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MOLECULAR BASIS OF ENVIRONMENTALLY INDUCED BIRTH DEFECTS

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■ **Abstract** Exposure of the developing conceptus to selected environmental agents can lead to deleterious and often times lethal birth defects. These malformations result in serious emotional and financial consequences to families and societies worldwide. As we continue to progress technologically, we face challenges from the introduction of new pharmacological agents and chemical compounds into the environment. This results in a concomitant need to more fully understand the relationship between in utero exposure to environmental teratogens and the risk of congenital malformations. The goal of this review is to provide a current perspective of the major concepts related to the molecular basis of environmentally induced birth defects. Starting with a discussion of commonly occurring birth defects, we consider important fundamental facets of embryonic development, teratology, and gene-environment interactions. The review then summarizes our current understanding of the molecular mechanisms involved in selected birth defects following exposure to pharmacological compounds, including thalidomide, retinoids, and valproic acid. Understanding these signaling pathways may lead to the development of safer pharmaceutical compounds and a reduction in the number of infants born with preventable birth defects.

INTRODUCTION

Congenital malformations are the leading cause of infant mortality in the United States (1). Malcoe and coworkers (2) recently demonstrated that the presence of any malformation (including those considered unlikely to be lethal) diagnosed

during the first year after birth increased mortality 9-fold for black infants and 18-fold for white infants. These malformations are associated with enormous emotional and fiscal costs. For example, the estimated lifetime cost for children born each year in California with spina bifida exceeds $58,375,000. Similarly, those born with a conotruncal heart defect have medical costs exceeding $287,000,000, and for orofacial clefts the costs are nearly $86,000,000 (3). These figures can be multiplied by 10 to roughly estimate lifetime medical costs for these same malformations among children born each year throughout the United States. Clearly, the public health impact of congenital defects cannot be overstated. Yet despite our best efforts, the prevalence of such adverse pregnancy outcomes has not been dramatically reduced over the past decade. While folic acid has reduced neural tube defect frequency, overall birth defects of all kinds remain fairly static.

For the most part, we are uncertain of the etiologic nature of the vast majority of congenital malformations in infants. It has been estimated that some 5% to 10% of all birth defects are due to an in utero exposure to a known teratogenic agent or maternal factor (4). As such, an increasing importance has been placed on trying to understand the role environmental agents play during early mammalian development. This is critically important because there is growing concern that human populations are being exposed to an ever-increasing number of potentially harmful agents through the environment, including the contamination of our foodstuffs by agricultural chemicals and petrochemicals. It is estimated that several thousand new compounds synthesized each year or produced as industrial byproducts reach our environment (5). Perhaps 10% of these agents persist in appreciable amounts as potential environmental toxicants. While our efforts to monitor these compounds have not kept pace with their introduction, Schardein (6) estimates that over 3300 chemicals have now been tested for their teratogenic potential, and approximately 37% exhibit some evidence of teratogenicity. Similarly, Shepard (7) documents more than 2500 compounds in his catalog of teratogenic agents. Of these, 1200 produce congenital defects in experimental animals, yet only 40 are generally considered to be human teratogens. The 40 known human teratogens include: infectious agents, physical factors, maternal metabolic imbalances, drugs, and environmental chemicals. The huge gap between the two estimates suggests that a great deal more needs to be known about the teratogenic potential of environmental chemicals in human populations.

Since virtually any chemical can be considered to have a teratogenic potential at some dose level, it is important to understand the basic mechanism by which environmental agents disrupt normal embryonic development. The two primary processes important to consider are teratogenesis and mutagenesis. These two means of inducing birth defects may involve overlapping targets, yet there remain significant differences between them. For example, mutagenesis produces heritable changes in the genetic material and ultimately involves alterations in either

the primary message, conformation, and/or regulation of a specific target DNA (8). On the other hand, teratogens cause noninherited malformations, inducing congenital defects by altering fundamental embryological processes, many of which may be linked to alterations in gene function. Because we can simplify the regulation of early morphological development as the production and recognition of various cellular signals, teratogen-induced disruptions may be caused either by aberrant signal production or by altering a cell's recognition and response to a given signal. The interference with these processes may directly disrupt or modulate the levels of the key molecular components of critical developmental pathways (9).

Teratogens are believed to act in a number of different developmental pathways exploiting multiple targets and mechanisms to alter normal embryogenesis (5). To date, relatively little is known about the consequences of a teratogenic insult on the molecular homeostasis of the developing embryo. Such information is only now being acquired with the advent of the recently developed tools of molecular biology. What is apparent from a number of different experimental systems is that a teratogenic insult can disrupt important developmental processes likely to be controlled by multiple genes utilizing a complex regulatory structure. One approach successfully applied to investigate complex developmental events and their genetic regulation has been to disrupt normal morphogenesis using specific teratogenic agents and then carefully to analyze the morphological, physiological, and molecular consequences.

