of TBARS per milligram of protein.

Statistical Analysis

The data were analyzed with Student's unpaired *t*-test and taking a *P*-value of ≤ 0.05 as significant.

RESULTS

To investigate the effect of cyclohexanol on the development of preneoplastic lesions in rat liver we analyzed different treatment groups in search of GGT expression, an enzyme widely used as a tumor marker in the liver during chemical carcinogenesis, because its expression is an early event in this process [18]. GGT-positive liver foci were not detected in the Group 1 with a detection parameter for focus size larger than 0.01 mm². Average quantity of GGT-positive liver foci from Group 2, hepatocarcinogen-

esis model of Semple-Roberts, was taken as 100%. To study the tumor co-initiator role of cyclohexanol the animals received single doses of cyclohexanol 12 h before and after DEN administration (Group 3). In this group, the number of GGT foci was smaller than Group 2 with no statistically significant increase in area. The evaluation of cyclohexanol potential as a tumor initiator, administered without DEN (Group 4), reported diffuse low-intensity GGT-positive stained areas. On the other hand, evaluation of cyclohexanol as a tumor co-promoter, co-administrated with 2-AAF in 12 h intervals (Group 5), demonstrated a significant increase (compared to Group 2) by 38, 25, and 331% in the number of foci per cm³, number of foci per liver and percentage of GGT-positive area, respectively. Finally, the analysis of cyclohexanol as a tumor promoter, administered without 2-AAF (Group 6), only induced small GGT positive foci, which represented only 13 % of the number and 0.09% of the area, of the foci observed in Group 2 (Table 1, Figure 1). A control group which included PBS instead of cyclohexanol did not show any traces of preneoplastic lesions (data not shown). Therefore, cyclohexanol by itself induced the appearance of small liver preneoplastic lesions.

This GGT analysis showed that Group 5 had the strongest effect on generation of GGT positive foci



Figure 1. Effects of cyclohexanol on the expression of the tumoral marker GGT on liver slices. Histochemically stained sections showing the effect of cyclohexanol on the induction of GGT-positive liver foci 25 d after experiment initiation. Liver sections representative of each treatment: Groups 1–6.

compared to Group 2, therefore the analysis of all following parameters were carried out on Group 5 with the appropriate controls.

Effect of Cyclohexanol During Hepatocarcinogenesis Treatment on GST-P

In addition to GGT assay, the Group 5, which showed a significant increase in GGT positive foci compared to Group 2, was analyzed by measuring the GST-P expression. The GST-P tumor marker is considered among the most sensitive for detection of altered hepatic foci induced by most chemicals [19]. We evaluated the magnitude of preneoplastic lesions by GST-P 25 d after experiment initiation in Groups 1, 2, and 5. GST-P expression analysis by Western blot assay in liver cytosolic proteins indicated by significant increases in Groups 2 and 5 compared to Group 1. It is noteworthy that the cyclohexanol (Group 5) administration increased by 3.1-fold the GST-P expression in comparison to Group 2 (Figure 2).

Histopathology

The microscopic findings showed that 25 d after experiment initiation the normal microscopic architecture displayed by the livers of Group 1 (not



Figure 2. Expression of liver cytosolic GST-P. Representative Western blot assay of cytosolic extracts for analysis of the GST-P protein from livers of rats sacrificed 25 d after experiment initiation. The bar graphs indicate the relative amounts of GST-P after normalization with respect to the amount of actin loaded. Data are mean \pm SD (n=6 per group, ** $P \le 0.001$ in comparison with Group 2).

treated) was completely distorted in Groups 2 and 5. In the livers of Groups 2 and 5, most of the lesions were altered hepatic foci consisting of focal eosinophilic areas composed of atypical hepatocytes with compression of the adjacent parenchyma. Cyclohexanol exposure in Group 5 increased the presence of preneoplastic nodules compared to Group 2. In addition, multinodular alterations were evident leading to the formation of confluent conglomerates (Figure 3).

Group 1

Group 5







5

Figure 3. Effects of cyclohexanol on the development of preneoplastic liver. Representative histological sections from livers of rats sacrificed 25 d after experiment initiation. H&E-stained sections showing preneoplastic nodules. Arrows show the periphery of the nodules. Photographs were taken at $40 \times$ magnification.

