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Chapter 6. Carcinogenicity of TCDD in Animals

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Chapter 6. Carcinogenicity of TCDD in Animals

Health Assessment for
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
and Related Compounds

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Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, D.C.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Ah	Aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
ALT	L-alanine aminotransferase
AST	L-asparate aminotransferase
BDD	Brominated dibenzo-p-dioxin
BDF	Brominated dibenzofuran
BCF	Bioconcentration factor
BGG	Bovine gamma globulin
bw	Body weight
cAMP	Cyclic 3,5-adenosine monophosphate
CDD	Chlorinated dibenzo-p-dioxin
cDNA	Complementary DNA
CDF	Chlorinated dibenzofuran
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DCDD	2,7-Dichlorodibenzo-*p*-dioxin
DHT	5α-Dihydrotestosterone
DMBA	Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

LIST OF ABBREVIATIONS (cont.)

DRE	Dioxin-responsive enhancers
DTG	Delayed type hypersensitivity
DTH	Delayed-type hypersensitivity
ED_{50}	Dose effective for 50% of recipients
ECOD	7-Ethoxycoumarin-0-deethylase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EROD	7-Ethoxyresurofin 0-deethylase
EOF	Enzyme altered foci
FSH	Follicle-stimulating hormone
GC-ECD	Gas chromatograph-electron capture detection
GC/MS	Gas chromatograph/mass spectrometer
GGT	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
GST	Glutathione-S-transferase
HVH	Graft versus host
HAH	Halogenated aromatic hydrocarbons
HCDD	Hexachlorodibenzo-p-dioxin
HDL	High density lipoprotein
HxCB	Hexachlorobiphenyl

LIST OF ABBREVIATIONS (cont.)

HpCDD	Heptachlorinated dibenzo-p-dioxin
HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HxCDD	Hexachlorinated dibenzo-p-dioxin
HxCDF	Hexachlorinated dibenzofuran
ID_{50}	
I-TEF	International TCDD-toxic-equivalency
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)
LH	Luteinizing hormone
LDL	Low density liproprotein
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MFO	Mixed function oxidase
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK	Natural killer

LIST OF ABBREVIATIONS (cont.)

NOAEL	No-observable-adverse-effect level
NOEL	No-observed-effect level
OCDD	Octachlorodibenzo-p-dioxin
OCDF	Octachlorodibenzofuran
PAH	Polyaromatic hydrocarbon
PB-Pk	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyl
OVX	Ovariectomized
PBL	Peripheral blood lymphocytes
PCQ	Quaterphenyl
PeCDD	Pentachlorinated dibenzo-p-dioxin
PeCDF	Pentachlorinated dibenzo-p-dioxin
PEPCK	Phosphopenol pyruvate carboxykinase
PGT	Placental glutathione transferase
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
ppm	Parts per million
ppq	
ppt	Parts per trillion
RNA	Ribonucleic acid
SAR	Structure-activity relationships

LIST OF ABBREVIATIONS (cont.)

SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SRBC	Sheep erythrocytes (red blood cells)
$t_{½}$	Half-time
TCAOB	Tetrachloroazoxybenzene
TCB	Tetrachlorobiphenyl
TCDD	Tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factors
TGF	Thyroid growth factor
tPA	Tissue plasminogen activator
TNF	Tumor necrosis factor
TNP-LPS	lipopolysaccharide
TSH	Thyroid stimulating hormone
TTR	Transthyretrin
UDPGT	UDP-glucuronosyltransferases
URO-D	Uroporphyrinogen decarboxylase
VLDL	Very low density lipoprotein
v/v	Volume per volume
w/w	Weight by weight

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6. CARCINOGENICITY OF TCDD IN ANIMALS

6.1. INTRODUCTION

There is more scientific information relevant to the use of animal cancer data for the estimation of human risks than was available in 1988. However, much of the tumor incidence data in experimental animals was available in 1988 to demonstrate that TCDD is a carcinogen at multiple sites in both sexes of rats and mice. Some of the cancers occurred following low doses. Since 1988, TCDD has been shown to be a carcinogen in hamsters and some of the tumor incidence data in rat liver has been reevaluated during the last 3 years.

In the last few years there have been several studies which have impact on the evaluation of cancer studies in experimental animals. For example, the evidence is now considerably stronger that TCDD does not damage DNA directly through the formation of DNA adducts. However, there are proposed mechanisms for the possibility that TCDD might alter the DNA damaging potential of some endogenous compounds including estrogens. In addition, there have been numerous reports on TCDD-mediated modifications of growth factor pathways and cytokines in experimental animals and cell systems. Some of the altered systems include those for epidermal growth factor, transforming growth factor α, estrogen, glucocorticoids, tumor necrosis factor α, interleukin 1 B, plasminogen inactivating factor and gastrin. Many of these pathways are involved in cell proliferation and differentiation and provide plausible avenues to research the mechanisms responsible for the carcinogenic actions of TCDD. These effects are consistent with the general accepted conclusion that TCDD acts as a tumor promoter in multistage models for chemical carcinogenesis and is virtually devoid of initiating activity in these models. It is important to note that "tumor promotion" is an operational and not a mechanistic term and there are likely multiple mechanisms of tumor promotion. Each of these mechanisms may be fundamentally different from the other.

Over the last few years there has been growing consensus that most, if not all, of TCDD's biochemical and toxic effects require interaction with the Ah receptor. The properties of the Ah receptor and the mechanisms whereby this receptor regulates gene expression will be evaluated in other chapters. However,

formation of the Ah receptor-TCDD complex is only the first of many steps involved in the production of a biochemical and toxic effect. Although we are gaining increasing detail of the subsequent steps, we know very little about some components of the Ah receptor mediated responses. It is clear, however, that cell specific factors other than the Ah receptor must be involved in determining tissue responses once TCDD binds the Ah receptor.

Evaluation of dose-response is one of the more issues that impact dioxin risk assessments. The focus of the controversy centers on whether the effects of dioxin would exhibit a threshold or not. It now appears that for some responses there is a proportional relationship between receptor occupancy and response which is evidenced by a linear relationship between target dose and effect over a wide dose range. However, different dose response relationships are seen for different responses so it is likely inappropriate to use a single surrogate marker to estimate dioxin's risks. Furthermore, these data reveal there is no unifying dose-response relationship for all Ah receptor mediated events.

Another controversial area in risk assessment is whether experimental animal models are appropriate for estimating human risks. During the last few years there has been increasing evidence that biochemical and toxic responses resulting from human exposure to TCDD and its structural analogs appear to be similar to responses in experimental animals. However, there is also increasing awareness that interindividual variation in human responses to dioxin are a complicating factor in risk assessment; it appears there are responsive and non-responsive individuals to numerous environmental chemicals including TCDD.

Much of the controversy surrounding dioxin risk assessment reflects the selection of methods; threshold or linear multistage. We now know considerably more about mechanism of action of dioxin and this knowledge may permit the construction of biologically-based models which removes some of the uncertainty in current risk estimates. These approaches and recent advances on mechanisms of tumor promotion and dose-response relationships for biochemical and biological events relevant to the carcinogenic actions of dioxin will be discussed in more detail in the following sections of the paper.

6.2. ANIMAL BIOASSAYS FOR CANCER

There have been seventeen long-term bioassays for carcinogenicity of TCDD in several species. All seventeen produced positive results. It is clear that TCDD is a multistage carcinogen in both sexes of rats and mice (Huff et al., 1991; Zeise et al, 1990). It is also a carcinogen in the hamster which is considered the most resistant species to the acute toxic effects of TCDD. The seventeen studies are summarized in Table 6-1 including information on species, sex, dose and tumor site. Some of the studies are especially relevant to risk assessment. Detailed evaluations of these studies are given in the following paragraphs.

6.2.1. Kociba Study. The most cited cancer bioassay for TCDD was published in by Kociba et al. (1978). It was a lifetime feeding study of male and female Sprague-Dawley rats using doses of 1, 10 and 100 ng/kg/day. There were 50 males and 50 females in each group. Data derived from these studies provide the basis for many of the risk assessments for TCDD. The most significant finding was an increase in hepatocellular hyperplastic nodules and hepatocellular carcinomas in female rats. The carcinomas were significantly elevated above the control incidence at the 100 ng/kg/day dose, whereas increased incidences of hyperplastic nodules were evident in the 10 ng/kg/day dose group. There have been two reevaluations of the Kociba slides of liver sections (Squire, 1985; Sauer, 1990). The Squire review was requested by EPA as an independent review of the slides. The Sauer review used diagnostic criteria for liver tumors described by Maronpot et al., (1986). Liver tumor incidences for the three evaluations are compared in Table 6-2. Although there are some quantitative differences in the evaluations, the lowest detectable effect is consistently 10 ng/kg/day for liver tumor incidence. In the 10 ng/kg/day dose group hyperplastic nodules of the liver were observed in female rats (18 Kociba, 27 Squire). Two females had carcinomas of the liver. In the recent reevaluation of liver lesions by Sauer (1990), nine females were identified with hepatocellular adenomas and none with carcinomas; thus only one-third of the previously observed tumors were confirmed. There was no detectable increase in liver tumor incidences in male rats (Table 6-1) in any of the dose groups. The mechanism responsible for dioxin-mediated

TABLE 6-1

Sites for Increased Cancer in Animal Bioassays*

Species/Strain	Sex	Site
Rats/Sprague-Dawley	male	tongue nasal turbinates/hard palate
	female	lung nasal turbinates/hard palate liver
Mice/Osborne-Mendel	male	thyroid adrenal cortex
	female	liver adrenal cortex subcutaneous fibrosarcoma
Mice/B6C3F1	male	liver
	female	subcutaneous fibrosarcoma liver thyroid
Mice/B6CeF1	male/	thymic lymphomas
	female	liver
Syrian Golden	male	facial skin carcinoma

*Source: Kociba et al., 1978; NTP, 1982; Della Porta et al., 1987; Rao et al., 1988

TABLE 6-2

Dif.erent Evaluations of Kociba Liver Tumor Data in Female Rats[a,b]

Study	Tumor Type	Control	Dose (ng/kg/day)		
			1	10	100
Kociba	hyperplastic nodule	8/86 p<0.001	3/50 p=0.8	18/50 p<0.001	23/50 p<0.001
	hepatocellular carcinoma	1/86 p<0.001	0/50 --	2/50 p=0.3	11/50 p<0.001
	hyperplastic nodule; hepatocellular carcinoma	9/86 p<0.001	3/50 p=0.7	20/50 p<0.001	34/50 p<0.001
Squire	hyperplastic nodule; hepatocellular carcinoma	16/68 p<0.001	8/50 p=0.7	27/50 p<0.001	33/47 p<0.001
Sauer	hepatocellular adenoma	2/86	1/50	9/50	14/50
	hepatocellular carcinoma	0/86	0/50	0/50	4/50
	hyperplastic nodule; hepatocellular carcinoma	2/86	1/50	9/50	18/50

[a]Source: Kociba et al., 1978

[b]p-Values for Fisher's exact test are given beneath the incidence data for TCDD-treated animals; Mantel-Haenszel trend test are given beneath the control incidences

sex specificity for hepatocarcinogenesis in rats is not clear but may involve estrogens and this is discussed in the section on tumor promotion.