DEFINITIONS OF CONGENITAL ANOMALIES

It is important to recognize that not all congenital defects are malformations. Congenital defects can be classified as either malformations, deformations, or disruptions (10). This classification scheme has been developed so that consideration is given to the means by which the anomaly occurred. This has implications with respect to assessing the prognosis, as well as how the health care team might approach recurrence risk counseling. For example, a malformation develops owing to a problem that is intrinsic to the embryologic differentiation or development of a structure. Deformations are anomalies that occur owing to an alteration in the shape and/or structure of a body part that had previously undergone normal differentiation. The vast majority of deformations are thought to be the result of intrauterine molding and involve the musculoskeletal system. The final classification of morphological birth defects are called disruptions. In these situations, a structural defect results from the destruction of a previously normally formed structure. This could be the result of either aberrant amniotic bands or through an interruption in the vascular supply. Thus there was no defect in the assembly phase, but the problem that disrupted normal morphogenesis occurred later in development and resulted in a birth defect.

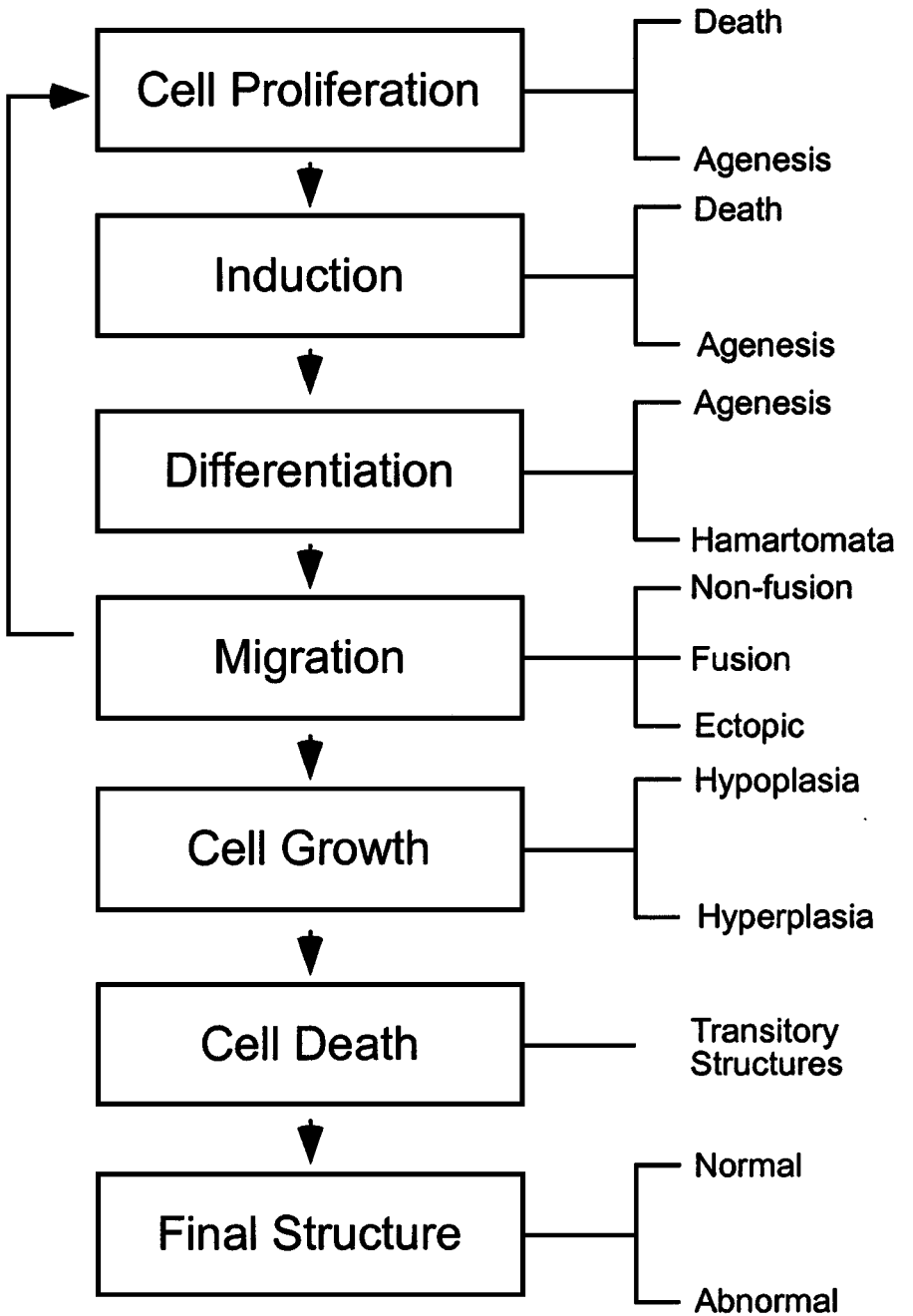
From a practical standpoint, aside from the ability to provide accurate recurrence risk information to the family, recognition that certain structural defects are malformations implies an error intrinsic to the developing embryo. These malformations represent a single primary defect in development that is usually of a multifactorial etiology. This often implies the interaction between an environmental factor (teratogen) and a subset of maternal and/or embryonic genes. Since teratogens may adversely affect a nearly unlimited number of genes, it is important to consider those developmentally regulated genes expressed during critical periods of mammalian embryogenesis. Although not all congenital anomalies are malformations, the focus of this review is the molecular mechanisms underlying birth defects that can be classified as malformations.

