

Carcinogenesis: A Late Effect of Irreversible Toxic Damage during Development

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Intrauterine and early postnatal life are periods of exceptionally high susceptibility to certain kinds of chemical carcinogens. The most potent known transplacental carcinogens are direct acting alkylating agents. Most nonreactive compounds, which require enzymes for metabolic conversion into chemically reactive "proximate carcinogens," are less effective because the required enzymes are present at low levels in the fetus, and many proximate carcinogens are too reactive to reach the fetus when formed in maternal tissues. Despite this, many carcinogens which require metabolic activation are very active transplacentally, as the intrinsic susceptibility of rapidly dividing fetal cells compensates effectively for comparatively low tissue levels of reactive metabolites. Transplacental carcinogens of all kinds are most effective late in gestation, generally after organogenesis has begun and after the period of greatest susceptibility to teratogens. Only a small number of known carcinogens have been tested for transplacental carcinogenic activity.

The great majority of tumors induced transplacentally in the well-studied rodent and lagomorph species (mouse, rat, Syrian hamster, and rabbit) have morphologic features of adult, rather than embryonal, tissues. A given agent tends to induce in a given species largely the same types of tumor when given transplacentally as when administered directly to postweaning animals, unless its carcinogenic effect in the latter is ascribable to some peculiarity of distribution, metabolism, or physiology. In a second species, the spectrum of tumors induced either before or after birth may be quite different.

For bioassay of suspected carcinogens, the significance of perinatal carcinogenesis lies in the facts that the fetal and preweaning rodent is an extremely sensitive indicator of carcinogenic activity, and that the facile adaptability of fetal cells to tissue culture and their rapid expression *in vitro* of properties of neoplastic transformation make possible a rapid *in vivo/in vitro* screening system for chemical carcinogens.

Introduction

That the newborn mouse is more susceptible than adult mice to oncogenic viruses was firmly established in the early 1950's by Gross (1). In contrast, subsequent demonstrations of a similarly heightened neonatal susceptibility to chemical carcinogens were greeted with some reserve (2), chiefly because the effect was not always great and appeared not to be general.

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However, the relatively recent discovery of the spectacular transplacental carcinogenic activity of certain direct-acting alkylating agents, notably ethylnitrosourea (ENU) (3), convincingly demonstrated that the period of postembryonic development was one of great intrinsic susceptibility to chemical carcinogens. This susceptibility has since been shown to apply not only to direct-acting alkylating agents, but to a broad range of other classes of chemical carcinogens as well (3-27) (Table 1).

There are a few agents which have been shown to be carcinogenic only in neonatal rodents, but no carcinogens are known which act only on the

Table 1. Representative transplacental carcinogens: small organic molecules.

Compound	Species	Target Organ	Reference
Enzyme-independent alkylating agents			
Methylnitrosourea	Rat	Nervous system	(4)
Ethyl nitrosourea	Rat	Nervous system	(3, 4)
Ethyl nitrosourea	Mouse	Lung, liver	(5)
Ethyl nitrosourea	Hamster	Nervous system	(6)
Ethyl nitrosourea	Rabbit	Kidney	(7)
<i>n</i> -Propyl nitrosourea	Rat	Nervous system	(8)
<i>n</i> -Butyl nitrosourea ^a	Rat	Nervous system	(8)
Methyl nitrosourethan ^b	Rat	Various	(9)
Methyl azoxymethanol	Rat	Various	(10)
Dimethyl sulfate	Rat	Nervous system	(11)
Diethyl sulfate	Rat	Nervous system	(11)
Propane sulfone	Rat	Nervous system	(12)
Methyl methane-sulfonate	Rat	Nervous system	(13)
Enzyme-dependent alkylating agents			
Dimethylnitrosamine	Rat	Kidney	(14)
Diethylnitrosamine	Rat	Kidney	(15)
Diethylnitrosamine	Mouse	Lungs, liver	(16)
Diethylnitrosamine	Hamster	Trachea	(17)
1,2-Diethylhydrazine ^c	Rat	Nervous system	(18)
Procarbazine, <i>N</i> -isopropyl- α -(2-methyl-hydrazino)- <i>p</i> -toluamide	Rat	Nervous system	(19)
Azoethane ^c	Rat	Nervous system	(18)
Azoxyethane ^c	Rat	Nervous system	(18)
Cycasin, methylazoxy-methyl- β -D-glucoside	Rat	Various	(20)
Polynuclear aromatic hydrocarbons			
Benzo[a]pyrene	Mouse	Lung, skin	(22)
7,12-Dimethylbenz[a]anthracene	Mouse	Lung, various	(21)
7,12-Dimethylbenz[a]anthracene	Rat	Nervous system, kidney	(23)
Estrogens			
Diethylstilbestrol	Human	Vagina	(24)
Diethylstilbestrol	Mouse	Vagina	(^d)
Diethylstilbestrol	Hamster	Vagina	(^e)
Miscellaneous			
Ethyl carbamate	Mouse	Lung	(25, 26)
Aflatoxin	Rat	Liver	(26, 27)