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Effect of Cyclohexanol on CYP2E1 Expression

We evaluated the CYP2E1 expression by Western blot in rat liver microsomes to determine the effects of cyclohexanol on CYP2E1 expression in the hepatocarcinogenesis protocol. CYP2E1 expression increased 100% at 24 h after the last dose of 2-AAF as compared to Group 2 (Figure 4A). On the other hand, when liver preneoplastic lesions occurred 25 d after experiment initiation, the CYP2E1 expression was similar between treated and untreated animals (Groups 2 and 5) (Figure 4B).

Effect of Cyclohexanol on Lipid Peroxidation

Since it has been shown that lipid peroxidation plays roles that are important in the early stages of hepatocarcinogenesis, and is an expression of oxidative stress in cells [8,20], we determined TBARS as a global approach. Lipid peroxidation levels in liver homogenate increased more than 100% in Groups 2 and 5 at 24 h after the last dose of 2-AAF as compared to Group 1. However, no statistically significant differences were observed between Groups 2 and 5 (*P*-value ≥ 0.5) (Figure 5A). Likewise, 25 d after protocol initiation, the lipid peroxidation levels were not modified significantly in Group 5 (**P*value ≥ 0.5) as compared to Group 2 (Figure 5B).

Effect of Cyclohexanol on Cell Proliferation

To investigate if the increase of liver preneoplastic lesions were associated with an increase in the cell proliferation levels, Ki-67 and PCNA markers were analyzed 24, 48, 72 h after PH and 25 d after cyclohexanol treatment, in Groups 1, 2 and 5. PCNA staining did not reveal a significant difference in the proliferative index between Groups 2 and 5 (Figure 6A). Also, no significant difference, in the proliferative index, between Groups 2 and 5 was found in the Ki-67 and PCNA proliferation markers analyzed by Western blot (Figure 6B).

Effect of Cyclohexanol on the Bcl-2, Bcl-X_L, and Bax Mitochondrial Levels in Hepatocarcinogenesis

Because it is known that overexpression of Bcl-2like proteins and downregulation of proapoptotic Bcl-2 relatives can enhance tumor progression [21], we decided to investigate the effects of cyclohexanol on members of the Bcl-2 family by a Western blot assay of liver mitochondrial extracts at the end of the hepatocarcinogenesis protocol. In either Groups 2 or 5, there was no significant alteration in either Bcl-2 or Bcl-X_L expressions. Likewise, the Bax level was maintained in Group 2, which was similar to Group 1. However, we observed a remarkable downregulation of 10.5-fold of Bax proapoptotic protein in Group 5, compared to Group 2 (Figure 7).

DISCUSSION

The use of animal models has become an effective alternative for investigating the carcinogenic potential of chemical compounds [22]. In this study we used the hepatocarcinogenesis model of Semple-Roberts to analyze the cyclohexanol effect as a co-carcinogen. Because this model of chemical hepatocarcinogenesis can be divided into three stages: initiation, promotion, and progression [23],





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Figure 6. Effect of Cyclohexanol on the proliferation markers PCNA and Ki-67. (A) Representative pictures of liver samples stained for PCNA obtained 25 d after experiment initiation. The photographs show a 200-fold magnification. (B) Western blot of nuclear extracts for analysis both PCNA and Ki-67. The bar graphs indicate the relative amounts of PCNA and Ki-67 after normalization with respect to the amount of lamin B that was loaded. Data are mean \pm SD (n = 4 per group, *P*-value is ≥ 0.05 in comparison with Group 2).



Figure 7. Effect of cyclohexanol on the Bcl-2, Bcl-X_L, and Bax mitochondrial levels. Western blot of liver mitochondrial extracts obtained 25 d after experiment initiation for analysis of Bax, Bcl-2, and Bcl-X_L corresponding to Groups 1, 2, and 5. The bar graphs indicate the relative amounts of Bax, Bcl-2, and Bcl-X_L after normalization with respect to the amount of cytochrome oxidase (cyt ox) used as a control for loading. Data are mean $\pm \frac{\text{SD}^{\text{O6}}(n=4)}{\text{P}^{\text{O6}}(n=4)}$ per group, ***P ≤ 0.0001 in comparison with Group 2).

it allows studies of chemopreventive agents in the sequence from nodule to cancer [24,25].