DEVELOPMENTAL PROCESSES

The period of embryonic development in the human begins at fertilization and technically ends after week eight, at which time the fetal stage of development begins (11). Week one begins with fertilization, and during this week, the initial cell divisions and formation of a single germ cell layer embryo occurs. By the end of week two, the embryo consists of two germ layers. By the end of week three, all three germ layers (endoderm, ectoderm, and mesoderm) are present, as well as the establishment of the cranial-caudal and dorsal-ventral axes of the embryo. In week four, all the major organ systems are beginning to form. Weeks five through eight are referred to as the period of organogenesis, when the principal development of the organs and limbs occur. At the end of this period, the embryo has progressed to the fetal stage and has all the recognizable body structures. As is clear from the above, most of the critical morphogenic processes are taking place in the first two months postconception. At this time, the embryo is especially vulnerable to teratogenic insult. While environmental insults can also adversely affect a developing fetus, it is during the first eight weeks (embryogenesis) that teratogens or teratogenic exposure have their major impact upon the structural form of the developing embryo.

The development of embryonic structures result from a well-orchestrated series of rather complicated molecular, biochemical, and physical events that eventually lead to what we recognize as a fully developed infant. The development of a structure begins with cell proliferation that enables the embryo to attain a critical mass or density (Figure 1). These earliest of embryonic primordia can subsequently be

Figure 1 Potential consequences of environmental insult during development. The boxes represent different cellular and tissue processes during development. Lines extending to the right illustrate potential deleterious outcomes upon exposure to harmful environmental teratogens and conditions.



(after Chernoff & Jones 1983)

acted upon by inductive forces. Such forces or signals are able to promote cellular differentiation, thereby providing the necessary anlagen for the development of the final embryonic structures. Many of the inductive signals are secreted growth factors passed between cell populations of interacting tissues. These growth factors can include the members of fibroblast growth factor (FGF), epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF) families (12–18).

One of the most critical of the signals between two opposing cell populations involves epithelial-mesenchymal interactions. The mesenchymal cells influence the epithelium, which can differentiate and secrete factors that subsequently influence the mesenchyme. Such interactions continue until a target organ develops with organ-specific cell populations. The classic example of an inductive interaction between two embryonic tissues is the formation of the lens of the eye. Here, contact between the ectoderm and optic vesicle induces a lens placode, which invaginates and differentiates to form the lens. Although lens induction has been studied for nearly a century, the underlying mechanisms are not well understood, and no extracellular molecules mediating interaction between the optic vesicle and the ectoderm have as yet been identified. A correlation has been recently established between the expression of the bone morphogenic protein-4 (Bmp4) gene in the optic vesicle and surface ectoderm, and the timing of lens induction in the mouse embryo (19). By analysis of Bmp4 homozygous knockout embryos that survive to gestational day 9.5, it was shown that the mutant optic vesicle fails to induce a lens placode. Significantly, the defect can be rescued by localized application of Bmp4 protein to mutant eye vesicles developing in organ culture. These and other functional studies still in progress have led to a new model for lens induction. According to this model, the lens is induced by the coordinated activity on the competent surface ectoderm by Bmp4 and other factors secreted by the optic vesicle.

Differentiation, or the acquisition of cellular specificity that creates diversity among the cells of the developing embryo, is generally preceded by nuclear processes in which the cell becomes committed to a selected fate (Figure 1). These processes include methylation of specific genomic regions in selected cell populations, as well as the selective turning on and off of specific genes by cell-specific transcription factors (20–23). Cellular differentiation is most often followed by a migratory phase, in which cells actually move to a targeted region within the developing embryo. The progression from a fertilized single cell to fully developed fetus is dependent upon these migrating cells obtaining the proper positional information, ensuring that the embryo's antero-posterior axis is formed correctly (9). Once established, the cells may either repeat this morphogenetic program or mature into the final fetal structure through a process of localized cell growth and apoptosis (24).

Should environmental agents or teratogens interact with the aforementioned developmental program at any time, the consequences could be embryonic demise or the induction of a developmental defect. For example, should teratogenic

exposure inhibit cell proliferation in the early embryo prior to the time when these cells have differentiated, the outcome is likely to be either embryonic death or the failure of a structure to form (Figure 1). One might anticipate that the embryo would die if this were to occur prior to implantation, whereas later in development, the lack of cellular proliferation might once again impede the development of a specific organ or structure. Somewhat later in development, it is possible that a teratogenic insult might alter the inductive signals of a population of cells, either by inhibiting the production of the signal or by rendering the target cells unresponsive to the message. Depending on when this occurs in early embryogenesis, it is likely to result in early embryonic loss or agenesis of a specific structure (Figure 1). When there is an environmentally induced alteration in cellular differentiation, the resulting embryo may present with a missing structure, or one abnormally constructed containing abnormal tissue types, such as in a hamartomata and teratomata (10).