^a Higher homologs are inactive.

^b Intraplacental injection.

^c The dimethyl homolog is inactive.

^d Data of J. McLachlan, Proceedings of the Conference on Transplacental Carcinogenesis, January 19-21, 1976, NCI Monograph, in preparation.

^e Data of M. Rustia, Proceedings of the Conference on Transplacental Carcinogenesis, January 19-21, 1976, NCI Monograph, in preparation.

fetus. The same factors of metabolism, genetics, etc. which determine the carcinogenic activity of a compound in postnatal animals also pertain to the fetus, with the added complications for transplacental carcinogenesis that (a) the placenta may modify the distribution and further metabolism of a compound and its maternal metabolites, determining exposures for fetal tissues which may be both quantitatively and qualitatively different from those sustained by maternal tissues; (b) enzymes of drug metabolism required for activation of potential carcinogens may be absent and weakly or noninducible in fetal tissues until just before birth; and (c) differentiating and rapidly dividing fetal cells differ from adult cells in their intrinsic susceptibility to carcinogens. This paper will summarize certain features of carcinogenesis in prenatal and early postnatal life, chiefly in rodent species, which should be of general interest to toxicologists. More details can be found in recent reviews (28-31).

Transplacental Carcinogenesis

Chemical Characteristics of Transplacental Carcinogens

The single most comprehensive generalization currently accepted about most chemical carcinogens which are small nonpolymeric organic molecules is that they act by forming covalent bonds to intracellular target molecules. These reactions are nucleophilic substitution reactions in which the carcinogen is the "nucleus," or electrically positive reactant, while the intracellular receptor is an electron-rich entity, often a heteroatom (N,O,S) with an unshared electron pair (32). Under biological conditions such reactions are usually irreversible. Some chemical carcinogens, notably the direct-acting alkylating agents, can participate in such reactions without metabolic modification, or spontaneously form decomposition products which are intrinsically reactive. Others, including the polynuclear aromatic hydrocarbons, nitrosamines, aromatic amines, and in general the vast majority of known chemical carcinogens, require enzyme-mediated structural alteration—or activation—to form chemically reactive metabolites or "proximate carcinogens." Transplacental carcinogens generally (a) are direct-acting agents, (b) are activated by enzymes present in fetal target tissues, or (c) are transformed by maternal (or

placental) enzymes into reactive metabolites sufficiently stable to reach the fetus.

Direct-acting alkylating agents are the most active known transplacental carcinogens (Table 1), and of these, the most potent are the alkylnitrosoureas. ENU, administered as a single dose, induces neurogenic tumors in rats (3) and lung tumors in mice (5), with an apparent efficiency 50 times as great in the fetal rat and 15 times as great in the fetal mouse as in adults (Tables 2 and 3). The actual relative quantities of carcinogen reaching fetal tissues relative to adult tissues have not been accurately determined, but preliminary data indicate that the dose to the fetus is lower than that to adults. The fetus is thus probably even more susceptible to ENU than these figures indicate.