Kociba et al. (1978) had reported that chemically-related preneoplastic or neoplastic lesions were not found in the 1 ng/kg/day dose group. However, Squire identified two male rats in the 1 ng/kg/day dose group with squamous cell carcinoma of the nasal turbinates/hard palate and a separate male squamous cell carcinoma of the tongue. These are both rare tumors for Sprague-Dawley rats and these sites are targets for TCDD implying that the 1 ng/kg/day may not represent a no observed effect level (NOEL).

In addition to the liver, tongue, nasal turbinates and hard palate, increased lung tumor incidences were observed in female rats (seven Kociba, nine Squire). The increase, at the high dose (100 ng/kg/day), was statistically significant for keratinizing squamous cell carcinomas.

One of the more interesting findings in the Kociba bioassay was reduced tumor incidences of the pituitary, uterus, mammary gland, pancreas and adrenals. For example, carcinomas of the mammary gland occurred in 8/86 of the control female rats whereas the incidence was 0/49 in the 1 ng/kg/day dose group. However, the incidence of mammary gland carcinomas in the medium- and high-dose groups was similar to that of control rats suggesting that protection against breast cancer might be a low-dose effect. These findings coupled with the sex specificity of TCDD induced liver tumors emphasizes that the carcinogenic actions of TCDD involve a complex interaction of hormonal factors. Moreover, it appears likely that cell specific factors modulate TCDD/hormone actions relevant to cancer.

6.2.2. NTP Study (Osborne-Mendel Rats and B6C3F1 Mice) (NTP, 1982a). Groups of 50 male rats, 50 female rats and 50 male mice received doses of 10, 50 or 500 ng/kg/week TCDD by gavage in two administrations each week for two years; groups of 50 female mice were given 40, 200 or 2000 ng/kg/week. These exposures correspond to average daily doses of 1.4, 7.1 or 71 ng/kg/day for rats and male mice and to doses of 5.7, 28.6, or 286 ng/kg/day for female mice so the doses were roughly similar to those used in the Kociba dietary study. There were no statistically significant dose-related decreases in survival in any sex-species group.

Tumor data in the NTP bioassay are summarized in Tables 6-3 and 6-4. TCDD-induced malignant liver tumors in the high-dose female rats and in male and female mice. These can be considered to result from TCDD exposure since they are relatively uncommon lesions in control Osborne-Mendel rats (male 1/208; female 3/208), are seen in female rats and mice of both sexes and their increasing incidence with increasing dose is statistically significant (Cochran Armitage trend test, p=0.004). Since liver tumors were increased in both sexes of mice, this effect is not female specific as observed in rats. Interestingly, liver tumor incidences were decreased in female rats in both the NTP and Kociba low doses (not statistically significant compared to controls). For example, the combined control incidence data were 11/161 compared to 4/99 (4%) in the low-dose group.

The incidences of thyroid gland (follicular cell) tumors were increased in all three dosed groups in male rats. Because the responses in the two highest dose groups are highly significant, the elevation of incidence in the lowest dose group (Fisher exact p value=0.42) is considered to be caused by exposure to TCDD. Thus, for this study the LOEL is 1.4 ng/kg/day and a NOEL was not achieved within the specified dose range suggesting that thyroid tumor incidence may be the most sensitive site for TCDD-mediated carcinogenesis.

TCDD induced neoplasms of the adrenal gland in high-dose female rats. Fibrosarcomas of the subcutaneous tissue were significantly elevated in high-dose female mice and possibly female rats. One additional tumor type, lymphomas, were seen in high-dose female mice. Lung tumors were elevated in high-dose female mice; the increase was not statistically significant when compared with concurrent controls but the increase was dose related (Cochran Armitage trend test p=0.004).

Therefore, TCDD is a multisite carcinogen and it increased neoplasms in rats and mice of both sexes. As in Kociba et al. (1978), liver tumors were observed with greater frequency in treated female rats, but the male thyroid appears to be the most sensitive (increased tumor incidence doses as low as 1.4 ng/kg/day).

6.2.3. Syrian Golden Hamster. Groups of 10-24 male Syrian Golden hamsters were given two to six intraperitoneal or subcutaneous injections of TCDD over a 4-week period at doses of 50 or 100 μg/kg TCDD in dioxane (Rao et al., 1988). The

TABLE 6-3

Tumor Incidences in Male and Female Osborne-Mendel Rats Given TCDD by Gavage for 2 Years[a,b]

Target Organ/Tumor Type	Sex	Dose (ng/kg/day)			
		0	1.4	7.1	71
Thyroid follicular cell adenoma	males	1/69 p=0.006	5/48 p=0.042	6/50 p=0.021	10/50 p=0.001
Liver neoplastic nodule		0/74 p=0.005	0/50 --	0/50 --	3/50 p=0.06
Adrenal cortex adenoma		6/72 p=0.26	9/50 p=0.09	12/49 p=0.015	9/49 p=0.09
Liver neoplastic nodule	females	5/75 p<0.001	1/49 --	3/50 --	12/49 p=0.006
Adrenal cortex adenoma or carcinoma		11/73 p=0.014	9/49 p=0.4	5/49 --	14/46 p=0.039
Subcutaneous fibrosarcoma		0/75 --	2/50 p=0.16	3/50 p=0.06	4/49 p=0.023

[a]Source: NTP, 1982

[b]p-Values under the tumor incidence data of controls are from Cochran Armitage test for dose-related trend and p-values under TCDD-treated groups are from Fisher's exact trend test

TABLE 6-4

Tumor Incidences in Male and Female B6C3F1 Mice Given TCDD by Gavage for 2 Years[a,b]

Target Organ/Tumor Type	Sex	Dose (ng/kg/day)			
		0	1.4	7.1	71
Liver	male				
carcinoma		8/73 p=0.002	9/49 p=0.19	8/49 p=0.28	17/50 p=0.002
adenoma		7/73 p=0.024	3/49 --	5/49 p=0.6	10/50 p=0.09
Subcutaneous fibrosarcoma	female	1/74 p=0.007	1/50 p=0.6	1/48 p=0.6	5/47 p=0.032
Liver					
carcinoma		1/73 p=0.008	2/50 p=0.4	2/48 p=0.4	6/47 p=0.014
adenoma		2/73 p=0.11	4/50 p=0.2	4/48 p=0.2	5/47 p=0.08
Thyroid follicular cell adenoma		0/69 p=0.016	3/50 p=0.07	1/47 p=0.4	5/46 p=0.009
Lymphoma		18/74 p=0.011	11/50 --	13/48 p=0.4	20/47 p=0.029

[a]Source: NTP, 1982

[b]p-Values for controls represents Cochran-Armitage trend test and p values for TCDD-treated groups derived from Fisher,s exact test.

experiments were terminated after 12-13 months. The 100 μg/kg groups (total dose of 600 μg/kg) from both injection routes developed squamous cell carcinomas of the skin in the facial region: 4/18 (22%) from the intraperitoneal injection and 3/14 (21%) from the subcutaneous injection. The lesions were large (1.5-3 cm) with extensive necrosis and some metastasized to the lung. The earliest neoplasms were detectable 8 months after the initial injection. Similar lesions were not seen in hamsters receiving two intraperitoneal injections of 100 μg/kg TCDD or six subcutaneous injections of dioxane vehicle and none have been reported over the past 10 years in this laboratory. An extensive study by Pour et al. (1976) identified only one skin papilloma in 533 control Syrian hamsters. This report demonstrates that the hamster, a non-responsive species (for acute toxic effects) is susceptible to the carcinogenic actions of TCDD at doses well below the maximum tolerated dose.

6.2.4. B6C3 and B6C mice (Della Porta et al. 1987). TCDD was administered intraperitoneally in corn oil at doses of 0,1,30 and 60 μg/kg to groups of 89-186 B6C3 and B6C mice of both sexes once weekly for 5 weeks starting at the day 10 of life, and the animals were observed until 78 weeks of age. Histopathological observations were limited to the liver, kidney and organs with apparent or suspected pathological changes. Thymic lymphomas were induced at the 60 μg/kg level in both sexes of both hybrids and at 30 μg/kg in all but female B6C3 mice. Neoplasms of the liver occurred in male B6C3 at 30 μg/kg and female B6C3 mice at 60 μg/kg. In a separate experiment, groups of 42-50 B6C3 mice were exposed to 0, 2.5 and 5.0 μg/kg TCDD in corn oil by gavage once weekly for 52 weeks starting at 6 weeks of age. The study was stopped at 110 weeks. Increased incidences of liver tumors were related to TCDD exposure at both dose levels.

In summary, there is convincing evidence in the scientific literature that TCDD is a potent multisite carcinogen in both sexes of several species and carcinogenic effects have been observed at doses over two orders of magnitude less than the maximum tolerated dose.

6.2.5. Carcinogenicity of Related Compounds (NTP, 1980)

A mixture of two isomers of HCDD (1,2,3,6,7,8 and 1,2,3,7,8,9) were given by gavage twice weekly for 2 years to Osborne-Mendel rats and B6C3F1 mice. The doses of HCDD were 0, 1.25, 2.5 or 5 μg/kg/week in rats and male mice. Doses for

female mice were 0, 2.5, 5 and 10 μg/kg/week. There was no affect of administration of TCDD on survival of either sex of rats or mice (NTP, 1980). Results are summarized in Table 6-4 and revealed that HCDD increased liver tumors in both sexes of rats and mice although female rats seemed to be more sensitive than male rats (significant increases detected in female rats in the 1.25 μg/kg/week dose group; equivalent to 180 ng/kg/day). Therefore, HCDD is approximately 1/20 as potent a liver carcinogen as TCDD.