Should the adverse environmental interactions occur during the critical period of embryogenesis when there are large numbers of migrating cells, it is possible that the teratogenic impact would be that certain structures might develop in abnormal locations. If cells fail to migrate altogether, the embryo may have congenital malformations or have fused structures secondary to the adhesion of adjoining tissues (24). The failure of cell growth in the embryo following migration may result in undersized or hypoplastic organs or structures. This may be the result of fewer cells overall or an actual diminution of the individual cell's size. On the other hand, should the teratogenic insult produce excessive cell growth, one might anticipate that the resulting structure would be hyperplastic. Given that the foundation for the structure is already established at this point in embryogenesis, teratogen-induced changes in cell growth would lead to malformations, rather than agenesis. Finally, apoptosis, or programmed cell death, is critically important in providing the final sculpting to the embryonic structure or organ. When cells do not respond to normal signals or cues and fail to die, the embryo may retain what are normally transient structures. Thus, compounds that alter normal apoptotic signals are very often teratogenic (Figure 1).

It is possible to view common teratogen-induced malformations in the context of alterations of the above-described pathway of developmental processes. For example, with neural tube closure defects such as spina bifida or anencephaly, we can trace back to problems that arise in these essential morphogenetic events. In the neurulation stage embryo, the notochord sends inductive signals to the overlying ectodermal cells, which begin to differentiate into those cells that contribute to the neural crest and those that support and contribute to the neural folds. This process occurs at different levels of the neural tube, in accord with the concept of multiple closure initiation sites (25). The final formation of the closed neural tube involves localized areas of pronounced cellular growth and proliferation, as well as programmed cell death. Interference with this process by teratogenic exposure will prevent the neural tube from closing and result in the birth of an infant with a neural tube defect.

GENE ENVIRONMENT INTERACTION CONCEPTS

Gene Expression Abnormalities

During development, much like in the adult state, changes in the rates of gene expression are occurring in response to the physiologic needs of the organism. The appropriate temporal and spatial expression of suites of developmental genes is absolutely critical to normal development and physiology (20, 21). In addition, the developing organism needs to respond at the transcriptional level to a myriad of environmental factors relating to the mother, such as her nutritional state and potentially to the presence of environmental toxins. As was previously mentioned, disruption of a developmental process such as proliferation of individual cells, differentiation of cells to form tissues, and migration of cells can all result in the birth of an infant with a congenital malformation. It follows then that an environmental agent may directly alter the transcriptional response of a gene encoding a protein involved in any of the above-mentioned processes and may have important consequences for normal embryogenesis. Conversely, an environmental agent that interferes with a protein involved in a signal transduction pathway that normally culminates in the transcriptional regulation of developmental genes may also cause a birth defect.

Several agents believed to be teratogenic and to directly influence embryonic gene transcription do so in part by acting as ligands for the steroid/thyroid/retinoid superfamily of transcription factors (26, 27). These proteins mediate their effects by binding specific DNA elements located in promoter and enhancer elements of genes, and so they modulate the actions of RNA polymerase II. The proteins are important mediators of embryonic and fetal development. Examples of teratogenic substances that act through the steroid receptor superfamily include the retinoids, estrogen-like compounds, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (28–31). The molecular actions and teratogenic consequences of retinoids are discussed in more detail in a subsequent section of this review.

While the data linking retinoids to human congenital malformations is clear, the relationship of environmental estrogens and TCDD to birth defects is far more speculative. Exposure to diethylstilbestrol following maternal pharmacological consumption during pregnancy was responsible for a threefold increase in the incidence of hypospadia in human males (32). Although the incidence of these congenital malformations was increased, fertility and libido changes were not observed in the adult males that were exposed in utero. While exposure to estrogenic-like compounds present in the aquatic environment from industrial waste products are believed to have induced malformations in other animals, the effect on humans is far less clear. It has been proposed that exposure to pesticides through gardening can increase the risk for cryptochidism (33). Exposure to the contaminants polychlorinated dibenzodioxins and polychlorinated dibenzofurans (dioxins) is believed to be developmentally toxic. The dioxin TCDD can act as a ligand for the aromatic (aryl) hydrocarbon receptor (AhR) (34, 35), and binding to the AhR

results in the transcriptional upregulation of the cytochrome P4501A1 gene. This in turn results in the increased metabolism of the toxin. There is, however, evidence that this compound may be acting as an endocrine disrupter by interfering with estrogen responsive pathways (36).

A developmental signaling pathway highly susceptible to environmental insult is the evolutionarily conserved sonic hedgehog (SHH)-patched(PTCH)-GLI pathway (37). SHH is a secreted signaling protein that performs a critical role in patterning of the head and brain. It is produced during embryogenesis by the cells of prechordal plate, and it results in the induction of ventral forebrain from the neural plate (38). SHH is also secreted by cells that ultimately give rise to the midline facial structures. The SHH pathway begins with a proteolytic cleavage of SHH, a process involving cholesterol. Following proteolytic cleavage, mature SHH binds to the PTCH receptor, and subsequent activation of the GLI transcription factors occurs.