In the homologous series of *n*-alkylnitrosoureas, maximal activity is found with the ethyl and propyl compounds, while the methyl and butyl compounds are distinctly less active and higher homologs are inactive (4,8,31). Only the methyl and ethyl derivatives of most other classes of alkylating agents have been studied; in all cases, ethylating agents were more potent transplacental carcinogens than methylating agents. Probably this reflects in part an optimal combination of chemical reactivity (which tends to decrease as alkyl chain length increases) and solubility properties (oil/water partition coefficients tend to increase with increasing alkyl chain length). Possession of a key

structural element (e.g., a nitrosamide group) is thus not sufficient *per se* to confer transplacental carcinogenic activity on a compound.

Biosynthesis of Reactive Intermediates or "Proximate Carcinogens"

Enzyme-dependent alkylating agents, such as the dialkylnitrosamines and the 1,2-dialkylhydrazines, generally require enzyme-mediated oxidative metabolism of the *N*-dealkylase type for formation of proximate carcinogens (Fig. 1, pathways A and D). The formation of α -hydroxy derivatives of the dialkylnitrosamines has been inferred from the fact that aldehydes are isolated, along with alkylation products, from microsomal reaction mixtures *in vitro*. The intermediates themselves, such as methyl(hydroxymethyl)nitrosamine [$\text{CH}_3\text{N}(\text{NO})\text{CH}_2\text{OH}$, DMN-OH] from dimethylnitrosamine [$\text{CH}_3\text{N}(\text{NO})\text{CH}_3$, DMN, Fig. 1, path A, $\text{R}=\text{R}'=\text{H}$] are too short-lived to isolate. It is virtually certain that transplacental carcinogenic effects of such compounds are due to formation of proximate carcinogenic metabolites solely within fetal target tissues, where such enzymes are present at levels much lower than in corresponding adult tissues (33). A common result is that compounds such as DMN are active only very late in gestation, and are less active in the fetus than in the adult (14) (Table 3). However, the principle that ethylating agents are more effective transplacental carcinogens than methylating agents applies also to enzyme-dependent compounds. Diethylnitrosamine, subject to the same metabolic requirements as its dimethyl homolog, is as least as effective transplacentally in the Syrian hamster fetus as in the adult (34) (Tables 2 and 3).

It has recently become possible to investigate the modes of action of DMN more directly, following the synthesis of methyl (acetoxymethyl) nitrosamine [$\text{CH}_3\text{N}(\text{NO})\text{CH}_2\text{OCOCH}_3$, DMN-OAc], the stable acetyl derivative of its postulated active metabolite, DMN-OH (35). DMN, when given to rats in a single dose either to adults or transplacentally, induces renal tumors almost exclusively. DMN-OAc is readily hydrolyzed by esterases, and was expected to produce tumors in a wide variety of tissues as a consequence of local generation of DMN-OH. When given to rats in a single intraperitoneal injection, however, it yielded intestinal tumors exclusively (36), which we believe is due to its rapid destruction in rat serum and its much greater stability in bile. The offspring of

Table 2. Comparative oncogenic effects of direct-acting and enzyme-dependent alkylating agents administered transplacentally to the fetus or directly to adults.