Dermal application of the same HCDD mixture as described above (NTP, 1982b) were given to Swiss Webster mice for 104 weeks (thrice weekly). For the first 16 weeks, doses of 5 ng/application were used. Thereafter doses of 10 ng/application were used. No HCDD-exposure-related carcinogenic responses were noted.

Dibenzo-*p*-dioxin given in the diet for 2 years at concentrations of 0, 5,000 and 10,000 ppm did not increase carcinogenic responses in Osborne-Mendel rats or B6C3F1 mice (NCI, 1979a). DCDD in the diet of Osborne-Mendel rats for 110 weeks or B6C3F1 mice for 90 weeks at levels of 0, 5,000 or 10,000 ppm did not increase neoplasms in male or female rats or in female mice. In male mice, increased incidences of lymphoma or hemangiosarcoma were observed in the low-dose group and neoplasms of the liver were observed in both dose groups (NCI, 1979b). The more highly chlorinated CDDs and CDFs have not been studied in long-term animal cancer bioassays. Many of the CDDs and CDFs bioaccumulate and exhibit toxicities similar to those of TCDD and are considered as carcinogens (EPA Science Advisory Board, 1989; CDHS, 1985).

6.3. MECHANISMS OF TCDD-MEDIATED CARCINOGENICITY

There is substantial evidence that TCDD is not a direct genotoxic agent. Since "genotoxic" and "non-genotoxic" are controversial and often misused terms it is prudent to describe accurately the scientific criteria used to call a chemical "genotoxic" or "non-genotoxic" (IARC, 1992). Some of the criteria for designating TCDD a non-genotoxic agent are that it does not bind covalently to DNA (does not form DNA adducts), is negative in short-term tests for genotoxicity and is a potent promoter and weak initiator in multistage models for chemical carcinogenesis. In a recent study (Turtletaub, 1990) using accelerator mass spectrometry, DNA adducts were not detected in rodent tissue following exposure to TCDD. This method is extraordinarily sensitive, being capable of detecting

one adduct in 10^{12} normal nucleotides. For comparison, approximately one adduct in 10^6 normal nucleotides are found in rodent tissues following carcinogenic doses of benzo(a)pyrene (7,8 diol-9,10 epoxide deoxyguanosine DNA adduct), methylnitrosurea (O^6 methylguanine) or NNK (O^6 methylguanine).

Another criterion for designating TCDD a non-genotoxic carcinogen is that numerous studies have demonstrated that TCDD is negative in the *Salmonella*/Ames test in the presence or absence of a MFO activating system. These negative studies have encompassed 13 different bacteria strains with tests performed in nine laboratories (Wassom et al., 1977; Kociba, 1984; IARC, 1982; Giri, 1987; Shu et al., 1987). NTP (1984) concluded that TCDD was non-mutagenic using its battery of tests for genetic toxicity. Additionally, several scientific panels have stated that false negatives for TCDD genetic toxicity are highly unlikely (EPA Science Advisory Board, 1984). TCDD has been found to promote the transformation of C3H/10T1/2 cells; it was concluded that this response did not reflect TCDD's ability to directly damage DNA (Abernethy et al., 1985). In human populations accidentally or occupationally exposed to TCDD, there is no consistent evidence for increased frequencies of chromosomal aberrations in workers exposed to TCDD (Shu et al., 1987).

Although DNA is negative in genetic toxicity tests, recent reports have demonstrated that TCDD (50-100 μg/kg) induces single strand breaks in Sprague-Dawley rats, presumably as a consequence of increased lipid peroxidation (Wahba et al., 1988, 1989). In another set of studies, increased frequency of sister chromatid exchanges were observed in lymphocytes of people exposed to PCDFs in Taiwan when those lymphocytes are challenged with α-naphthoflavone (Lundgren et al., 1986, 1988). The mechanism responsible for this effect is that the PCDFs cause increased rates of metabolic activation of α-naphthoflavone to DNA reactive metabolites. These findings are consistent with the idea that TCDD's ability to induce drug-metabolizing enzymes (CYP1A1 and 1A2) may lead to increased rate of formation of DNA reactive metabolites of some carcinogens, most notably the polycyclic aromatic hydrocarbons and aromatic amines. However, there is evidence that the opposite effect occurs in some cases since *in vivo* exposure to CYP1A1 inducers actually leads to a decrease in DNA adducts in target tissue following

in vivo exposure to PAHs such as benzo(a)pyrene (Cohen et al., 1979; Parkinson and Hurwitz, 1991). It can reasonably be concluded that TCDD exposure may increase the rate of DNA adduct formation for some carcinogens but decrease the rate for others and that predictions should not be made without experimental data on DNA adduct concentrations in control and TCDD-treated animals.

A final criterion for designating TCDD a non-genotoxic carcinogen is that it is a potent tumor promoter and weak initiator in two-stage models for liver (Pitot et al., 1980; Graham et al., 1988; Lucier et al., 1991; Clark et al., 1991a; Flodstrom and Ahlborg, 1991) and skin (Poland et al., 1982). These findings will be discussed in more detail in the section on tumor promotion including plausible mechanisms for the tumor promoting actions of TCDD such as TCDD-mediated increases in cell proliferation rates of genetically-altered cells.

It is now accepted by the scientific community that most if not all of TCDD's toxic and biochemical effects including tumor promotion are Ah receptor dependent and that TCDD provides an example to evaluate the issues relevant to risk assessment for receptor-mediated carcinogens. The steps involved in Ah receptor-mediated events are reviewed in the chapter on Mechanisms (Whitlock).

6.4. INITIATION-PROMOTION STUDIES

The multistage nature of chemical carcinogenesis is being defined by an increasing understanding of the discrete steps required to produce a genetically-altered cell which is clonally-expanded and ultimately progresses to a tumor (IARC, 1992; Barrett and Wiseman, 1987; Swenberg et al., 1987; Barrett, 1992) (Figure 6-1). Briefly, the process involves damage to a specific site on DNA, a round of cell replication to fix that damage into the genome, clonal expansion of the genetically-altered cells (tumor promotion), followed by additional genetic damage and rounds of cell replication (tumor progression). Figure 6-1 schematizes the multistage nature of cancer. Birth and death rates of genetically-altered cells compared to normal cells is the centerpiece of risk assessment models which recognize the multistage nature of chemical carcinogenesis (Moolgavkar and Knudson, 1981; Portier, 1987). The roles of proto-oncogene activation and tumor suppression genes have provided clues in attempts to dissect out discrete steps in cancer. It is also clear that cell proliferation is an essential component of chemical carcinogenesis, for without it DNA

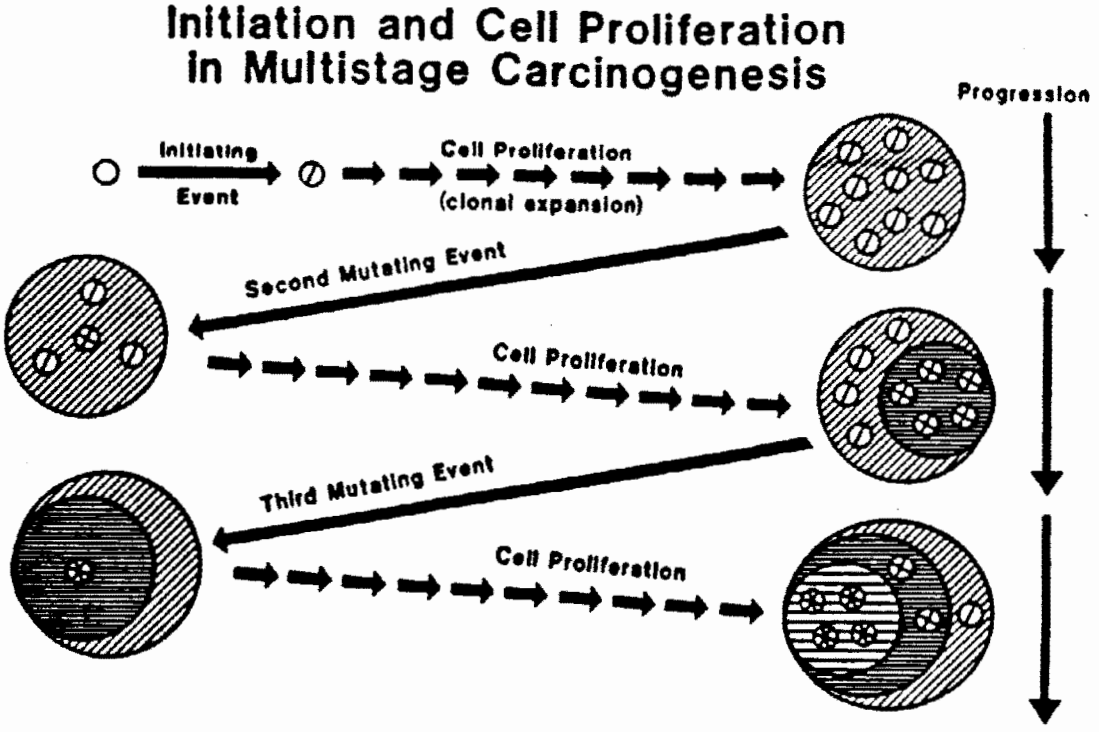

FIGURE 6-1

Schematic representation of multistep carcinogenesis including the roles of genetic damage and cell proliferation. It is important to note that several DNA damaging steps and several cell proliferation steps are likely involved during the complete process of chemical carcinogenesis.

damage would not be fixed into the genome and clonal expansion of genetically-altered cells would not occur.

Concurrent with our increased understanding of the mechanistic underpinnings of chemical carcinogenesis, multistage models have been developed to identify the particular stage or stages in which carcinogens act to increase tumor incidence. There is a wealth of information on liver initiation/promotion protocols in the scientific literature (Pitot and Sirica, 1980; Farber, 1984; Pitot and Campbell, 1987). These protocols frequently employ a single initiating dose of a chemical which damages DNA, followed by enhancement of cell replication (partial hepatectomy or cytotoxicity) to fix that damage into the genome (initiation) and then chronic exposure to a chemical which produces clonal expansion of the genetically-altered cells (promotion). Increased tumor incidence is produced by chemicals which act at either stage. It is important to note that "initiation" and "promotion" are operational and not mechanistic terms since both stages are likely comprised of multiple steps. Nevertheless, the protocols have provided valuable information in our attempts to understand chemical carcinogenesis. Detailed descriptions of initiation/promotion protocols in liver and skin are provided elsewhere (Pitot and Campbell, 1987; Dragan et al., 1991; Pitot et al., 1987; Farber, 1984; Slaga et al., 1982; Peraino et al., 1981; Ito et al., 1980).

