# Tumor Promotion by 2,3,7,8-TCDD: Identification of Biomarkers and Characterization of Dose-Response Relationships

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## INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and its structural analogs are ubiquitous environmental pollutants and they produce a broad spectrum of biochemical and toxic effects in animals and humans[1]. TCDD is a potent multisite carcinogen in rodents and most human risk assessments for dioxins are based on the carcinogenic effect in rat liver. The mechanism of dioxin action is not clearly understood but it is generally accepted that most, if not all, effects require an initial interaction with the Ah-receptor.

Of major importance for estimating dioxin's risk in humans is low dose effects and this issue has generated considerable controversy. Our research has focused on the characterization of dose response relationships for Ah-receptor dependent biochemical effects in the liver (enzyme induction and effects on cellular receptor systems) following chronic exposure to TCDD in female Sprague-Dawley rats. Dose response relationships of biochemical effects were compared to more complex biological responses like rate of cell replication and number and size of enzyme altered foci. Biochemical responses such as induction of different enzymes are sensitive markers of exposure and reflect interactions of the Ah-receptor/TCDD complex with dioxin responsive genes. However, their relevance to toxic endpoints is often unclear. Complex biological responses involve interactions of multiple events and might reflect more persistent changes and might correlate better with toxic endpoints. In order to identify biomarkers which reflect a more persistent TCDD mediated change in the liver we conducted a study where rats received TCDD for 30 weeks followed by a recovery period of 30 weeks. Changes in the livers as well as liver- and body weights were compared to changes after 30 weeks of continuous TCDD treatment. In another approach to identify markers for the mechanism of action and markers for adverse health effects we looked at specific TCDD induced changes in presumably preneoplastic lesions compared to surrounding tissue by immunohistochemical techniques.

METHODS

Female Sprague-Dawley rats received a single necrogenic dose of diethylnitrosamine (DEN 175 mg/kg) followed by biweekly oral gavage of various doses of TCDD (0.1 to 125 ng/kg/day) Controls received corn oil (vehicle). After 30 weeks of tumor promotion the animals were sacrificed, tissues frozen and paraformaldehyde-fixed for immunohistochemistry studies. Animals of the highest dose group were allowed to survive for an additional 30 weeks without dosing (60 we study). The 60 we study included age matched naive controls. Parameters measured included induction of CYP1A1 protein by RIA and immunolocalization as well as mRNA quantification by reverse transcriptase polymerase chain reaction (RT-PCR)[2]. Changes in EGF receptor were quantified by binding assay. The mRNA for other dioxin responsive genes (TGFa, PAI2, UGT) was also quantified. Changes in proteins for EGFR, TGFa, estrogen receptor were detected by immunolocalization studies. Cell proliferation was measured by incorporation of bromodeoxyuridine in DNA and expressed as % cells labelled (LI). Foci of cellular alteration were calculated from sections immunohistochemically stained for the placental isozyme of glutathione S-transferase (PGST+ foci).

RESULTS AND CONCLUSIONS

Dose Response Relationships (30 we study) (Table 1):

Induction of CYP1A1 protein as well as mRNA are very sensitive markers for TCDD exposure. The low dose behaviour seems to be proportional between exosure and response[3] which is confirmed by pharmacokinetic modeling of the data[4]. Decrease in EGFR binding is significant at a dose of 10ng/kg/d[5] and pharmacokinetic modeling of the data also showed a proportional response to exposure[4]. PGST+ foci show a trend of increase but the % of liver occupied by foci is significantly different from controls just at the highest dose. However, there is significant interindividual variation within each dose group and even at lower doses there are a few high responders[7]. The labelling index seems to decrease at lower doses and is significantly increased at the highest dose but there is also a high interindividual variation. It is also important to note, that in hepatic focal lesion the labelling index is significantly higher (30-40%) than in surrounding tissue[7].

In summary these studies demonstrate that dose response relationships are different for various biochemical and biological TCDD mediated effects implicating that the shape of dose-response curves for effects of receptor mediated carcinogens, like TCDD, can not be predicted solely on the basis that the response is receptor mediated. In addition these studies show, that some of the TCDD mediated changes can not be detected on the transcriptional level but they are detectable on the protein level, e.g. mRNA levels for TGFa seem not to be effected by TCDD in rat liver but preliminary results by immunohistochemical techniques show an increase in TGFa protein.

**Table 1**: Dose response relationships for 2,3,7,8-TCDD mediated effects in livers of DEN initiated female Sprague-Dawley rats after 30 weeks of chronic exposure. Values expressed as % of controls.

| | Dose TCDD (ng/kg/day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 0.1 | 0.3 | 1 | 3.5 | 10 | 35 | 125 |
| UGT-1 (mRNA) | 100 | 134 | 99 | 216 | 226* | --- | --- | 262* |
| PAI-2 (mRNA) | 100 | --- | --- | --- | --- | --- | --- | 105 |
| TGFa (mRNA) | 100 | --- | --- | --- | --- | --- | --- | 120 |
| EGFR (protein) | 100 | --- | --- | --- | 106 | 90 | 64* | 39* |
| CYP1A1 (mRNA) | 100 | 165 | 345* | 2900* | 5710* | --- | --- | 26400* |
| CYP1A1 (protein) | 100 | 139 | 230* | 1009* | 2376* | 8700* | 16300* | 22050* |
| PGST+ foci (Vol. Fract.) | 100 | --- | --- | --- | 149 | 175 | 163 | 391* |
| LI (%) | 100 | --- | --- | --- | 62* | 61 | 121 | 272* |