A perturbation of the SHH-PTCH-GLI signaling pathway during embryogenesis can result in the midline defect known as holoprosencephaly (HPE) (39). HPE is a developmental disorder that exhibits a wide range in the severity of its phenotypic expression, which may present as mildly as a single central incisor, or as dramatically as cyclopia (40–43). It occurs at a rate of 1 per 16,000 live births, although evidence exists that in aborted fetuses, the rate of HPE is closer to 1 in 250 conceptuses. The etiology of HPE is complex and it is widely believed that both environmental and genetic variables are important. Mutations have been observed in a variety of genes including SHH, but not all HPE patients have mutations in known HPE genes or have cytogenetic anomalies (43). Perturbation of the SHH developmental pathway can also occur by a biochemical (teratogenic) interruption of the SHH signaling pathway. Compounds demonstrated to interrupt normal function of SHH include the steroidal alkaloids cyclopamine and jervine. Cyclopamine and jervine are components of the plant *Veratrun californicum* (also known as the corn lily), and they can result in cyclopia among the offspring of grazing animals (44, 45). The compounds are believed not to interrupt the processing of SHH, but rather to interfere with the ability of the target tissues to respond to the SHH signals. Although these two compounds induce HPE in the fetuses of grazing animals, no direct link between these compounds and the occurrence of HPE in humans has been established. Other genes involved in cholesterol biosynthesis, such as 7-dehydrocholesterol reductase, the gene causing Smith-Lemli-Opitz syndrome, can also result in malformations of the midline facial structures when they are mutated (46–49). This further demonstrates that exposure to compounds that affect cholesterol metabolism may ultimately interfere with SHH signaling and result in a congenital malformation.

Teratogenic Exposure and the Susceptible Genotype

There are substantial epidemiological data to illustrate that the interplay between environmental and genetic factors in the development of congenital malformations is significant. Socioeconomic, geographic, ethnic, and maternal health

considerations are all important precipitating factors for the development of birth defects (50–53). As might be expected, the genotype of the fetus is also important for contributing to the development of birth defects. Human epidemiological studies suggesting that the genotype is important are supported by a rich literature of experimental animal studies. For example, it has often been demonstrated that the rates of congenital anomalies differ between different strains of mice in response to the same dose of a given teratogen. These different hierarchies of susceptibility are explored in greater detail in a subsequent section of this review.

Prime examples of congenital malformations in which it is strongly suspected that both genetic and environmental factors are important include both neural tube defects (NTDs) and craniofacial anomalies (54–56). While the most common forms of NTDs include spina bifida and anencephaly, the clinical spectrum of NTDs also includes craniorachischisis and iniencephaly. Due to the complex etiology of NTDs, the identification of genes involved in the susceptibility to NTDs is difficult. Recent evidence linking folic acid to the prevention of NTDs has stimulated a great deal of new research in order to determine the protective mechanism of this B-vitamin (56–61). Although supplemental folic acid may reduce the incidence of NTDs by up to 70%, the molecular mechanisms by which it exerts its protective effect remain unknown and are a matter of considerable speculation. The striking ability of folic acid to reduce the incidence of NTDs has resulted in the mutation and polymorphism screening of genes encoding proteins directly involved in folic acid metabolism and uptake. These include folate receptor alpha (FRα), reduced folate carrier (RFC), the 5,10-methylene-tetrahydrofolate reductase (MTHFR), cystathionine B-synthase (CBS), methionine synthase (MS), methionine synthase reductase (MTRR), and methylenetetrahydrofolate dehydrogenase (MTHFD) (59, 62–75). To date few polymorphisms that have been identified appear to be related causally to the NTDs or serve as risk factors. The gene that codes for the MTHFR enzyme has been most often described as a potential risk factor for neural tube defects. The MTHFR gene has been mapped to the short arm of chromosome 1 (1p36.3), consists of 11 exons, and is known to be alternatively spliced. There is a common polymorphism that occurs at nucleotide C677T and occurs in the homozygous state in between 10% and 25% of the population (65, 71, 73, 76). The mutation involves a change from a cytosine to a thymine, which results in an alanine-to-valine amino acid substitution. Individuals who are homozygous for this allele have 50% to 60% lower enzyme activity at high temperatures (it is a thermolabile form of the wild-type enzyme). Most importantly, homozygotes for this allele have slightly elevated homocysteine concentrations if their folic acid intake is low. Individuals heterozygous for this polymorphism have enzyme levels that are intermediate between the low activity of mutant homozygotes and the high activity of the homozygous wild-type individuals. Another variant in the MTHFR gene is known as the A1298C polymorphism (77). The point mutation occurs in exon 7, which results in a single amino acid substitution of glutamate for an alanine. With this polymorphism, the enzyme's activity is reduced, but not to the extent that it is with the 677 gene variant. Individuals who are homozygous for this

polymorphism do not have elevated homocysteine levels. Those individuals who are heterozygous for each of the variants behave just like the C677T homozygotes, in that they have elevated homocysteine levels in the presence of low folate intake. It is important to realize that such compound heterozygotes are very rare and are unlikely to be of much concern from a genetic screening perspective.

In 1995, a Dutch group led by Henk Blom reported a threefold increase in the risk for NTDs among infants who were homozygous for the C677T variant (73). In 1998, they enlarged their sample size and found a reduction in the risk for spina bifida to 1.7-fold over that of individuals not having this polymorphism. As a result of these initial reports, there was a flood of studies to determine the frequency of this variant allele in different populations around the world and to see if there was an association between its prevalence and the risk for NTDs. Some studies in the United States and in Europe reported that homozygotes for the C677T allele had a two- to sevenfold increased risk for spina bifida (65). However, there were numerous other studies that failed to find any association whatsoever between this variant and an increased risk for an NTD (65). The highest allele frequencies were observed in Italians and in U.S. Hispanic populations, with the frequency of this polymorphic allele exceeding 40%. The lowest frequency of the allele was found in U.S. Blacks and in Sub-Saharan African populations, where it was found in 6% to 14% of the individuals sampled. The European populations had the frequency of this mutant allele clustered between 30% and 38% (65).