6.4.1. Two-Stage Models in Rat Liver. Pitot et al. (1980) reported that TCDD was a potent liver tumor promoter when rats were initiated with a single dose of DEN followed by chronic TCDD exposure (0.14 and 1.4 μg/kg subcutaneously once every 2 weeks for 7 months). These doses are equivalent to 10 and 100 ngTCDD/kg/day (the medium- and high-dose in the Kociba bioassay). Histological evaluation revealed that five of seven animals which had received DEN and the high TCDD dose had hepatocellular carcinomas. No liver tumors were evident in rats receiving DEN only, DEN/low-dose TCDD or TCDD only (high or low-dose). EOF in liver were also evaluated in this study and these are considered to represent preneoplastic lesions since increases in EOF are associated with liver cancer in rodents (Maronpot et al., 1989; Popp and Goldsworthy, 1989; Pitot et al., 1989; Williams, 1989). The EOF data was consistent with the tumor data in that a large proportion of the liver was occupied by the preneoplastic lesions (43%) in animals receiving DEN and the high dose of TCDD. A much smaller portion of the

liver was occupied by EOF in the other groups. This work provides strong evidence that TCDD is a potent tumor promoter in liver with no detectable initiating activity within the specified experimental framework.

A second set of studies (Graham et al., 1988; Lucier et al., 1991; Clark et al., 1991a; Dragan et al., 1992) have confirmed and extended Pitot's findings including data on the mechanistic basis for TCDD's tumor promoting effects in rat liver. These studies also used DEN as the initiator and have demonstrated that TCDD's liver tumor promoting actions are ovarian dependent. This finding is consistent with two-year bioassays which showed that TCDD is a hepatocarcinogen in female rats but not male rats. In the tumor promoting studies (Graham et al., 1988; Lucier et al., 1991) DEN was used as the initiating agent and TCDD (biweekly doses of 1.4 μgTCDD/kg equivalent to 100 ng/kg/day for 30 weeks) was used as the promoter. There were four groups of intact female rats (controls, TCDD only, DEN only and DEN+TCDD). The same four groups were also used following ovariectomy. Data revealed that TCDD was a much weaker liver tumor promoter in OVX rats (Table 6-5). For example, there were 387 GGT foci/cm^3 in intact rats compared to 80 in OVX rats in the DEN+TCDD groups. Corresponding differences were evident in the proportion of liver occupied by GGT foci; 0.37% in DEN/TCDD intact rats compared to 0.08% in DEN + TCDD OVX rats. Few or no foci were found in the control or TCDD only groups. PGT is being used increasingly as a phenotypic marker of enzyme altered foci (Ito et al., 1989) and results with this marker of preneoplasia were similar to those for GGT in that ovariectomy protected against the liver tumor promoting actions of TCDD. The influence of ovariectomy on liver tumor incidence was evaluated in a parallel experiment using the same treatment groups in which TCDD was administered for 60 weeks. In the intact DEN + TCDD rats, liver tumor incidence was 13/37 with a total of 32 tumors compared to 7/39 (11 total tumors) in DEN + TCDD OVX rats. Both hepatocellular adenomas and carcinomas were evident along with a smaller incidence of hepatocholangiomas and hepatocholangiocarcinomas.

The mechanisms responsible for the protective effect of ovariectomy is not clear but ovarian influences on liver TCDD retention does not seem to be involved; liver TCDD concentrations were ~20 ppb in both intact and OVX rats

TABLE 6-5

Preneoplastic Foci and Cell Proliferation After 30 Weeks of TCDD Tumor Promotion[a]

	S/C	S/TCDD	DEN/C	DEN/TCDD
GGT + foci/cm^3				
intact	6	5	44	387[b]
OVX	0	0	30	80
GGT + foci (vol fraction)[c]				
intact	0.01	0.01	0.03	0.37[b]
OVX	0	0	0.03	0.08
BrdU-labeling index[c]				
intact	0.3[b]	6.0[b]	0.8	7.3[b]
OVX	1.1	1.0	1.1	0.7

[a]Source: Clark et al., 1991

[b]Significantly different from OVX

[c]Percentage of hepatocytes undergoing replicative DNA synthesis in 1 week following 30 weeks of TCDD exposure

S/C = Controls; S/TCDD = TCDD only; DEN/C = DEN only no TCDD; DEN/TCDD = DEN initiated and TCDD promoted

(Lucier et al., 1991) which is similar to liver concentrations reported by Kociba et al. (1978) using the same dose of TCDD (100 ng/kg/day) but for 2 years rather than 60 weeks. One plausible mechanism may be related to cell proliferation since TCDD did not stimulate cell proliferation rates in OVX rats whereas a mean increase of 20-fold was apparent in intact rats receiving 100 ng TCDD/kg for 30 weeks (Table 6-5) (Lucier et al., 1991). There was considerable interindividual variation in both cell proliferation rates and enzyme altered foci in the DEN/TCDD groups. Comparisons of the two data sets revealed a strong positive correlation between enzyme altered foci and cell proliferation, although the importance of this finding is diminished by the fact that cell proliferation was quantified in non-lesioned hepatocytes. The mechanism whereby ovarian hormones and TCDD interact to produce cell proliferation in hepatocytes may involve growth factor pathways. Consistent with this idea, TCDD produced a loss of plasma membrane EGF receptor in intact rats but not OVX rats (Clark et al., 1991a). EGF is thought to provide a mitogenic stimulus in hepatocytes and play a key role in hepatocarcinogenesis (Vickers and Lucier, 1991; Velu, 1990; Shi and Yager, 1989; Eckl et al., 1988). A schematic representation of a plausible mechanism for the role of estrogen in TCDD-mediated liver cancer in rats is given in Figure 6-2.

Another possible mechanism for the influence of the ovaries is that TCDD induces cytochrome P-450 1A2 which could lead to DNA reactive metabolites of 17β-estradiol, the naturally-occurring estrogen. P-4501A2 catalyzes the formation of catechol estrogens which are considered by some to be DNA reactive precursors (Metzler, 1984; Li and Li, 1990).

The CDFs and other CDDs are also liver tumor promoters. In a recent study (Flodstrom and Ahlborg, 1991), enzyme-altered foci were increased in female Sprague-Dawley rat livers by an initiating dose of DEN followed by TCDD, 1,2,3,7,8-pentachlorodibenzo-p-dioxin or 2,3,4,7,8-pentachlorodibenzofuran were used as the promoting agent. Comparative potencies indicated that the two CDDs were nearly equipotent and the PCDF about 1/10th as potent as TCDD. These results are consistent with the idea that the hepatocarcinogenic actions of TCDD and its structural analogs are Ah receptor dependent.

6.4.2. Rat Lung. Since the lung and respiratory tract may be target sites for TCDD carcinogenesis in humans (Fingerhut et al., 1991), it is of interest to

POSSIBLE SEQUENCE OF EVENTS INVOLVED IN ESTROGEN DEPENDENT TCDD PROMOTION OF LIVER TUMORS

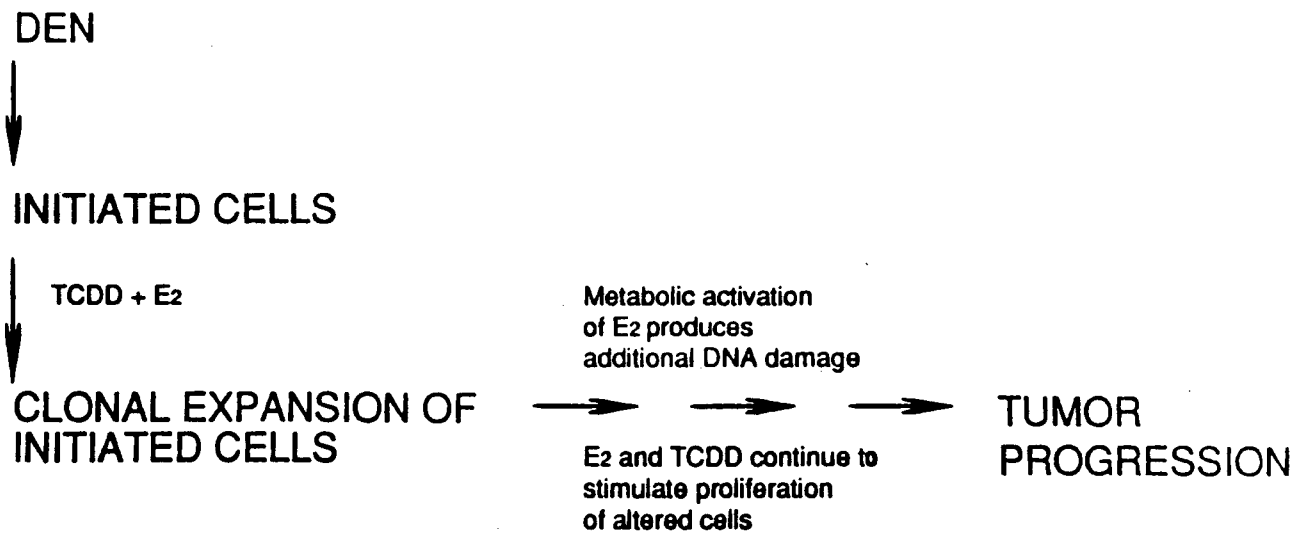

FIGURE 6-2

Operational model of TCDD/estrogen interactions relative to tumor promotion in a two-stage model of hepatocarcinogenesis. Clonal expansion of initiated cells may reflect stimulation of mitogenesis through receptor-mediated events involving EGFR, ER and the Ah receptor.

evaluate whether TCDD is a tumor promoter in rodent lung. The only published report on lung tumors used DEN as the initiating agent and TCDD (100 ng/kg/day for 60 weeks) as the promoting agent (Clark et al., 1991a). Both intact and OVX rats were used and the results were surprising. In contrast to liver tumor promotion, lung tumors were seen only in DEN/TCDD OVX rats (4/37). No lung tumors were present in DEN/TCDD intact rats or in DEN only TCDD only, or control rats with or without ovariectomy). The background incidence of lung tumors in rats is very low so the lack of tumors in controls was not unexpected. The four tumors in DEN/TCDD intact rats were comprised of two squamous cell carcinomas and two adenocarcinomas. These tumors were analyzed for the presence of activated oncogenes by the NIH 3T3 transfection and nude mouse tumorigenicity assays (Reynolds et al., 1992). A transforming gene of rat origin was detected in all tumors but Southern blot analysis indicated that it is an unknown oncogene. This unknown oncogene was not detected in the DEN/TCDD rat liver tumors.