* significantly different from controls by Student's t-test $p<0.05$

60 Week Study (Table 2):

TCDD tissue concentrations were measured after 30 weeks of dosing and after 30 weeks dosing followed by 30 weeks of recovery. Tissue concentrations in livers after the recovery period are still significantly elevated over control values and the calculated elimination half life for TCDD in the rat liver is appr. 25 days. Biochemical changes, induction of CYP1A1 and decrease in EGFR, are slightly different from controls in the 60 we study but the changes are significantly lower than after 30 we of continous exposure. The labelling index is still high in livers of treated animals even after the recovery period indicating that this response by that time is independent of TCDD tissue concentration. The labelling index is 2.8-fold increased over age matched controls as compared to a 4.2-fold increase over controls at the end of 30 weeks of dosing. PGST+ foci, are quantified as foci per $mm^2$ as well as the volume fraction (%foci/volume liver). The results show that after the recovery period there is still a high percentage in PGST+ foci in the livers. The volume fraction is significantly increased over controls and 2-3 fold increased over livers of rats sacrificed after 30 weeks of chronic treatment. This indicates that the overall number of foci decreases but certain PGST+ foci continue to grow significantly despite decreasing TCDD tissue levels. Consistent with this finding is the increase in absolute and relative liver weights.

Preliminary results looking at various TCDD mediated changes in serial sections of fixed liver tissue by immunohistochemistry indicate that effects are different in PGST+ foci and in the foci surrounding tissue. EGFR seems to be decreased in foci as compared to surrounding tissue whereas estrogen receptor seems to be elevated at least in some foci.

In summary this study shows that some TCDD induced biochemical responses in the rat liver (CYP1A1 induction and decrease in EGFR binding) are reversible and might be directly dependent on the presence of TCDD whereas more complex responses (cell proliferation and growth of enzyme altered foci) are independent of the TCDD tissue concentration at later time points.

**Table 2**: Comparison of 2,3,7,8-TCDD mediated effects in livers of DEN initiated female Sprague-Dawley rats after 30 weeks of continous exposure and 30 weeks exposure followed by 30 weeks of recovery.

| | 30 Weeks | | 60 Weeks | |
|---|---|---|---|---|
| | control | TCDD | control | TCDD |
| TCDD levels (ppb) | --- | 19.9 ± 4.8 | $<1x10^{-3}$ | $64 \pm 22 x10^{-3}$ |
| EROD (pmol/mg/min) | 628 ± 435 | 6159 ± 2219 | 193 ± 56 | 247 ± 72 |
| LI (%) | 3.4 ± 1.9 | 14.3 ± 8.3 | 3.1 ± 3.2 | 8.6 ± 3.6 |
| PGST+: | | | | |
| Foci/sq.mm | 0.31 ± 0.46 | 26.5 ± 15.7 | 0.88 ± 1.07 | 17.2 ± 11.3 |
| %Foci/vol. | 0.01 ± 0.01 | 2.23 ± 1.47 | 0.32 ± 0.97 | 6.05 ± 4.34 |
| LW/BW x 100 | 3.4 ± 0.2 | 4.5 ± 0.3 | 3.2 ± 0.2 | 6.8 ± 1.55 |

REFERENCES

1. E. K. Silbergeld, and T. A. Gasiewicz, Dioxins and the Ah receptor, *Amer. J. Indust. Med.* 1989;16:455-474.

2. J. P. Vanden Heuvel, F. L. Tyson, and D. A. Bell, Construction of recombinant RNA templates for use as internal standard in quantitative RT-PCR, *BioTechniques* 1993;14:395-398.

3. A. M. Tritscher, J. A. Goldstein, C. J. Portier, Z. McCoy, G. C. Clark, and G. W. Lucier, Dose-response relationships for chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in a rat tumor promotion model: quantification and immunolocalization of CYP1A1 and CYP1A2 in the liver, *Cancer Research* 1992;52:3436-3442.

4. C. Portier, A. Tritscher, M. Kohn, C. Sewall, G. Clark, L. Edler, D. Hoel, and G. Lucier, Ligand/Receptor binding for 2,3,7,8-TCDD: Implications for risk assessment, *Fundam. Appl. Toxicol.* 1993;20:48-56.

5. C. H. Sewall, G. W. Lucier, A. M. Tritscher, and G. C. Clark, TCDD-Mediated changes in hepatic epidermal growth factor receptor may be a critical event in the hepatocarcinogenic action of TCDD, *Carcinogenesis* 1993 (in press).

6. R. R. Maronpot, J. F. Foley, K. Takahashi, T. Goldsworthy, G. Clark, A. M. Tritscher, C. J. Portier, and G. W. Lucier, Dose response for TCDD promotion of hepatocarcinogenesis in rats initiated with DEN: Histologic, biochemical, and cell proliferation endpoints, *Environ. Health Perspect.* 1993 (submitted).