Initially, our hypothesis of carcinogenic/cocarcinogenic hazards by cyclohexanol derived from the fact that cyclohexanol increases CYP2E1, which in turn could increase ROS. Oxidative stress has been shown to trigger damage to cellular membranes and nuclear DNA, which results in lipid peroxidation and oxidative DNA damage, respectively [26]. Therefore, we hypothesized that cyclohexanol administered before and after DEN, or 2-AAF administration, would induce damage to DNA prior to the carcinogens' administration, and in this way could increase the development of preneoplastic lesions. In this study, the livers of animals initiated with cyclohexanol, instead of DEN, presented a diffuse pattern of GGT lesions similar to that produced by N-ethyl-N-nitrosourea (ENU) [8], a direct mutagen which generates DNA-ethyl adducts, but does not produce liver tumors in rats unless given under

oxidative stress conditions, such as PH or phenobarbital treatment [23,27]. Although our data suggests that cyclohexanol does not have tumor-initiator properties, the similarities between the lesions generated by cyclohexanol and ENU may reflect a mechanism similar in generating hepatic damage. Hence, it would be interesting to determine if cyclohexanol could generate liver tumors similar to ENU under others oxidative stress conditions, such as phenobarbital treatment. In addition, it would be also interesting to use different doses of cyclohexanol during the initiation stage to investigate its ability to directly initiate tumors and produce damage to the DNA structure similar to ENU.

Since no studies have been conducted to evaluate the co-promoter/promoter effect associated with cyclohexanol administration, an novel feature of our study was the demonstration that cyclohexanol, administrated 12 h before and 12 h after 2-AAF, caused a striking increase in the number and size of liver preneoplastic lesions. Although it is not clear how the cyclohexanol contributed to the increase in liver preneoplastic lesions, it is possible that cyclohexanol increases the tumor promoter activity of 2-AFF. According to Miller and Miller (1981) first necessary step for 2-AAF activation is N-hydroxylation to form the proximate carcinogen N-OH-AAF. This step occurs in the liver and is mediated by cytochrome P450 enzymes, in particular the isoform CYP1A2 $[27] \longrightarrow$ will be of the interest to identify, in our system, if cyclohexanol induce, the activity of other CYP enzymes, in particular CYP1A2.

In the present study, cyclohexanol administration without 2-AAF, induced the appearance of GGT positive liver foci. Although the number of foci promoted with cyclohexanol is low, compared with the number of foci promoted with 2-AAF, there is the potential that cyclohexanol itself promotes preneoplastic lesions. In this context, cyclohexanol could form adducts similar to other tumor promoters, and if the adducts remain un-repaired at the time of DNA replication, a mutation may result. This hypothesis may be supported by previous reports that have shown an enhanced mutagenic effect by an important group of nitrosamines in animals previously treated with cyclohexanol [6]. Future studies will be necessary to determine if the cyclohexanol effect is similar to other tumor promoters.

Although the levels of CYP2E1 and lipid peroxidation were similar, between the animals treated and not treated with cyclohexanol 25 d after protocol initiation, an evident change in CYP2E1 expression was observed when cyclohexanol was administered 24 h after the last 2-AAF dose. Hence, the possibility of CYP2E1 could take part in this co-promotion property is considered. In accordance with this hypothesis, recent studies of CYP2E1-expressing hepatoma and rat hepatocyte cell lines have shown that CYP2E1 increased cellular resistance to

oxidant-induced death = 0] which may increase the tumor development.

Abnormal proliferation of cells is the main feature of carcinogenesis, and is evidence of a tumor growth. Thus, we analyzed the PCNA and Ki-67 proliferation markers in the promotion stage of this experimental model. In our experiment, we found that neither PCNA nor Ki-67 expression tended to increase in the groups treated with cyclohexanol and analyzed 25 d after experiment initiation. Further analyses were performed 24, 48, and 72 h after PH, however no changes were found. These results, however, do not invalidate the consideration that cyclohexanol could increase the proliferative status at different times than those analyzed in this study.

Alteration in the ratio between proapoptotic and anti-apoptotic members of the Bcl-2 family, rather than the absolute expression level of any single Bcl-2 family member, can determine apoptotic sensitivity [31] Ir results revealed a marked reduction of Bax mitochondrial levels in the animals treated with cyclohexanol that could increase the number and size of liver preneoplastic lesions through a decrease in apoptosis sensitivity independently of Bcl-2 and Bcl-X_L levels. This hypothesis is supported by a previous report indicating that downregulation of Bax is associated with tumoral progression by enhancing survival of otherwise doomed cells, which enables accumulation of additional mutations [32]

Although further studies will be necessary to determine the participation of cyclohexanol in the development of liver preneoplastic lesions, our results are the first to show the co-carcinogenic effect of cyclohexanol in the development of liver preneoplastic lesions. It is also important to note that cyclohexanol administration in this model of hepatocarcinogenesis can be a useful example in studying the carcinogenicity or co-carcinogenicity of other frequently used industrial compounds and to analyze the mechanism(s) that could be involved.

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