The rodent tumorigenicity data provide clues to the complex hormonal interactions that produce site specific carcinogenic actions of TCDD. Liver tumors are ovarian dependent whereas the ovaries appear to protect against TCDD-mediated tumor promotion in lung. Therefore, the rat tumor data is consistent with the NIOSH study which revealed TCDD-related increases in respiratory tract tumors but no statistically significant increases in liver tumors in a population comprised mostly of men.

6.4.3. Mouse Skin. Initiation/promotion studies on skin have demonstrated that TCDD is a potent tumor promoter in mouse skin as well as rat liver. Poland et al. (1982) administered a single dermal initiating dose of MNNG to hairless mice followed by twice weekly doses of TCDD (3.75, 7.5, 15 or 30 ng) or TPA (1 or 3 μg) for 20 weeks. TCDD promoted the development of papillomas at all doses and the response was dose dependent (100% of the animals had tumors in the high-dose TCDD group). Control animals or animals receiving MNNG or TCDD only exhibited only a low incidence of tumors. These studies demonstrate that TCDD is at least two orders of magnitude more potent an agent than TPA in mouse skin (Poland et al., 1982). It appears that the skin tumor promoting actions of TCDD are Ah receptor dependent. Moreover, tumorigenic responses segregate with the hr locus

and biochemical responses such as CYP1A1 induction can occur without carcinogenesis (Poland and Knutson, 1982; Poland et al., 1982).

Other studies have tested TCDD as an initiator and TPA as a promoter in CD-1 mice (DiGiovanni et al., 1977). Results revealed that TCDD had weak or no initiating activity in this system. In order to better understand the possible influence of TCDD-mediated induction of cytochrome P-450 on the carcinogenicity of PAHs, TCDD was co-administered with benzo(a)pyrene or dimethylbenzathracene to mice followed by promotion with TPA (Cohen et al., 1979). Results revealed that TCDD decreased tumor incidence of both PAHs compared to controls. However, co-administration of TCDD with 3-methylcholanthrene to mice produced tumor incidences similar to those produced by 3-methylcholanthrene alone (Kouri et al., 1978). These results are consistent with the findings that TCDD induction of drug metabolizing enzymes is associated with both metabolic activation as well as deactivation of PAHs (Lucier et al., 1979).

The relative toxicity and tumor promoting capacity of two CDFs (2,3,4,7,8-CDF and 1,2,3,4,7,8-CDF) has been investigated in hairless mice (Hebert et al., 1990). These studies used a treatment protocol similar to that of Poland et al. (1982) including the use of MNNG as the initiating agent and varying doses of TCDD, 2,3,4,7,8-CDF or 1,2,3,4,7,8-CDF for 20 weeks. Proliferative lesions (squamous cell papilloma, squamous cell carcinoma or hyperproliferative nodules) were quantified. Results demonstrated that 2,3,4,7,8-CDF was 0.2-0.4 times as potent as TCDD and the 1,2,3,4,7,8-CDF was 0.08-0.16 times as potent as TCDD. These data suggest that the tumor promoting potencies of structural analogs of TCDD, like promotion of liver tumors, reflect relative binding properties to the Ah receptor. However, this is an effect of chronic exposure so rates of metabolism/clearance would obviously impact on correlations between Ah receptor binding and tumo promotion.

Taken together, results on initiation/promotion protocols indicate that TCDD is an extraordinarily potent promoter of liver and skin tumors (Pitot et al., 1987) and they provide strong evidence that the carcinogenic actions are Ah receptor-mediated. A summary of studies on tumor promotion by TCDD or the polychlorinated biphenyls is given in Table 6-6. Plausible mechanisms of actions

TABLE 6-6

Summary of Positive Tumor Promoting STUDIES ON TCDD and CDFs

Species/Sex	Initiator	Promoter	Site	Reference
Rat/female	DEN	TCDD	Liver	Pitot et al, 1980
Rat/female	DEN	TCDD	Liver	Graham et al, 1988
Rat/female	DEN	TCDD	Liver	Lucier et al, 1991
Rat/female	DEN	TCDD	Liver	Clark et al, 1991
Rat/female	DEN	TCDD	Liver	Flodstrom et al, 1991
Rat/female	DEN	TCDD	Liver	Flodstrom et al, 1991b
Rat/female	DEN	PCDFs	Liver	Flodstrom et al, 1991b
Rat/female	DEN	TCDD	Liver	Dragan et al, 1992
Rat/female	DEN	TCDD	Liver	Lucier et al, 1992
Mice/female hairless	MNNG	TCDD	Skin	Poland et al, 1982
Mice/female hairless	MNNG	PCDFs	Skin	Hebert et al, 1990
Rat/female (ovariectomized)	DEN	TCDD	Lung	Clark et al, 1991
Rat/female (ovariectomized)	DEN	TCDD	Lung	Reynolds and Lucier, 1992

responsible for the tumor promoting actions of TCDD and the impact of these mechanisms on dose response relationships will be presented in the next section.

6.5. BIOCHEMICAL RESPONSES

There is an expanding list of effects that are produced by TCDD in experimental animals and in cell systems. These effects include those which may alter normal cell regulatory processes such as cell proliferation and differentiation, metabolic capacity, and hormonal pathways. This section on biochemical responses will summarize some of the changes produced by TCDD including discussion of (a) possible relevance of the response to TCDD-mediated cancer, (b) whether the response is Ah-receptor mediated, (c) whether information is available on the role of transcriptional activation, (d) dose-response relationships, and (e) whether animal models are consistent with human responses. This chapter will not attempt to evaluate all of the biochemical and molecular responses to TCDD but will focus on the ones that are either the most relevant to carcinogenic responses and/or have received the most study. The responses selected for evaluation are cytochrome P-4501A1 (CYP1A1), cytochrome P-4501A2 (CYP1A2), EGFR, ER, and UDPGT. Table 6-7 lists many of the biochemical changes produced by TCDD in *in vivo* and/or *in vitro* and some information on mechanisms of action.

6.5.1. CYP1A1 and 1A2. The most studied response to TCDD has been induction of cytochrome P-450 isozymes (Whitlock, 1990; Silbergeld and Gasiewicz, 1989; Poland and Knutson, 1982). The first reports of P-450 induction *in vivo* and *in vitro* appeared in 1973 (Lucier et al., 1973; Greig and DeMatteis, 1973; Poland and Glover, 1973) and hundreds of papers have been published on the subject since that time. These papers have dealt with various aspects of TCDD-mediated induction of P-450 such as isozyme specificity, time-course, structure-activity relationships, molecular mechanisms of transcriptional activation of the CYP1A1 gene, identification of transcriptional activating factors, tissue and cell specificity and dose-response relationships. The molecular mechanisms responsible for enzyme induction are described in the chapter by Whitlock in this volume.

The mechanistic relationship of CYP1A1 and 1A2 induction to cancer or any other toxic endpoint following dioxin exposure has not been demonstrated, yet considerable controversy exists on this subject (Roberts, 1991). Since CYP1A1

TABLE 6-7

Classification of Members of the Ah Gene Battery*

Class	Gene/Product	Secreted Protein
Activation of gene transcription; Ah receptor-mediated	Cyp1A1, Cytochrome P_1450	-
	Gst-Ya, glutathione S-transferase	-
	Nmo-1, menadione oxidoreductase	-
Activation of gene transcription, AhR agonist-mediated	Clone 1, unknown gene	-
	Cyp1A2, cytochrome P_2450	?
	PAI-2, plasminogen activator inhibitor-2	+
	T-ALDH, aldehyde deydrogenase	-
Induction of mRNA levels; AhR agonist-mediated	Clone 141, unknown gene	?
	c-erb A related, hormone receptor	-
	GST-Yb, glutathione S-transferase	-
	GST-Yc	-
	αhCG, human chorionic gonadotropin	+
	IL-β, interleukin-1β	+
	MDR-1, multidrug-resistance	-
	Testosterone 7 α-hydroxylase	-
	TGF-α, Transforming growth factor-α	+
Induction of enzyme activity; Ah receptor-mediated	ODC, ornithine decarboxylase	-
	Ugt-1, UDP-glucuronyl transferase	-
	EGFR, epidermal growth factor receptor	-
	ER, estrogen receptor	-
	Gastrin	+
	TNF-α, tumor necrosis factor-α	+
Induction of enzyme activity; AhR agonist-mediated	ALAS, δ-aminolevulinic acid synthetase	-
	Aryl hydrocarbon binding protein	-
	Choline kinase	-
	60-kd microsomal esterase	-
	Malic enzyme	-
	Phospholipase A_2	-
	Protein kinase C	-
	Enzyme $pp60^{c\text{-}src}$, tyrosine kinase	-

*Source: Sutter and Greenlee, 1991

functions to catalyze the metabolic activation of many chemicals such as the polycyclic aromatic hydrocarbons to DNA reactive metabolites, it has been postulated that induction of CYP1A1 might enhance the carcinogenic actions from a given exposure level to many PAHs. However, usually, preinduction of CYP1A1 diminishes the carcinogenic potency of PAHs such as 3-methylcholanthrene, benzo(a)pyrene and dimethylbenzoanthracene if exposure to the inducing agent is short term (Parkinson and Hurwitz, 1991; Wattenberg, 1985; Cohen et al., 1978; Wattenberg, 1978; Miller et al., 1958). Induction also protects against the carcinogenic actions of aflatoxin, diethylnitrosamine, arylamines and urethane. Protection occurs at numerous cancer sites including liver and lung. Several lines of evidence support the idea that enzyme induction is the mechanism responsible for the protective effect. First, treatment of mice, deficient in the Ah receptor, with inducers does not protect against PAH-mediated cancer (Kouri et al., 1978). Second, the ability of inducing agents to protect against cancer is positively correlated with their potency as inducing agents (Wattenberg and Leong, 1970; Arcos et al., 1961). Third, the inducing agent must be administered at least one day prior to treatment which allows sufficient time for the inducer to produce elevated levels of CYP1A1 (Parkinson et al., 1983; Wheatley, 1968).