Botto & Yang (65) performed a meta-analysis on the data from over 20 studies and reported a pooled odds ratio for NTD risk among C677T homozygotes as 1.8, with a slightly lower odds ratio for individuals who were heterozygous for the T-allele. It is important to note that not all of the epidemiological studies found a positive association between this allele and an increased NTD risk. Shaw and colleagues performed one of the largest studies ever conducted: They reported that there was a tendency toward an increased odds ratio (1.2), although not significantly so ($P > 0.05$) relative to the reference group, which was homozygous wild-type genotype and which used maternal vitamins. In this study, when the mother took no multivitamins and the baby had the TT genotype, there was an even higher odds ratio (1.6), although it was not statistically significant. The same was true to a lesser extent when the mother took vitamins and the infant was heterozygous. The Shaw study was designed to identify any interactions between maternal vitamin use, fetal genotype, and the risk for spina bifida (78). The results are consistent with a hint of evidence for an interaction between genotype and maternal vitamin use, as the risk for NTDs appears to increase among the offspring of women who did not take folic acid.

Craniofacial anomalies such as cleft lip (CL) and/or cleft palate (CP) comprise another group of congenital anomalies in which environmental factors are believed to play a central role in their etiology. CL and CP malformations are categorized as either syndromic or nonsyndromic (79–82). The term nonsyndromic excludes those CL/P patients with other accompanying physical malformations, as well as those with no obvious maternal environmental exposures. Current estimates

of the relative frequency of syndromic versus nonsyndromic CL/P is 30% and 70%, respectively. CL/P are etiologically heterogeneous major malformations, with reported birth prevalences of 1 to 2 per 1000 livebirths in California (83). The causes of nearly all isolated clefts, and many that occur in association with other malformations, are largely unknown. In only a small proportion of infants with orofacial clefts is a distinct pattern of associated malformations recognized. The main categories of recognized etiologies include cytogenetic abnormalities, Mendelian single gene disorders, and those attributable to teratogenic exposure, but the proportion of clefts attributable to each of these categories is relatively small (84). Among the cases of clefts with an unknown etiology, it is often suspected that genetically determined host susceptibility factors, along with environmental exposures, interact to increase their expression. Such interactions have proven to be difficult to identify and measure in human studies.

The development of the structures of the face involves a multitude of different signaling pathways that utilize both growth and transcription factors to regulate morphogenetic events (85–87). Evidence to illustrate that single gene mutations can result in CL/P comes from the identification of a number of syndromic CL/P genes that have been identified through positional cloning (41, 88, 89). Conversely, an environmental contribution to the development of CL/P is clear in that nutritional deficiencies as well as maternal exposure to certain drugs including thalidomide, valproic acid, and phenytoin induce CL/P (90–92). There is a solid body of evidence to demonstrate that environmental exposure to alcohol and cigarette smoking is a risk factor for the development of CL/P (93, 94). As is the case with NTDs, the challenge is to identify the specific gene(s) involved in these gene-environment interactions leading to the malformations.

Women who smoke during early pregnancy may be at a greater risk of delivering infants with orofacial clefts. In one of the most clear-cut examples of a gene-environment interaction leading to a birth defect that exists in the literature, Shaw and coworkers (94) demonstrated that specific allelic variants of the transforming growth factor-alpha (TGFα) gene are risk factors for the development of orofacial clefts. A large-scale study was conducted to determine if a relationship existed between maternal cigarette smoking, the genotype of the conceptus at the TGFα locus, and the development of CL/P. The study confirmed that maternal cigarette smoking is a risk factor for CL/P. The infant is approximately two times as likely to develop isolated cleft lip with or without cleft palate and to develop isolated cleft palate if the mother smokes >20 cigarettes per day. If the genotype of the infant at the TGFα locus was considered, the odds ratio for the development of cleft lip was increased by 3- to 11-fold. These data illustrate that as suspected, a gene-environment interaction may be contributing to the development of clefts due to smoking. In a follow-up to the original study, Shaw et al. (95) demonstrated that periconceptional maternal multivitamin use decreased the risk factor for cleft lip and palate development due to the smoking/TGFα allele combination. This provided solid evidence that gene-nutrient interactions are a significant risk factor for orofacial clefting in infants.

PROPOSED MOLECULAR MECHANISMS OF KNOWN TERATOGENS

Thalidomide

The literature on birth defects associated with in utero exposure to thalidomide contains over two thousand citations, in keeping with the fact that this is the most infamous of all pharmaceutical teratogens (96). In spite of intensive investigation over the past forty years, the mechanism by which thalidomide disrupts normal embryogenesis remains a topic of considerable controversy. Perhaps as a result of its checkered history, a large number of hypotheses have been put forward over the years in an attempt to explain its teratogenic mechanism of action. Twenty-four different hypotheses were reviewed by Stephens, who found that 13 of the hypotheses were fundamentally incorrect (97). Of the remaining 11 hypotheses, some were supported by experimental data, albeit not sufficiently conclusive or convincing. Several other hypotheses remain to be adequately tested. The hypotheses could generally be classified into those proposing that thalidomide affects DNA replication or transcription, its impact on the synthesis and/or function of growth factors or integrins, and finally, thalidomide's effects on angiogenesis, chondrogenesis, cell death, or cellular injury.