Compound and dose	Species (gestation period)	Parameter of carcinogenesis	Reference
Direct acting alkylating agent			
Ethylnitrosourea, 60 mg/kg	BD IX Rat (23 days)	Multiplicity of neurogenic tumors	(3)
Ethylnitrosourea 60 mg/kg	Swiss mouse (21 days)	Multiplicity of lung tumors	(5)
Enzyme-dependent alkylating agents			
Dimethylnitrosamine, 1 mg/kg/day \times 7	Rat (22 days)	Fraction of rats with kidney tumors	(14)
Diethylnitrosamine, 45 mg/kg	Syrian hamster (15 days)	Fraction of hamsters with respiratory tract tumors (trachea, larynx, lungs)	(33)

Table 3. Comparative oncogenic effects of direct-acting and enzyme-dependent alkylating agents administered transplacentally to the fetus or directly to adults.*

		Ref.	Results by day of treatment														Adult
			Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	
Ethylnitrosourea	Rat	(3)	0	0	0	0	0.1	1.4	1.2	1.6	1.9	2.4	2.7	2.4	2.7	2.4	0.06
Ethylnitrosourea	Mouse	(5)	—	—	—	—	0.3	—	5.5	9.5	26.5	21.3	11.8	—	—	—	1.8
Dimethylnitrosamine	Rat	(14)	←————— 0 —————→										←————— 0.04 —————→			0.79	
Diethylnitrosamine	Syrian hamster	(33)	0	0	0	0	0.4	0.4	0.71	0.95							0.82

* Animals and dosages as detailed in Table 2.

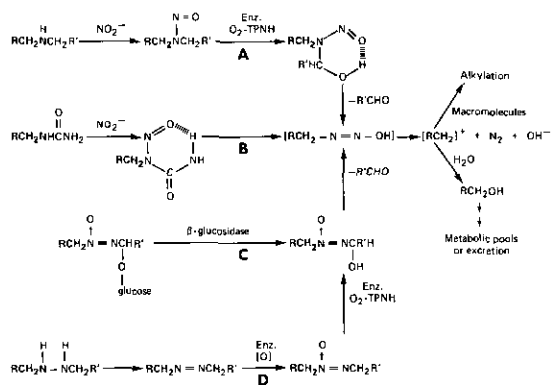


FIGURE 1. Metabolic paths, some requiring enzymes, all of which lead to a common reactive alkylating species from a variety of transplacental carcinogens. Path A: secondary amine \rightarrow dialkyl nitrosamine \rightarrow alkyl- α -alkanol nitrosamine; Path B: Alkylurea \rightarrow alkyl nitrosourea; Path C: cycasin (R = H) \rightarrow alkyl azoxyalkanol; Path D: 1,2-dialkylhydrazine \rightarrow azoalkane \rightarrow alkoxyalkane \rightarrow alkyl azoxyalkanol. Reproduced by permission from Rice (29).

rats given DMN-OAc intravenously during the last 10 days of pregnancy develop brain and kidney tumors, and very rarely intestinal tumors (Rice et al., in preparation). These results are compatible with the postulate that the induction of tumors by DMN reflects endogenous formation of DMN-OH within affected organs, since the stable "carrier" derivative, DMN-OAc, is clearly capable of causing tumors in other organs if it is able to reach them.

Cycasin (methylazoxymethyl- β -D-glucoside) is a natural product, a constituent of the nut of the cycad, *Cycas circinalis*. This substance is harmless when injected into adult rats; it is excreted quantitatively in the urine. But if fed, the gluco-

side linkage is hydrolyzed by bacterial glucosidases in the intestine, liberating the moderately stable proximate carcinogen, methylazoxymethanol (MAM; Fig. 1, path C, R = R' = H). Cycasin and MAM are both transplacental carcinogens in the rat (10,20), the former when given orally. The stability of MAM is much greater than that of DMN-OH (note that the two are structural isomers), which undoubtedly accounts for the wide variety of tumors produced by both MAM and cycasin when given transplacentally or to adults. The failure of the carcinogens dimethylhydrazine and azoxymethane to induce tumors transplacentally except on the last day of gestation (37), despite their potent activity in adults and their required metabolism to MAM (Fig. 1, path D, R = R' = H), is in marked contrast to the potent transplacental carcinogenicity of diethylhydrazine, azoethane, and azoxyethane (18). The stability of the proximate carcinogen cannot be the limiting factor in this case. It is possible that (a) fetal enzymes which readily metabolize the ethyl homologs are less active toward methyl substrates, and that enzymes which do act on the methyl substrates do not appear until just before birth or (b) MAM generated in maternal tissues generally reacts *in situ* and is not as efficiently released into the maternal blood stream as its diethyl homolog. Neither of these possibilities has been tested.