The most probable mechanism responsible for the protective effect of enzyme induction is that it leads to decreased concentrations of promutagenic DNA adducts in target tissues. These findings appear to contradict the knowledge that CYP1A1 is required for the metabolism of PAHs, aflatoxin and several other carcinogens to DNA reactive arene oxides (Guengerich, 1988; Levin et al., 1982; Conney et al., 1982). For example, the promutagenic DNA adduct of benzo(a)pyrene appears to be 7,8-diol-9,10 epoxide metabolite adducted to deoxyguanosine, and formation of this metabolite requires two separate actions of CYP1A1. The contradiction can be resolved by analysis of the entire metabolic pathways for chemical carcinogens whose potencies are decreased by pretreatment with inducing agents. In addition to CYP1A1-mediated increases in metabolic activation, this cytochrome also converts PAHs to inactive metabolites (Thakker et al., 1985; Pelkonnen and Nebert, 1982). Moreover, induction of uridine diphosphoglucuronly-transferase also occurs coordinately with CYP1A1 induction (Lucier et al., 1986).

This enzyme also detoxifies PAHs and many other carcinogens and facilitates their excretion from the body (Thakker et al., 1985; Nemoto and Gelboin, 1976). Therefore, it appears that TCDD-mediated enzyme induction increases the rate of detoxification of some carcinogens to a greater extent than it increases the rate of formation of DNA damaging metabolites.

Although there is not clear mechanistic link between CYP1A1 induction and cancer, it is important to note that many CYP1A1 inducers are themselves carcinogens when encountered in chronic dosing regimens so the protective effect of inducing agents is limited to short-term exposure. For example, benzo(a)-pyrene, 3-methylcholanthrene and TCDD are CYP1A1 inducers and multisite carcinogens (Vanden Heuvel and Lucier, 1992; Levin et al., 1982; Slaga et al., 1979; Sims and Glover, 1974).

The relationship of CYP1A2 induction to the carcinogenic actions of other compounds is less clear than it is for CYP1A1. For example, CYP1A2 catalyzes the formation of catechol estrogens from 17β-estradiol (Graham et al., 1988). The catechol estrogens are considered as possible toxic metabolites in that they could lead to increased free radical damage to cellular macromolecules such as DNA (Li and Li, 1990; Metzler, 1984). This mechanism could be in part, responsible for the findings that TCDD is a hepatocarcinogen in female rats but not male rats and that ovariectomy protects against the hepatocarcinogenic actions of TCDD. Also consistent with the hepatocarcinogenicity data is the observation that CYP1A2 is induced in liver but not in extrahepatic organs with the possible exception of the nasal mucosa (Goldstein and Linko, 1984). In contrast, CYP1A1 induction occurs in virtually every tissue of the body which is consistent with the observation that the Ah receptor is found in a wide variety of cell types.

There are a number of studies described in the scientific literature on dose response relationships for TCDD's effects on CYP1A1 and 1A2 (DeVito et al., 1991; Lin et al., 1991a; Kedderis et al., 1991; Harris et al., 1990; Goldstein and Safe, 1989; Abraham et al., 1988; Lucier et al., 1986; Vecchi et al., 1983; Poland and Glover, 1980; Kitchin and Woods, 1979; Lucier, et al., 1973; Poland and Glover, 1973). These studies include single and chronic dosing schedules (Tritscher et al., 1992; Graham et al., 1988; Sloop and Lucier, 1987), time-course evaluations and species comparisons. Dose response relationships have

been evaluated by quantitation of CYP1A1 and 1A2-dependent enzyme activities, mRNA levels by Northern blot analysis, quantitation of CYP1A1 and 1A2 protein by radioimmunoassay and also by immunolocalization in tissue sections. All of the above methods have yielded consistent results. The single dose ED_{50} for CYP1A1 or 1A2 induction is approximately 0.5-1.5 μg TCDD/kg in both rats and mice. In a chronic exposure situation, the ED_{50} is in the range of 5-10 ng/kg/day (Tritscher et al., 1992). The limit of detection for enzyme induction varies depending on the method used for quantitation; i.e. P-450 dependent enzyme activities, mRNA, or protein. Recently, it was shown (VandenHeuvel et al., 1992) that TCDD-mediated increases in CYP1 in mRNA were detectable following a single dose of 0.1 ng/kg which produces a TCDD liver concentration equivalent to a chronic dose of 2-5 pg/kg/day.

Evaluations of various data sets for TCDD-mediated dose response relationships have revealed some interesting information. One way of analyzing data for linearity or non-linearity of dose response for receptor-mediated events is the Hill equation (Hayashi and Sakamoto, 1986). A Hill coefficient of 1 suggests a linear relationship between exposure and dose throughout the experimental dose range and would predict a proportional relationship between target tissue concentration of TCDD and biological response at all dose levels. This would imply that the response had no practical threshold or "no effect level." Hill coefficients greater than 1 would indicate sublinearity in dose response, whereas a Hill coefficient of less than 1 would indicate supralinearity for response in the low-dose region. Analysis of both single exposure as well as chronic exposure data for CYP1A1 and CYP1A2 induction in rat or mouse liver indicate a Hill coefficient of slightly greater than 1 for CYP1A1 and slightly less than 1 for CYP1A2 (Portier et al., 1992; Kohn et al., 1992). Although these analyses involve an extrapolation beyond the range of experimental data, they are consistent with the hypothesis that there is not a practical threshold for TCDD-mediated induction of CYP1A1 and 1A2.

Immunological detection of induced CYP1A1 and 1A2 in liver sections obtained from rats exposed chronically to TCDD suggest hepatocyte heterogeneity in response to TCDD (Tritscher et al., 1992; Bars and Elcombe, 1991). For example,

relatively low doses of TCDD (1 ng/kg/day) appear to induce maximally some cells around the centrilobular region. Increasing doses of TCDD increase the number of cells responding rather than the amount of induction in responding cells. These data, which document cell differences in sensitivity to induction, complicate evaluation of dose response relationships. For example, some hepatocytes appear to be maximally induced by low doses to TCDD whereas other hepatocytes exhibit no detectable P-450 induction response by these same doses. As discussed earlier a mechanistic link between P-450 induction and cancer has not been established. Evaluation of P-450 induction and TCDD-mediated cell proliferation by immunocytochemical methods in rat liver reveal that cells which express CYP1A1 and 1A2 are different from those exhibiting TCDD-mediated increases in DNA replication (Lucier et al., 1992).

Placentas from Taiwanese women exposed to rice oil contaminated with polychlorinated dibenzofurans (PCDFs) have markedly elevated levels of CYP1A1 (Lucier et al., 1987; Wong et al., 1986). Comparison of these data with induction data in rat liver suggest that humans are at least as sensitive as rats to the enzyme inductive actions of TCDD and its structural analogs (Lucier, 1991). Consistent with this contention, the *in vitro* EC_{50} for TCDD-mediated induction of CYP1A1-dependent enzyme activities is approximately 1.5 nM when using either rodent or human lymphocytes (Clark et al., 1992). However, binding of TCDD to the Ah receptor occurs with a higher affinity in rat cellular preparations compared to humans (Lorenzen and Okey, 1991; Okey, 1989). This difference may be related to the greater lability of the human receptor during tissue preparation and cell fractionation procedures (Manchester et al., 1987). In any event, it does appear that humans contain a fully functional Ah receptor (Cook and Greenlee, 1989) as evidenced by significant CYP1A1 induction in tissues from exposed humans and this response occurs with similar sensitivity as observed in experimental animals.

6.5.2. EGFR. EGF is a potent mitogen and it stimulates the generation of mitotic signals in both normal and neoplastic cells (Stoscheck and King, 1986; Carpenter and Cohen, 1979). Several lines of evidence suggest that the EGF receptor and its ligands, including transforming growth factor-α possess diverse

functions relevant to cell transformation and tumorigenesis (Velu, 1990; Marti et al., 1989; Mukku and Stancel, 1985). In fact, the mechanism of action for several tumor promoters such as phenobarbital and the phorbol esters is thought to involve the EGF receptor pathway (Stoscheck and King, 1986). A schematic representation of the proposed mechanism for EGF-stimulated mitogenesis is given in Figure 6-3.

Several studies have shown that TCDD decreases the binding capacity of the plasma membrane EGF receptor for its ligand without a change in K_d (Clark et al., 1991a; Lin et al., 1991a; Abbot and Birnbaum, 1990; Astroff et al., 1990; Sunahara et al., 1989; Hudson et al., 1985; Madhukar et al., 1984). One study utilized a range of TCDD doses (3.5-125 ng/kg/day) for 30 weeks to evaluate the effects of chronic TCDD exposure on EGF receptor in rat liver plasma membranes (Sewall, 1992). There was a clear dose-response relationship for TCDD's effects on the total binding capacity of the EGF receptor although TCDD did not produce a change in binding affinity of the receptor. The maximal effect was a three-fold decrease in the concentration of plasma membrane EGF receptor and the ED_{50} was ~10/ng/kg/day based on administered dose and ~2 ppb TCDD based on liver TCDD concentration. These values are similar to the ED_{50} for induction of CYP1A1 and CYP1A2 for 30-week exposures. The dose-response data, like the data for CYP1A1 and CYP1A2 induction, was subjected to curve fitting analyses using the Hill Equation (Portier et al., 1992). This analysis indicated that a Hill coefficient of one provided the best fit suggesting that there is a linear relationship between target tissue dose and the magnitude of response for effects on the EGF receptor. Although, Hill analysis of dose response data for TCDD's effects on the EGF receptor, CYP1A1 induction, and CYP1A2 induction are inconsistent with the idea of a threshold, the lowest dose used in these experiments was 100 pg/kg/day so it is possible that dose-response relationships are different in the very low-dose region (1-10 pg/kg/day) encountered as background human exposures.

