Hepatocarcinogenesis in a Sprague-Dawley rat initiation/promotion model following discontinuous exposure to TCDD.

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Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and dioxin-like compounds are widely dispersed in the environment and their persistence results in long-term exposure to human populations[1]. In rodent models, TCDD is a multi-site, trans-species carcinogen in both sexes[2]. TCDD is the most toxic of the polychlorinated dibenzodioxin (PCDD) and polychlorinated dibenzofuran (PCDF) congeners and this is consistent with a similar mechanism of action via the aryl hydrocarbon receptor (AhR)[3].

TCDD is non-genotoxic and several studies have shown that it acts as a tumor promoter[4-6]. Maronpot showed that in a chronic 30 week initiation/promotion model there was a dose dependent increase in cell proliferation and foci formation in the livers of DEN initiated female Sprague-Dawley rats treated with up to 125 ng TCDD/kg/day[5].

Tumor promotion by TCDD is modulated by the presence of ovarian hormones in the female Sprague-Dawley rat[7]. Lucier showed that TCDD induced changes in cell proliferation required ovarian hormones, and that increases in altered hepatic foci (AHF) expressing gamma glutamyl transpeptidase (GGT) was higher in intact animals compared with ovariectomized animals[7]. In this study the modulation of foci formation by ovarian hormones was more pronounced for GGT positive foci than for foci expressing the placental form of glutathione transferase (PGST). PGST is a marker of foci that identifies almost 90% of all AHF whereas GGT staining identifies only 10% of AHF indicating that modultion of the promotional envirnoment promotion may have effcts on certain subsets of foci. Indeed it is known that different promoting agents result in the promotion of foci exhibiting different phenotypes[8].

One of the characteristics of tumor promotion is its reversibility upon withdrawal of the promoting agent. Several studies have shown that promotion of PGST positive foci formation was reversible upon cessation of treatment with TCDD[9,10]. The aim of the current study was to investigate the effect of discontinuous exposure to TCDD on the promotion of hepatocarcinogenesis in the female Sprague-Dawley rat.

Materials and Methods

Female Sprague-Dawley rats were housed three to a cage under conditions of controlled temperature (70 ± 0.5°F), humidity (50 ± 5%), and lighting (12 hour light/12 hour dark), and received food and water *ad libitum*. Animals were either initiated with 175mg diethylnitrosamine/kg body weight at 10 weeks of age (D) or received saline alone (S). Two or 18 weeks after initiation, animals were treated biweekly with 1750ng TCDD/ml/kg body weight by gavage for up to 60 weeks. Control animals received corn oil alone. For some groups, after 30 weeks of TCDD exposure, TCDD treatment was stopped and the animals subsequently received corn oil for the remainder of the study. TCDD was prepared by Radian Corporation (Morrisville, NC) in corn oil. Concentration (>96% of theoretical value) and purity (>99%) were confirmed by GC/MS analysis. One week prior to the termination of the study, animals were implanted with osmotic minipumps containing bromodeoxyuridine (BrdU). One week after the last treatment,

animals were killed with CO_2 inhalation. Representative sections of liver were fixed overnight in formalin and embedded in paraffin. Other sections of liver were removed, minced and aliquots frozen immediately in liquid nitrogen. Altered hepatic foci (AHF) expressing the placental form of glutathione-s-transferase (PGST) were detected immunohistochemically. PGST positive foci were quantitated by computer-assisted analysis of a digitized image of stained liver sections. Pathological evaluations were carried out by Experimental Pathology Laboratories Inc., RTP, NC

Because the data in several groups appeared to be highly skewed and not normally distributed, non-parametric methods were used to assess overall group differences (Kruskal-Wallis tests) and pairwise comparisons (Mann-Whitney U tests). Significant differences in tumor incidence were assessed by Fisher's exact test. Foci data is represented as the mean ± standard deviation.

Results and Discussion

In the 2 year bioassay of Kociba the incidence of liver tumors was 40% for combined adenomas and carcinomas, 31% for adenomas alone and 9 % for carcinomas alone in animals treated with 100ng TCDD/kg/day and only 2% for all liver tumors in control animals[11,12]. In this present study there were no observable tumors in non-initiated animals receiving either 125ng TCDD/kg/day or corn oil vehicle for 60 weeks (Table 1). Similarly there were no observable tumors in the DEN initiated animals treated with either corn oil or TCDD for 30 weeks. By comparison, there was a high incidence of liver neoplasms (adenomas and carcinomas combined) in animals that were initiated with a single dose of 175 mg DEN/kg and treated with either 125 ng TCDD/kg/day or corn oil for 60 weeks (79% and 55% respectively) (Table 1). The incidence of neoplasms was higher in DEN-initiated animals treated with TCDD for 60 weeks compared with those that received corn oil alone. This difference was not statistically significant, although the statistical power to detect any difference as a result of TCDD treatment was low due to the small sample size used and high background incidence rate. The multiplicity of adenomas alone was highest in DEN initiated group treated with TCDD for 60 weeks continuously.

