Modeling the Number and Size of Hepatic Focal Lesions Following Exposure to 2,3,7,8-TCDD

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1.0 Introduction

Data on the size and number of altered hepatic foci (AHF) were collected from two separate two-stage hepatocarcinogenesis studies in female Sprague-Dawley rats (Pitot et al, 1987; Tritscher et al, 1992). The studies consisted of multiple TCDD-exposed dose groups including both diethylnitrosomine (DEN) initiated and uninitiated animals. In one study, groups were observed after 229 days of TCDD exposure. In the other study, control animals were observed after 240 days and treated groups were observed after 180 days of TCDD exposure. The parameters in the first half of a two-stage mathematical model of carcinogenesis were estimated from these data.

The results suggest that TCDD stimulates the production of AHF (possibly through secondary mechanisms which increase the rate of formation of mutations that lead to these focal lesions) and promotes the growth of AHF. This finding suggests a mechanism for TCDD-induced carcinogenicity which we refer to as activation, labelling TCDD as an activator. The analysis also indicates there is an interaction between DEN and TCDD which results in dose-related formation of initiated cells throughout the study period. Best-fitting curves for TCDD-induced activation reached saturation levels at low doses of TCDD. For these data, best-fitting curves were all linear in the low-dose region.

2.0 Two-Stage Model Analysis

The methods originally described by Moolgavkar et al (1990) and modified for two-stage experiments by Luebeck et al (1991) were applied to the focal lesion data. The model (see Figure 1) is a simple one-stage model of lesion growth and model parameters estimated from these data are $\mu_1(d)$ (the rate (cell replication^{-1}) at which normal hepatocytes mutate to become initial cells in the AHF), γ_1 (the initial number of initiated cells resulting from the application of DEN), $\beta(d)$ (the rate (day^{-1}) of replication of initiated cells) and $\rho(d)$ (the ratio of the death rate (δ in Figure 1) to the birth rate for initiated cells). Note that the parameter μ_2 in Figure 1 is not estimated since it pertains to events which occur after the formation of AHF.

It is assumed that an average hepatocyte has a radius of 12 μm suggesting that the smallest detectable focus has 8 cells or a radius of 34 μm (the square root of $8x12^2$). It is also assumed that, at the start of the experiment, there are $X_0=1.9x10^9$ normal hepatocytes per liver and that normal cells undergo mitosis at the rate 0.00249 divisions per cell per day. These assumptions were studied in the analysis.

3.0 Animals and Treatment

Two different experiments are examined in this analysis; one from Tristcher et al (1992) and

the other from Pitot et al (1987). Tritscher et al used female Sprague-Dawley rats which were divided into 10 exposure groups with 8 to 10 animals per group. At 70 days, 5 of the groups received 175 mg/kg DEN by i.p. injection. Starting two weeks after this injection, four of these groups received TCDD by gavage in corn oil once every two weeks. Dosages of TCDD in these four groups were equivalent to 3.5, 10.7, 35.7 and 125 ng/kg/day. The remaining group received corn oil as a vehicle control. The other five groups received identical exposures and dosages of TCDD, but were not exposed to DEN, receiving 1 μL saline/g body weight as a control for the DEN. One week after the sixteenth dosing with TCDD, the rats were killed.

Pitot et al (1987) administered DEN (10 mg/kg) as a single bolus dosing 24 hours after a 7% partial hepatectomy. Dosing with TCDD was done biweekly with dioxin injected intramuscularly in corn. The resulting doses in the treated groups were 0.1,1,10 and 100 ng/kg/day. There was also an untreated group which received corn oil alone. Five additional groups were not exposed to DEN but were exposed to equivalent doses of TCDD. TCDD-treated animals were sacrificed 180 days following the onset of first exposure to TCDD and the control animals were sacrificed at 240 days following first exposure to the corn oil (TCDD vehicle).

4.0 Altered HepaticFoci

In the study by Tritscher et al, liver tissue was fixed at necropsy. Serial sections of liver were later stained for AHF which were positive for the placental form of glutathione-s-transferase (PGST+) using methods outlined elsewhere (Maronpot et al, 1993). PGST+ foci were quantified and recorded if their size exceeded a minimum of 8 contiguous hepatocytes using a computer assisted image analysis package. Also recorded were liver weights and sample sizes. Replicative DNA synthesis (S-phase) in non-focal hepatocytes (foci were determined histologically) were quantified by BrdU incorporation using a 7-day osmotic pump (see Maronpot et al, 1993). A sample of histological lesions in the control group and the high dose group (DEN initiated animals) were also quantified for cell replication.

In the study of Pitot et al (1987), similar methods were used to quantify and measure three additional AHF in three serial sections; those staining for γ-glutamyltraspeptidase (GGT), for canalicular ATPase (ATP) and for glucose-6-phosphatase (G6P).

5.0 Results

All four lesions from the two different studies produced similar quantitative results. The net results can be divided into two categories; those affecting the birth rate for initiated cells in the two-stage model and those affecting the mutation rate from the normal state to the initiated state. In addition, there was an interaction between TCDD and DEN which was statistically significant and could not be easily explained by a simple process like the model described in Figure 1.

