Use of reverse-transcription polymerase chain reaction to quantitate mRNA for dioxin-responsive genes in the low-dose region in rat liver.

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Introduction:

TCDD and related chemicals, upon binding to the Ah receptor, result in altered transcription of numerous genes. The most widely examined dioxin-responsive gene is cytochrome P4501A1 (CYP1A1). The induction of CYP1A1 is an extremely sensitive effect of dioxin although the relationship of this response to toxicity or carcinogenicity is unclear. Many genes which are induced by dioxin have effects on growth regulation or differentiation and these could represent steps in the hepatocarcinogenic effects of this class of compounds. These genes include transforming growth factor-α, plasminogen activator inhibitor-2, interleukin-1β and tumor necrosis factor-α.

An important issue in dioxin risk assessment is the shape of the dose-response curve in the low dose region. The purpose of the present study was to examine the dose-response relationships of several dioxin-responsive genes in rat liver following both single exposure as well as chronic exposure within the framework of a tumor-promotion model. We chose to examine the expression of several genes using a very sensitive method, reverse-transcription polymerase chain reaction (RT-PCR). Using competitive RT-PCR low expression genes in rat liver such as CYP1A1 could be easily detected and quantitated. The effects of TCDD in the low-dose region on CYP1A1, PAI2, TGF-α, TNF-α and IL1β are compared.

Methods

Single Exposure Studies: Female Sprague-Dawley rats (225-275 g) were given a single dose of TCDD by oral gavage in corn oil. The doses examined ranged from 0.05 ng TCDD/kg body weight to 10,000 ng TCDD/kg body weight. Four days posttreatment livers were excised and quickly frozen in liquid nitrogen. Total hepatic RNA was extracted using guanidine thiocyanate and DNA removed using conventional phenol/chloroform/isoamyl alcohol partitioning procedures[1].

Tumor Promotion Model: Female Sprague-Dawley rats (225-275 g) were given a single initiating dose of diethylnitrosamine followed by repeated oral doses of TCDD in corn oil[2]. The doses examined ranged from 0.1 ng/kg/day to 125 ng/kg/day. After

30 weeks of TCDD exposure, livers were excised, frozen and total RNA extracted as stated above.

RT-PCR methods: In order to accurately quantitate mRNA using PCR methods, internal standards are needed[3]. A novel PCR-based procedure was devised to synthesize recombinant mRNA (rcRNA) internal standards containing forward and reverse PCR primer sequences for several of the dioxin-responsive genes (target mRNA)[4]. A dilution series of rcRNA was added into rat liver total RNA samples prior to RT-PCR. The rcRNA and target mRNA PCR products were resolved on agarose gels, stained with ethidium bromide and quantitated by laser densitometry.

Results and Discussion

The use of rcRNA internal standards allows for correction of sample-to-sample variation, in mRNA content, reverse-transcription, and PCR amplification. This eliminates a major problem of quantitating mRNA using PCR. Using a 30-cycle PCR protocol we were able to detect as few as 10 molecules of rcRNA per reaction. In addition, RT-PCR was able to detect and quantitate constitutive expression of CYP1A1, a low expression gene in uninduced rat liver. The PCR-based method for synthesis of CYP1A1 rcRNA internal standards has been applied to other dioxin-responsive genes with equal success. Table 1 shows the genes which we currently have rcRNA and RT-PCR methods developed.

Table 1
Dioxin-Responsive Genes

Gene/Product	Transcriptional Activation	rcRNA Internal Standard
CYP1A1, cytochrome P_1450	+	+
CYP1A2, cytochrome P_2450	+	--
UGT1, UDP-glucuronyl-transferase	+	+
PAI2, plasminogen activator inhibitor-2	+	+
IL1ß, interleukin-1ß	?	+
TNF, tumor necrosis factor	?	+
TGF-α, transforming growth factor-α	?	+
EGFR, epidermal growth factor receptor	--	+
ß-Actin[1]	--	+

[1] Used as an external standard

As expected, induction of CYP1A1 was a very sensitive effect of dioxin administration. Following a single dose of 1 ng TCDD/kg body weight, the amount of CYP1A1 mRNA increased 6-fold over that of vehicle-treated rats. Maximal induction of approximately 10,000-fold was seen following treatment with 1 and 10 μg TCDD/kg body weight. The shape of the dose-response curve and the low-dose effects are currently being evaluated although preliminary analysis indicates that there is no evidence of a threshold for this response. The lowest dose in our study (50 pg/kg) produces tissue levels which are approximately equivalent to those produced by background exposures in humans to TCDD and structural analogs.

Plasminogen activator inhibitor-2 (PAI2) is transcriptionally activated in human keratinocytes by dioxin. However, preliminary experiments suggest this gene may not be induced by dioxin in rat liver. Following a single administration of 10 μg TCDD/kg body weight there was no induction of PAI2 mRNA as assessed by RT-PCR. Whether higher doses of dioxin are needed to see increases in PAI2 transcription in rat liver is currently being investigated.

The comparison between CYP1A1 and PAI2 illustrates that genes under direct control of the Ah receptor have quite different dose-response curves and tissue-specific regulation. In addition, better surrogates for assessing the carcinogenicity of dioxin are needed because CYP1A1 induction, although it is an extraordinarily sensitive indicator of exposure, may not reflect the tumor promoting activity of this compound. For this reason, we are currently examining low-dose effects of other dioxin-responsive genes in a tumor promotion model and correlating mRNA induction to cell proliferation and the development of putative preneoplastic lesions.

References

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