To examine the effect of periodic exposure on hepatocarcinogenesis, we analyzed tumor incidence after 60 weeks in animals exposed to TCDD for only 30 weeks starting either 2 weeks or 18 weeks following initiation with DEN. Liver tumor incidence in DEN initiated-animals that received TCDD for 30 weeks followed by corn oil for 30 weeks, was lower compared with animals that received TCDD continuously for 60 weeks (Fisher exact test $p<0.05$) or corn oil alone for 60 weeks (not significant). Total liver tumor incidence (adenomas and carcinomas combined) in animals treated with TCDD for 30 weeks, starting 18 weeks after initiation (recovery group) was not significantly different from corn oil controls or animals treated with TCDD for 60 weeks. (It is noteworthy that the incidence of adenomas alone was significantly lower in the recovery group compared with the animals receiving TCDD for 60 weeks (Fisher exact test, $p<0.05$), data not shown). There was no difference in tumor incidence between animals exposed to TCDD for 30 weeks 18 weeks after initiation, compared to those exposed to TCDD for 30 weeks, starting 2 weeks after initiation. TCDD levels in the livers of animals exposed for 60 weeks was 29 ppb (relative to wet weight) compared to 0.004 ppb in control animals. Liver burdens 15 weeks and 30 weeks after cessation of exposure were 0.92 ppb and 0.07ppb respectively.

We also analyzed the development of altered hepatic foci that express the placental form of glutathione transferase (PGST) (Table 2). Promotion with TCDD for 30 weeks led to a significant increase in the number of PGST positive foci (Mann-Whitney U-test $p<0.05$) over corn oil controls. The number of PGST positive foci in animals exposed to TCDD for 30 weeks followed by removal of TCDD exposure was significantly lower than in DEN-initiated age-matched control animals ($p<0.05$, Mann-Whitney U-test). This observation is consistent with observations made by Dragan that show that there is a decrease in foci number and increase in the size class of foci following cessation of TCDD promotion[10].

Altered hepatocytes formed during DEN-initiation can be promoted out to foci either by endogenous promotional agents or by exogenous promoters like TCDD. TCDD treatment may promote out a subset of initiated cells to produce foci that exhibit a programmed phenotype that is different to those that would be promoted by endogenous promoters. Therefore in rats, continual TCDD exposure may result in the development of several types of potential foci; "TCDD

responsive", "TCDD-non-responsive" (i.e. those promoted out by endogenous promoters) and "TCDD-independent" foci (i.e. TCDD responsive foci that progress into a state where they are no longer responsive to TCDD). There may also exist another type of potential foci that are "TCDD-inhibited", i.e. a TCDD-non-responsive foci whose development is inhibited by TCDD exposure. The number, size and type of foci in the liver would therefore reflect the promotional environment. On cessation of TCDD exposure there would be a disruption in this environment thereby leading to an alteration in development of foci type and subsequent changes in number size of foci. showed that on cessation of TCDD exposure there was a reduction in number of PGST foci but not GGT foci, i.e. GGT foci may represent TCDD non-responsive or TCDD-independent foci[10]. If certain types of foci have a greater ability to progress to tumors, alteration of the promotional environment (like withdrawal) may therefore result in an alteration in tumor incidence. The data presented in this paper support this hypothesis and suggest that discontinuous exposure to TCDD can modulate liver tumor incidence promoted either by TCDD or by endogenous agents.

Summary

We analyzed liver tumor incidence and altered hepatic foci in rats, 60 weeks after initiation with DEN and treatment with 125ng TCDD/kg/day for either 60 weeks or 30 weeks. We observed that liver tumor incidences in the group dosed for only 30 weeks was lower than in the DEN initiated groups receiving either 125ngTCDD/kg/day or corn oil alone for 60 weeks. The modulation of hepatocarcinogenesis by discontinuous exposure to TCDD support the hypothesis that changes in the promotional environment in the liver may alter the ability of subsets of foci to progress to tumors.

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References

(1) DeVito, M.J.; Birnbaum, L.S.; Farland, W.H.; Gasiewicz, T.A. Environ. Health. Perspect. 1995, 103, 820-831.