More recently, Stephens and coworkers (96, 98) proposed a model of the thalidomide embryopathy that neatly unifies previous hypotheses and provides a biologically sensible model system that accounts for the existing biochemical data, as well as explains the molecular specificity of this teratogen. In this model system, the insulin-like growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF-2) proteins work cooperatively in order to stimulate the production of alpha-5 and beta-3 integrin subunits. These integrin subunits have been previously demonstrated to function as angiogenesis factors, promoting vascularization in the developing limb bud, as well as select embryonic structures (99–101). The IGF-I and FGF-2 genes are transcriptionally regulated by the transcription factor Sp1. Sp1 binds guanine-rich GC box elements (GGGCGG) located in the promoter regions of select target genes. In this case, the genes are IGF-I and FGF-2, as well as their receptors. Stephens & Fillmore (96) propose that binding of thalidomide to the promoter prevents subsequent Sp1 binding, thereby downregulating the transcription of the two target genes. Even a subtle downregulation of critically important genes can adversely affect the developmental processes involved in embryogenesis.

Data in support of this hypothesis include a relatively robust literature that demonstrates that IGF-I can stimulate chondrogenesis and limb development (102–104). More recently, it has also been demonstrated that thalidomide can inhibit IGF-I stimulation of limb development under experimental conditions (102). The IGF-I gene promoter lacks both TATA and CCAAT boxes and is highly guanine-rich, containing several Sp1 binding sites (105). This makes the IGF-1 gene particularly vulnerable to intercalation by thalidomide. The drug is able to oxidize DNA at position 8, which faces the major groove of the DNA molecule and may serve

to facilitate intercalation at those sites. Similarly, FGFs also play significant roles in both limb development (106–108) and angiogenesis (109–111). Like the IGF-I gene, there are no TATA or CCAAT boxes on the FGF-2 promoter, but there are multiple Sp1 and early growth response protein (Egr-1) binding sites. The FGF-2 gene, then, represents another potential target of thalidomide interference in limb development.

The question of specificity of teratogenic effects can also be explained by thalidomide's proposed interaction with specific gene promoters. Clearly, the key morphological structures affected by in utero exposure to thalidomide are the limbs, ears, and eyes. As such, it is thought that the intercalation of thalidomide at guanine-rich sites in DNA is having its greatest impact at the promoter regions of genes critical to the development of these structures. Perhaps less than 9% of all gene promoters have neither a TATA nor a CCAAT box. These genes depend upon promoters with one or more GGGCGG sequences (112). Stephens & Fillmore propose that thalidomide intercalates into promoter regions that have long stretches of poly-G regions, as these are biophysically favorable sites for thalidomide binding (96). The specificity of thalidomide targets is further enhanced by the fact that since most GC regions in the Sp1 binding sites are constitutive, they would be unlikely to tolerate regulation during embryonic development (96).

Retinoids

It has long been appreciated that proper levels of retinoid compounds, including vitamin A, are essential for normal mammalian embryogenesis. They are important mediators of many morphogenetic processes including cell proliferation and differentiation. For the most part, the biological activity of retinoids appears to be mediated by members of the retinoid receptor superfamily (113). The various retinoid compounds serve as ligands for a number of different receptor subtypes found in the developing embryo. This includes two types of ligand-dependent transcription factors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (27, 114). The RARs consist of three subtypes encoded by different genes designated RARα, RARβ, and RARγ . Each subtype has a number of isoforms produced by alternative splicing and differential promoter usage. The RXRs are also comprised of three subtypes, RXRα, RXRβ, RXRγ, each of which is capable of forming heteroduplexes with the RAR subtypes. These receptors have well-defined expression patterns during embryogenesis (115, 116).

Retinoids cannot be synthesized de novo by higher animals and consequently must be consumed in the diet, usually via animal products that contain retinol and retinyl esters or from plant synthetic products that serve as their precursor products. They include both natural and synthetic forms of vitamin A, and they can adversely affect development either if they are too abundant or if the maternal diet is deficient (29). Retinoids are transported in the embryo by means of the retinol binding proteins. Basically, the embryo will express either cellular retinol binding protein (CRBP), which colocalizes with retinol and the products of retinoic

acid response element (RARE) containing genes, or cellular retinoic acid binding protein (CRABP), in those tissues that appear to be very sensitive to high levels of all-trans retinoic acid (117). The expression patterns of these two proteins are quite specific, even within individual developing structures. For example, the ectoderm of the developing limb bud expresses CRBP, while the supporting mesenchyme expresses CRABP. As a result, the two germ layer derivatives respond differently in response to alterations in the retinoid availability. When the embryo is subjected to reduced concentrations of vitamin A, the ectoderm is specifically targeted. On the other hand, excessive amounts of vitamin A produce apoptosis in the mesenchyme (29).