Dose-response data on EGFR were compared to dose-response relationships for TCDD-mediated increases in cell proliferation and growth of preneoplastic lesions within the framework of a two stage model for hepatocarcinogenesis in rats (Lucier et al., 1992). Results indicate that cell proliferation and the growth

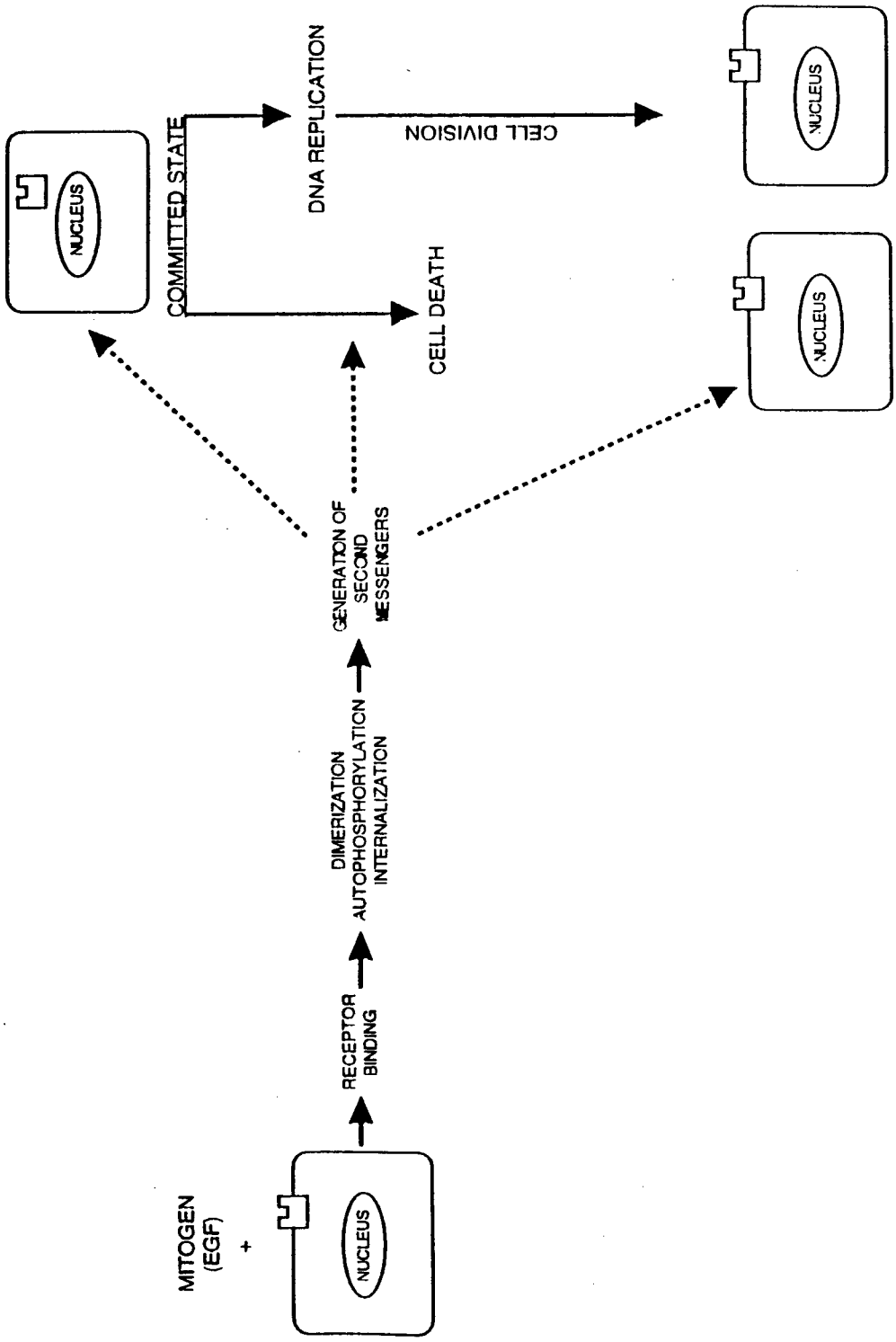

FIGURE 6-3

Plausible Mechanism for the Role of EGF-Mediated Stimulation of Mitotic Activity

of preneoplastic lesions are a less sensitive response to TCDD than is loss of plasma membrane EGF receptor. Therefore, the EGF receptor may be involved in the hepatocarcinogenic actions of TCDD but dose-response relationships for this effect may be different from dose-response relationships for liver cancer in rats. These data reflect the knowledge that several steps and/or several genes are involved in the modulation of coordinated biological responses.

The mechanism by which TCDD alters EGF receptor binding capacity is not fully understood although TCDD does not appear to decrease EGF receptor mRNA (Lin et al., 1991a; Osborne et al., 1988). Using congenic mice, deficient in the high affinity Ah receptor, TCDD's effects on the EGF receptor were shown to require the Ah receptor (Lin et al., 1991a). In control animals, the EGF receptor is distributed on the surface of the plasma membrane and is comprised of an external ligand binding domain, a transmembrane domain, and an intercellular domain (Velu, 1990; Carpenter, 1987). Ligands for the EGF receptor (EGF or TGF-α) in the intracellular space bind the EGF receptor producing a conformational change which stimulates the intercellular region to catalyze phosphorylation of the receptor itself as well as other proteins involved in cell regulation. The process results in internalization of the receptor characterized by an increase in cytosolic EGFR coupled with a decrease in membrane bound receptor. Effects of TCDD and CDFs on the number of binding sites for the plasma membrane EGF receptor are correlated with a concomitant decrease in EGF stimulated autophosphorylation of the EGF receptor indicating that TCDD produces a true functional change in the EGF receptor (Clark et al., 1991a; Sunahara et al., 1989; Nelson et al., 1988; Sunahara et al., 1988). It is important to note that addition of EGF to hepatocytes or several cell lines in culture produces a loss of plasma membrane EGF receptor coupled with a loss of EGF stimulated autophosphorylation (Velu, 1990; Carpenter, 1987). Therefore, TCDD produces an EGF receptor like response consistent with the idea that TCDD enhances the generation of cellular mitotic signals.

Although TCDD exposure mimics EGF actions in hepatocytes, TCDD itself does not bind the EGF receptor. The most plausible mechanism for effects on the EGF receptor involves the finding that TCDD induces production of TGF-α in hepatocytes as well as human keratinocytes (Choi et al., 1991). This response could

alter control of normal growth patterns since TGF-α binds the EGF receptor with high affinity leading to enhanced production of mitogenic signals. Alternatively, TCDD may affect EGF receptor transcription. In fact, TCDD has been shown to decrease uterine EGF receptor mRNA levels (Astroff et al., 1990). Receptor concentrations may also be altered by other events such as postranslational glycosolation, increased lysosomal degradation or alterations in signal transduction pathways such as protein kinases (Madhukar et al., 1988). It is also possible that TCDD alters phosphorylation of the EGF receptor by activation of protein kinase C resulting in decreased binding capacity of the plasma membrane EGF receptor. This effect occurs following exposure to the tumor promoter TPA and is associated with decreased autophosphorylation rates and EGF receptor internalization (Beguinot et al., 1985; Cochet et al., 1984). In any event, TCDD-mediated alterations in EGF receptor pathways may, in part, be responsible for the tumor promoting actions of TCDD by enhancement of mitotic signals.

The effects on the EGF receptor system may be mediated by estrogen action and it has been postulated that the estrogen and EGF receptor pathways are integrated by "cross talk" mechanisms (Ignar Trowbridge et al., 1992; Astroff et al., 1990). *In vivo* and *in vitro* studies have demonstrated that TCDD alters the estrogen receptor (DeVito et al., 1992; Lin et al., 1991a; Clark et al., 1991a; Umbreit and Gallo, 1988; Romkes et al., 1987) and estrogens can, in turn, alter EGF receptor binding and cellular distribution (Vickers and Lucier, 1991; Vickers et al., 1989; Mukke and Stancel, 1985). Moreover, studies conducted within the framework of a two-stage model for hepatocarcinogenesis have demonstrated that TCDD-mediated decreases in plasma membrane EGF receptor are ovarian dependent (Clark et al., 1991a; Sewall et al., 1992). These studies concluded that ovarian hormones are essential to the tumor promoting actions of TCDD in that TCDD does not induce hepatocyte proliferation or stimulate the growth of preneoplastic lesions in ovariectomized rats (see section on Initiation-Promotion studies).

There is evidence to indicate that TCDD and its structural analogs produce the same effects on the EGF receptor in human cells and tissues as observed in experimental animals. First, incubation of human keratinocytes with TCDD decreases plasma membrane EGF receptor and this effect is associated with increased synthesis of TGF-α (Choi et al., 1991; Hudson et al., 1985). Second,

placentas from humans exposed to rice oil contaminated with polychlorinated dibenzofurans, exhibit markedly reduced EGF stimulated autophosphorylation of the EGF receptor and this effect occurred with similar sensitivity as observed in rats (Lucier, 1991; Sunahara et al., 1987). The magnitude of the effect on autophosphorylation was positively correlated with decreased birth weight of the offspring.

6.5.3. UDPGT. Several studies have shown that TCDD induces synthesis of at least one isozyme of UDPGT (Lucier et al., 1973, 1974, 1986) by a mechanism which requires the Ah receptor (Bock, 1991). The gene UGT-1 regulates synthesis of the UDPGT isozyme which conjugates numerous substrates including 1-naphthol, p-nitrophenol and thyroxine (Burchell et al., 1991). This gene contains a TCDD responsive element which permits transcriptional activation following binding of the TCDD-Ah receptor complex. Other chemicals which bind the Ah receptor, such as 3-methylcholanthrene and benzo(a)pyrene also induce UGT-1 (Bock, 1991). UDPGTs are considered as a deactivation pathway for numerous environmental chemicals and endogenous compounds such as steroid hormones by rendering them water soluble and excretable as a consequence of the catalytic addition of a glucuronide moiety (Tephly and Burchell, 1990). Therefore, induction of UDPGT may, in part, be responsible for the finding that pretreatment with TCDD leads to diminished DNA adducts for PAHs and decreased concentrations of some steroid hormones.