(2) Huff, J.; Lucier, G.; Tritscher, A. *Annu. Rev. Pharmacol. Toxicol.* **1994**, 34, 343-372.

(3) Landers, J.P.; Bunce, N.J. *Biochem. J.* **1991**, 276, 273-287.

(4) Pitot, H.C.; Goldsworthy, T.; Campbell, H.A.; Poland, A. *Cancer Res.* **1980**, 40, 3616-3620.

(5) Maronpot, R.R.; Foley, J.F.; Takahashi, K.; Goldsworthy, T.; Clark, G.; Tritscher, A.; Portier, C.; Lucier, G. *Environ. Health. Perspect.* **1993**, 101, 634-642.

(6) Tritscher, A.M.; Goldstein, J.A.; Portier, C.J.; McCoy, Z.; Clark, G.C.; Lucier, G.W. *Cancer Res.* **1992**, 52, 3436-3442.

(7) Lucier, G.W.; Tritscher, A.; Goldsworthy, T.; Foley, J.; Clark, G.; Goldstein, J.; Maronpot, R. *Cancer Res.* **1991**, 51, 1391-1397.

(8) Pitot, H.C.; Dragan, Y.; Sargent, L.; Xu, Y.H. *Environ. Health Perspect.* **1991**, 93, 181-189.

(9) Tritscher, A.M.; Clark, G.C.; Sewall, C.; Sills, R.C.; Maronpot, R.; Lucier, G.W. *Carcinogenesis* **1995**, 16, 2807-2811.

(10) Dragan, Y.P.; Xu, X.H.; Goldsworthy, T.L.; Campbell, H.A.; Maronpot, R.R.; Pitot, H.C. *Carcinogenesis* **1992**, 13, 1389-1395.

(11) Kociba, R.J.; Keyes, D.G.; Beyer, J.E.; Carreon, R.M.; Wade, C.E.; Dittenber, D.A.; Kalnins, R.P.; Frauson, L.E.; Park, C.N.; Barnard, S.D.; Hummel, R.A.; Humiston, C.G. *Toxicol. Appl. Pharmacol.* **1978**, 46, 279-303.

(12) Goodman, D.G.; Sauer, R.M. *Regul. Toxicol. Pharmacol.* **1992**, 15, 245-252.

Table 1. Liver tumor incidences

Female Sprague-Dawley rats were initiated with 175 mg diethylnitosamime (DEN)/kg or received vehicle alone, followed by promotion with 125ng TCDD/kg/day for up to 60 weeks. The incidence is equal to the number of animals with adenomas and/or carcinomas divided by the total number of animals, and multiplicity is equal to the total number of neoplasms divided by the number of animals exhibiting a neoplasm.

Initiation	Promotion	Adenoma+carcinoma Incidence	Adenoma Multiplicity
Saline	corn oil (60 wks)	0/6	0
Saline	TCDD (60 wks)	0/6	0
DEN	corn oil (30 wks)	0/6	0
DEN	TCDD (30 wks)	0/6	0
DEN	corn oil (60 wks)	6/11 (55%)[1]	1.3
DEN	TCDD (60 wks)	11/14 (79%)[2]	2.0
DEN	TCDD (30 wks) +corn oil (30 wks)	2/13 (15%)*	3.1
DEN	corn oil (15 wks)+TCDD (30 wks)+ corn oil (15 wks)	5/12 (42%)[3]	1.0

* significantly different from rats treated continuously with TCDD for 60 weeks (Fisher exact test $p<0.05$).

[1]Incidence of carcinoma alone in this group was 2/11 (18%)

[2]Incidence of carcinoma alone in this group was 1/14 (7%)

[3]Incidence of carcinoma alone in this group was 3/11 (25%)

Table 2. PGST positive foci formation in TCDD treated rats

Initiation	Promotion	Volume fraction (%)	# foci/liver	mean Focus volume
Saline	corn oil (60 wks)	0.06 ± 0.09	548 ± 949	0.015 ± 0.016
DEN	corn oil (30 wks)	0.76 ± 0.41	9473 ± 6326	0.014 ±0.005
DEN	TCDD (30 wks)	6.8 ± 4.8	27715 ± 12009	0.046 ± .037
DEN	corn oil (60 wks)	3.8 ± 0.6	18747 ± 1447	0.05 ± 0.02
DEN	TCDD (30 wks) +corn oil (30 wk.)	2.8 ± 1.2	5536 ± 1614	0.13 ± 0.08
DEN	corn oil (15 wks)+TCDD (30 wks)+ corn oil (15 wks)	3.6 ± 4.4	6512 ±4394	0.298 ± 0.71

Values are represented as the mean ± standard deviation for n=6-12