Many substances which are not alkylating agents have demonstrable and sometimes striking transplacental carcinogenic activity. The predominance of alkylating agents in Table 1 is probably more a reflection of investigators' choices than of the true ratio of alkylating agents to other kinds of transplacental carcinogens. The polynuclear aromatic hydrocarbons, for example, have drawn the attention of only a few

laboratories. Benzo[*a*]pyrene (22), methylcholanthrene (38), and 7,12-dimethylbenz[*a*]anthracene (DMBA, 21) have all been shown to be weak transplacental carcinogens in mice, inducing predominantly lung tumors in low incidence. When DMBA was eventually studied in the rat, however, it was reported to be a highly effective transplacental carcinogen for the nervous system and kidneys (23), an observation we have confirmed and extended (S.R. Joshi, et al., in preparation). The target organs in each species were the same for the polynuclear aromatic hydrocarbons as for alkylating agents. The exact metabolic paths involved in activation of the polynuclear aromatic hydrocarbons to proximate carcinogens are not known, but are generally accepted to involve arene oxide intermediates formed enzymatically (40). Whether the proximate carcinogens responsible for the transplacental carcinogenic activity of these compounds are formed in fetal tissues or in adult tissues with subsequent transfer to the fetus has not been determined.

Diethylstilbestrol (DES) has the distinction of being the only substance known to be a transplacental carcinogen in man (24). Its effects appear so far to be limited to the vagina and cervix, the differentiation of which is modified by estrogen. This effect has been demonstrated recently in the mouse by J. McLachlan (in preparation) and in the Syrian hamster by M. Rustia (in preparation). In both rodent species the female genital tract was also the only organ system in which tumors developed. Although it is possible to envision reasonable metabolic paths which would convert DES into reactive metabolites like those of the aromatic hydrocarbon carcinogens, so far no evidence has been produced that such metabolites actually are formed. It may be that the carcinogenic activity of DES is ascribable entirely to its estrogenic activity, and that its mechanism of action is fundamentally different from that of other organic chemical carcinogens. This question is currently under active investigation.

Histologic and Biologic Features of Transplacentally Induced Tumors: Organotropism

Since certain pediatric tumors of man may be present at birth and must therefore have arisen during intrauterine life, transplacental carcinogenesis was originally looked upon as a possible source of such neoplasms, many of which are characterized by distinctively primitive histologic patterns (e.g., neuroblastoma,

medulloblastoma, Wilms' tumor). This apparently reasonable prediction, whatever its validity with respect to man, is not confirmed by experimental studies on rodents. The vast majority of tumors induced transplacentally in mice, rats, and Syrian hamsters are histologically and histogenetically equivalent to human tumors of adult life, and arise in these rodent species well after sexual maturity is attained. Three-fourths of all transplacentally induced neurogenic tumors observed in rats in a large German series (41) of over 1000 tumors were of the myelin-forming cells: schwannomas (neurinomas), oligodendrogliomas, and mixed gliomas in which neoplastic oligodendrocytes predominated. The only tumors of the neuron series observed in this species were olfactory esthesioneuroepitheliomas. No neuroblastic tumors of the retina, adrenal medulla, or cerebellum were seen. In contrast, the mouse, in which neurogenic tumors are much more difficult to induce, yields a few cerebellar medulloblastomas (but no other neuroblastic tumors) in response to neonatal (42) exposure to ENU. Kidney tumors are readily inducible in both species with many agents, either transplacentally or after birth, but in the mouse these are invariably of adult epithelial morphology, while in the rat a variable but small proportion of primitive nephroblastic tumors are seen. In the rabbit the latter are much more common (7). It appears that susceptibility to various embryonal tumors is a characteristic which varies from species to species, as in the case for many other kinds of inducible neoplasm.