The anticipated mode of action of TCDD prior to this analysis was that TCDD would increase the birth rate of initiated cells. Initially, nonparametric analysis were done to assess the shape of the dose-response for the rates in the two stage model. The nonparametric forms were used to identify parametric forms for the summary analysis given below. For all four AHF types, increases in the birth rates were observed and had the same basic pattern. In all four cases, the dose effect of TCDD on the birth rate was saturable and followed a function of the form

$$\beta_S(d) = \phi_0 + E_{max}\, d / (k_{0.5} + d).$$

where ϕ_0 is the birth rate for untreated animals, E_{max} is the maximum induced increase in the birth rate due to dioxin and $k_{0.5}$ is the dose of dioxin (in ng/kg/day) which results in half of the maximum effect. Table 1 provides these values for the four AHF types described above. All four lesion types show similar effects of TCDD on the growth of the lesion. In all four cases, the background growth rate is about 0.035 replications per day per cell (a labeling index of about 45%). The birth rates level off at about triple the growth rate of the TCDD-free lesions reaching the plateau at a very low dose and have about the same increase in growth due to TCDD as there is due to DEN. The unusually low values for $k_{0.5}$ for the GGT and G6P

lesions are due to these lesions achieving maximum growth rates at the lowest dose given. These results support the notion that TCDD promotes these lesions, but illustrates that the effect generally occurs for very small doses and is quickly saturated.

Table 1: Maximum likelihood estimates for the birth rate of AHF during chronic exposure to TCDD

Parameter	Tritscher et al (1992)	Pitot et al (1987)		
	PGST	ATP	GGT	G6P
ϕ_0 (constitutive birth rate)	0.0331	0.0372	0.0364	0.0383
E_{max} (maximum induction)	0.00639	0.0964	0.0138	0.0151
$k_{0.5}$ (half maximum dose)	9.93	1.35	0.001	0.0002
Added effect of DEN initiation	0.00641	0.0157	0.00911	0.0043

The effects of TCDD on the formation of new focal lesions is difficult to explain. Table 2 provides the estimated dose-response of TCDD on the net rate for formation of new AHF. The rates observed in untreated animals are reasonable and in the same range as those observed by Luebeck et al (1991) in female Wistar rats exposed to a combination of polychlorinated biphenyls (their values were 6.8 mutations per cm^3 for ATP lesions and 1.8 mutations per cm^3 for GGT lesions).The number of initiated cells due to DEN exposure prior to promotion (last row of Table 2) is very small and, in combination with the mutation rates, would seem to indicate that most lesions arise due to a continued effect of DEN rather than an initiated effect at the start of the experiment. However, from the analysis of the data of Tritscher et al (1992), the mutation rate in untreated animals is 8.293 mutations per cm^3 per day. If this rate pertained to the time prior to exposure to TCDD, this would result in a cumulative formation of 116.1 mutations per cm^3 at the start of promotion. Allowing for death of some of these lesions, the estimated value of 2.779 is still remarkably small and difficult to explain. The AHF due to the work of Pitot et al (1987) are more consistent (especially GGT lesions) with the rates in the untreated animals and would suggest the necrogenic dose applied by Tritscher et al may preferentially kill PGST+ cells. However, it should be noted that this estimated initiation value has very wide confidence bounds which include the projected values discussed above in all cases and which indicate a considerable lack of precision.Of greater concern is the effect of TCDD on the mutation rates seen in Table 2. A statistical test for the significance of this effect is highly significant ($p<0.001$) for all four AHF. Attempts to use the labeling data and organ size data of Maronpot et al (1993) to explain these results for the Tritscher et al data have yielded results which still significantly reject the hypothesis of no effect of TCDD on the mutation rate. There are several possibilities for how TCDD results in the increased production of AHF. One hypothesis is that TCDD alters the production of cytochrome P-450 1A2 (CYP1A2) which in turn results in an increase in the metabolism of estrogens to catechol estrogens (Graham *et al.*, 1988) and that further activation of these catechol estrogens can lead to cell damage (for example via free oxygen radicals) and eventually to mutations (Metzler, 1984). Yang et al (1993) found that human keratinocytes immortalized by adenovirus 12-simian virus 40 induced carcinoma when transplanted into nude mice following exposure of the keratinocytes to TCDD. This neoplas-

tic transformation correlates well with induction of aryl hydrocarbon hydroxylase suggesting an Ah-receptor mediated process.

Table 2: Mutation rates for the formation of AHF following exposure to TCDD

Dose (ng/kg/day)	Tritscher et al (1992)	Pitot et al (1987)		
	PGST[a]	ATP[a]	GGT[a]	G6P[a]
0.0	8.293	1.136	1.748	1.971
0.1	----	2.503	3.272	5.258
1.0	----	2.651	3.672	1.277
3.57	12.664	----	----	----
10.0-10.7	12.256	3.796	2.295	3.126
35.7	8.298	----	----	----
100.0-125.0	13.241	11.269	7.924	11.425
Initiation[b]	2.779	2.527	21.265	161.13

a.entries are best estimates and units are mutations per cm^3 of liver per day
b.entries are best estimates and represent the number of initiated cells per cm^3 of liver at start of promotion

The potential for a compound to increase the rate of production of initiated cells via indirect mechanisms not related to the birth and death rates of the initiated cells is a novel mechanism. In the case of TCDD, the mechanism seems to be an activation of natural metabolic pathways which result in an increased rate of mutations. Thus, we propose to label TCDD as an "activator" of carcinogenesis.

References

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