Conjugation of thyroxine by UGT-1 leads to deactivation and elimination of this thyroid hormone (Henry and Gasiewicz, 1987; Bastomsky, 1977). The decreased levels of thyroxine, associated with UDPGT induction produces decreased feedback inhibition of the pituitary gland which responds by secreting increased amounts of TSH (Sanders et al., 1988; Barter and Klaassen, 1992). Several studies have provided evidence that prolonged stimulation by TSH produces an oncogenic effect on the thyroid (Hill et al., 1989). Interestingly, rat liver EGF receptor may, in part, be regulated by thyroid hormones (Mukku, 1984). Increased incidence of thyroid tumors is the most sensitive endpoint in cancer bioassays as evidenced by a statistically significant increase at a dose of 1.4 ng/kg/day. Consistent with this hypothesis, short-term rodent studies have shown that TCDD and other inducers of hepatic UDPGT decreases thyroxine concentrations in blood which is

associated with increased levels of TSH (Barter and Klaassen, 1992; Henry and Gasiewicz, 1987).

Dose response studies for TCDD's inductive effects on hepatic UDPGT in rats have demonstrated that the single dose ED_{50} is approximately 0.7 μg/kg which is similar to the ED_{50} for CYP1A1 induction (Lucier et al., 1986). Furthermore, the shape of the dose response curve for both responses is similar. There is no data on UDPGT induction in long-term studies. Since humans contain the dioxin responsive UDPGT (UGT-1) (Burchell et al., 1991) and TCDD induces UDPGT in human hepatocyte cell cultures it is reasonable to assume that TCDD and its structural analogs would induce UDPGT in humans although laboratory data is needed to validate this assumption.

6.5.4. ER. Several lines of evidence have demonstrated that interactions of TCDD and estrogens are critical to some of the carcinogenic responses to TCDD. Although the precise mechanisms of those interactions have not been established, recent data indicate that TCDD effects on the ER and on estrogen metabolism are involved. The mechanisms for TCDD/estrogen interactions appear to be tissue specific. Of particular interest is the finding that TCDD increases liver tumor incidence in rats and at the same time decreases tumor incidence in organs such as the mammary gland, uterus and pituitary (Kociba et al., 1978). Threfore, TCDD/estrogen interactions will be examined separately for liver and other endocrine organs.

The liver contains a fully functional ER that possesses characteristics similar to those identified for ER in mammary gland and uterus (Mastri and Lucier, 1983; Powell-Jones et al., 1981; Eisenfeld et al., 1976). For example, the liver exhibits high affinity binding for 17β-estradiol and other potent estrogens, liver ER binding is specific for estrogens, the ligand receptor complex interacts reversibly with DNA, and this interaction leads to transcriptional activation of estrogen responsive genes. Synthesis of hepatic ER, unlike ER in other target tissues, is under pituitary control (Lucier et al., 1981). Treatment of rats with a single dose of TCDD decreases binding capacity of the hepatic ER and this effect is correlated with a decrease in ER protein (Zacharewski et al., 1991, 1992; Harris et al., 1990b; Romkes and Safe, 1988;

Romkes et al., 1987). TCDD also decreases rat hepatic ER in chronic exposure experiments with a 3-fold decrease evident following a dose of 100 ng/kg/day for 30 weeks (Clark et al., 1991b). TCDD also decreases hepatic ER binding in C57Bl6 mice but a much higher dose is needed to produce this effect in congenic mice deficient in the high affinity Ah receptor indicating that TCDD-mediated decreases in ER are dependent on the Ah receptor (Lin et al., 1991). Dose response studies in mice demonstrate that the single dose ED_{50} is ~0.7 μg TCDD/kg, similar to the ED_{50} for other biochemical endpoints such as CYP1A1 induction, loss of plasma membrane EGF receptor and induction of UDPGT. The observation that TCDD decreases hepatic ER is in apparent contradiction to the finding that TCDD increases hepatocyte proliferation since the ER is thought to produce mitogenic signals. However, quantitation of ER in control and TCDD-treated rats was done using preparations from liver homogenates. Immunolocalization studies are needed so that the relationship of ER concentrations to cell proliferation in normal and preneoplastic cells can be more carefully evaluated.

In addition to effects on hepatic ER, TCDD may influence estrogen action in another way. CYP1A2 efficiently catalyzes the conversion of estrogens to catechol estrogens in liver (Graham et al., 1988; Dannan et al., 1986). CYP1A2 is not found in extrahepatic tissues, with the possible exception of the nasal cavity, so catechol estrogen formation would be expected to occur only in liver. Catechol estrogens have been postulated to possess macromolecule damaging properties as a consequence of free radical generation (Li and Li, 1990; Metzler, 1984). Therefore, TCDD may increase the DNA damaging capacity of estrogens in liver as a function of CYP1A2 induction. This effect may, in part, explain the carcinogenic actions of TCDD in female rat liver and is consistent with the knowledge that ovariectomy protects against the hepatocarcinogenic actions of TCDD and that male rats are not susceptible to TCDD-induced liver tumors (Lucier et al., 1991; Kociba et al., 1978). It is important to note that cancer is more than a two-stage process and the stage specific actions of TCDD in multistage cancer models are not known, although TCDD-mediated cell proliferation and possible indirect genotoxic effects may be critical at more than one stage. A

hypothetical mechanistic scheme for TCDD-mediated liver cancer is shown in Figure 6-2.

The finding that chronic TCDD exposure decreases tumor incidences in pituitary, mammary gland and uterus may also reflect TCDD's effects on ER and estrogen metabolism. As discussed above, TCDD decreases uterine ER concentrations in cytosolic and nuclear fractions of rats and mice and these changes are associated with diminished estrogen action in *in vivo* as well as *in vitro* studies. TCDD also increases estrogen metabolism presumably as a consequence of CYP1A2 in liver and UDPGT induction in liver and extrahepatic tissues (Shiverick and Muther, 1982). Likewise, addition of TCDD to a breast cancer cell line (MCF-7) results in increased estrogen degradation (Gierthy et al., 1988). However, there are only small effects on serum 17-β estradiol levels following administration of TCDD to either rats or mice (Shiverick and Muther, 1983). Therefore, the effect on serum estradiol is considerably less sensitive than effects on the uterine receptor. This comparison has led investigators to conclude that the antiestrogenic actions of dioxins are primarily caused by effects on ER levels in reproductive tract tissues. Final evaluation on the role of estrogen metabolism awaits data on concentrations of estrogens in responsive cells of control and TCDD-treated rats which may be different from serum estradiol levels. In any event, it appears clear that TCDD does possess antiestrogenic properties which are likely important to decreased tumor incidences in some reproductive tract and endocrine organs. Numerous studies have documented that the estrogen receptor is found in virtually every tissue of the body although effects of TCDD on human estrogen receptor *in vivo* have not been studied.

6.5.5. Other Biochemical Endpoints. TCDD alters a number of other pathways involved in regulation of cell differentiation and proliferation. The specific relationships of these effects to multistage carcinogenesis is not known but the broad array of effects on hormone systems, growth factor pathways, cytokines and signal transduction components are consistent with the notion that TCDD is a powerful growth dysregulator. It is also consistent with the findings that TCDD alters cancer risks at a large number of sites possibly reflecting multiple mechansims of carcinogenicity. Biochemical/molecular/endocrine changes produced

by TCDD include the glucocorticoid receptor (Sunahara et al., 1989), tyrosine kinase (Madhukar et al., 1988), gastrin (Mabley et al., 1990), interleukin 1β (Sutter et al., 1991), plasminogen activator inhibitor (Sutter et al., 1991), tumor necrosis factor-α (Clark et al., 1991b), gonadotropin releasing hormone (Moore et al., 1989), testosterone (Moore et al., 1985), and LH (Mabley et al., 1992). The importance of these responses to the carcinogenic process should not be diminished by the lack of detail presented here. In every case studied, these responses have been shown to be dependent on the Ah receptor.

6.6. SUMMARY AND WEIGHT OF EVIDENCE FROM ANIMAL STUDIES

There have been 17 chronic studies designed to determine if TCDD is a carcinogen in experimental animals. All of these studies have been positive and demonstrate that TCDD is a multisite carcinogen, it is a carcinogen in both sexes and in several species including the Syrian hamster, it is a carcinogen in sites remote from the site of treatment and it increases cancer incidence at doses well below the MTD. In two stage models for liver and skin cancer, it is clear that TCDD is a potent promoting agent with weak or no initiating activity. This finding is not surprising since TCDD does not form DNA adducts and it is negative in short-term tests for genetic toxicity. The general consensus is that TCDD is an example of receptor-mediated carcinogenesis in that (1) interaction with the Ah receptor appears to be a necessary early step, (2) TCDD modifies a number of receptor and hormone systems involved in cell growth and differentiation such as the epidermal growth factor receptor and the estrogen receptor, and (3) hormones exert a profound influence on the carcinogenic actions of TCDD. For example, ovarian hormones are essential for the hepatocarcinogenic actions of TCDD in rats, whereas TCDD promotion of lung tumors in rats appears to occur only in the absence of ovarian hormones. Although tumor promotion data for the polychlorinated dibenzofurans and co-planar PCBs is limited, it appears that these compounds are liver tumor promoters with potencies dependent on their binding affinity to the Ah receptor.

Some of the central issues in the risk assessment of TCDD and its structural analogs are (1) characterization of the shape of the dose response curve for receptor-mediated events, (2) evaluation of the relevance of animal data in the estimation of human risks, and (3) the health consequences of background

exposures (1-10 pg TEQ/kg/day) of dioxin and its structural analogs. In regards to the shape of the dose response curve, it is clear from animal studies that there are different dose response curves for different TCDD effects which is consistent with the generally accepted dogma for steroid receptor-mediated responses (Lucier, 1992). In general, the biochemical/molecular responses such as cytochrome P-450 induction do not show evidence for a threshold although unequivocal conclusions cannot be made and the mechanistic link, if any, between biochemical responses and toxic effects have not been established. In fact, coordinated biological responses such as TCDD-mediated cell proliferation and growth of preneoplastic lesions (foci of cellular alteration in liver) appear to be less sensitive endpoints although evaluation of these responses is complicated by a high degree of interindividual variation: some animals do not exhibit any increase in cell proliferation in response to chronic TCDD exposure.

The mechanistic basis for interindividual variation is unclear and this lack of knowledge complicates approaches to estimate human risks from experimental animal data. However, several studies indicate that, mostly, humans appear to respond like experimental animals for biochemical and carcinogenic effects. However, data from epidemiology studies are difficult to evaluate because the carcinogenic effects, if any, resulting from background TCDD exposures are not known, although biochemical effects such as cytochrome P-450 induction may be produced by background exposures.

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