A corollary to this axiom is that a given agent, when administered transplacentally, frequently induces entirely different kinds of tumors in a series of closely related species (Table 1). There is also a general tendency for many different kinds of agents to induce a characteristic neoplasm or spectrum of neoplasms in a given species. Virtually any transplacental carcinogen except DES will produce lung tumors in mice, while the nervous system and kidneys appear especially vulnerable in rats.

Latency periods are not consistently shorter for a given type of tumor when the inducing treatment is administered to the fetus or neonate rather than to an adult.

Carcinogenesis during Infancy

As previously noted many, but not all, carcinogens are more effective in newborn or very young animals than in adults (2), despite the fact

that many of the drug metabolizing enzymes required for carcinogen activation are present in infant tissues at levels below those of adults. Aflatoxin B₁ for example, is a potent liver carcinogen in adult rats and in several other species, but adult mice are refractory to it; only newborn and infant mice are highly susceptible (43). Interestingly, aflatoxin is a rather weak transplacental carcinogen in the rat (27).

The changing patterns of enzyme activity during fetal and early postnatal development do not always result in a steadily increasing capability to activate carcinogens. Cycasin, as discussed earlier, ordinarily is activated by β -glucosidases of intestinal microorganisms, and for this reason is ineffective in adult rats when injected parenterally. However, there is a high level of a β -glucosidase in rat intestinal mucosa at birth and for the first few weeks of postnatal life. Subcutaneous injection of cycasin during this period leads to tumor induction by methylazoxymethanol liberated by this transient tissue enzyme (44). Since the carcinogenic metabolite is relatively stable, the tumors induced by it occur primarily in the kidney, remote from the site of biosynthesis.

When the variables associated with metabolism are eliminated, as when a direct-acting alkylating agent is involved, it is often found that not all tissues and organ systems in which tumors are induced by such a carcinogen are maximally susceptible in neonates. ENU in mice, for example, when given as a single intraperitoneal injection was most effective in neonates with respect to the liver, kidney, ovaries, and nervous system, but more effective in adults with respect to mammary gland, Harderian gland, stomach, and certain other tissues (45). Age-dependent shifts in organotropism are amplified in some cases by systemic physiologic factors, such as endocrine activity. For example, the mammary glands of young adult female rats of some strains are extremely susceptible to aromatic hydrocarbon carcinogens and are the principal organs affected by DMBA at this age (39).

In Vivo-In Vitro Bioassay Systems

The facile growth of fetal cells in tissue culture and the development of reliable morphologic indices of neoplastic transformation have provided a methodological approach to two desirable goals of bioassay screening for carcinogenic activity: utilization of the high susceptibility of fetal cells to improve sensitivity, and development of a

rapid, inexpensive *in vitro* test system. The transplacental host-mediated system of DiPaolo et al. (46), which has been further developed by Quarles and Tennant (NCI Monograph, submitted for publication), involves treating a pregnant hamster with a test compound. A primary culture is prepared from the minced fetuses of the treated animal, and after several passages the cultured cells are assayed for morphologic transformation (production of atypical colonies on plastic surfaces) or for ability to grow in soft agar. A number of known strong carcinogens, including polynuclear aromatic hydrocarbons and nitroso compounds, have been demonstrated positive in this system after a total elapsed time of only a few months. Quarles and Tennant have shown that nitroso derivatives of a number of commonly used pesticides are also positive. The system requires additional demonstrations of sensitivity to relatively weak carcinogens but offers great promise for a practical application of transplacental carcinogenesis to the expensive and time-consuming bioassay of suspected carcinogens.

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