The teratogenic potential of vitamin A was first recognized by Hale in 1933 (118), who described ocular agenesis among piglets born to a sow fed a vitamin A-deficient diet. Since that time, vitamin A has been shown to be teratogenic in a number of experimental animals, including mice, rats, guinea pigs, hamsters, rabbits, dogs, pigs, chicks, and monkeys (119). The possibility that there were genetically regulated differences in teratogenic sensitivity to retinoids was confirmed following the Thalidomide tragedy of the late 1960s when Nolen (120) demonstrated a differential response to all-trans retinoic acid for specific malformations in three albino rat strains. As is common among known teratogenic compounds, relatively minor changes in the gestational exposure period can result in profound shifts in sensitivity to retinoids, ranging from embryo lethality to some 40 different major and minor malformations that have been described (121). Generally speaking, the period of peak sensitivity for each of the types of congenital defects conformed to the time when the primordia for each specific structure were developing (29).

In addition to the data provided from experimental animal studies, there is strong evidence provided by clinical dysmorphologists that excessive retinoic acid exposure during early gestation can disrupt human embryonic development. A primary source of pharmacological exposure to retinoids is through the use of isotretinoin, a retinoid prescribed for severe recalcitrant cystic acne. A well-defined clinical syndrome has been described that includes craniofacial, cardiovascular, thymic, and central nervous system anomalies (122). Given this pattern of abnormality, it is highly suggestive that the human malformations produced by in utero retinoid exposure appear to be induced by disruptions of the cranial neural crest cells and an as-yet-unidentified CNS cellular population (123).

The mechanism of action for retinoid-induced teratogenesis is basically unknown, although much has been learned over the last two decades (29). One possibility is that the retinoids are inappropriately activating RARs, and thus adversely affecting selected developmental processes. It is highly probable that the pathogenesis of retinoid teratogenesis can be attributed to alterations in several distinct developmental processes, including: cell proliferation (124), pattern formation (125), cellular induction and differentiation (126, 127), neural crest cell migration (128), apoptosis (129), and induced inflammation (130). In contrast to the lack of information on the teratogenic mechanism of action, there is a great deal known

about the strict structural requirements for retinoids in order for them to have a teratogenic potential. It is well established that teratogenic retinoid compounds must have either a polar terminus with an acidic pK_a or a functional group that can be biotransformed to such a terminal group (131). In addition, the alternative side chain of the molecule must confer lipophilicity, have more than five carbon atoms, and maintain pi electron delocalization across the entire molecule. While it is possible to significantly alter the β-cyclogeranylidene ring without decreasing teratogenic activity of the retinoid compounds (132), increasing the conformational constriction at C7 to C9 increased teratogenic potency unless the bond adjacent to the β-cyclogeranylidene ring could rotate. Under these conditions, the teratogenic activity was decreased; however, a general increase in conformational restriction did not alter potency (133). In terms of teratogenicity, the charge transfer properties within the molecule were important in the determination of its biological activity. Finally, the incorporation of dimethyl substituents at the C1 and C4 positions of all-trans RA enhanced teratogenic potency significantly.

RAR receptors have well-defined expression patterns during embryogenesis (115, 116) and anything that disrupts this pattern is capable of significantly altering normal morphogenetic events. Administration of an RAR antagonist provided only limited protection against malformations induced by an RARα specific agonist in the highly inbred mouse strain NMRI at gestational day 8.25. While it protected against anal atresia and micrognathia, it failed to be effective for exencephaly (134). On the other hand, the administration of an RAR antagonist at gestational day 8 actually induced craniofacial defects (135). These observations demonstrate the importance of endogenous retinoids and their balance to normal craniofacial morphogenesis.

Utilizing transgenic and knockout mice provides an enhanced understanding of the role of these receptors in early embryogenesis. For example, it appears that embryos have varying sensitivities to specific malformations that occur when the embryos are exposed to high levels of retinoic acid, depending upon the functionality of their RARs. Whereas mice with all functional RARs have craniofacial and skeletal malformations when exposed to teratogenic concentrations of retinoic acid on gestational days 8 and 9, embryos lacking a functional RARγ were resistant to some of the skeletal but not the craniofacial anomalies (136). Embryos that were heterozygous for the null allele retained some partial resistance to RA-induced posterior truncations, which suggests that critical levels of RARγ must be present to fully induce these defects. The embryos completely lacking RARγ proceed through embryogenesis normally, but these embryos are resistant to RA-induced malformations of the posterior region. Paradoxically, embryos nullizygous for RXRα exhibit normal limb development but were resistant to limb defects produced by RA (137). It can be said that specific RARs have well-defined roles in teratogenesis. RARγ is necessary for posterior truncation anomalies and may be involved in mediating cranial malformations. RXRα appears to be essential for the development of limb defects. In contrast, the RARβ null mutants were as susceptible to excess RA as were wild-type embryos (138). In conclusion, the ability

of excess retinoid to result in anomalies depends upon the availability of specific receptors in the developing embryo.

In conclusion, the interplay between retinoids and the developing embryo is complex and involves not only the ligands, but the various receptors as well. The temporal and spatial expression of the receptors, the binding proteins, and the availability of the different retinoid compounds in the appropriate concentrations all contribute to the normal homeostasis and development of the organism. It follows then that alterations in retinoid compound concentrations can result in problems during embryogenesis and subsequently result in embryos with congenital malformations.

Valproic Acid

Valproic acid (VPA) is a widely prescribed anticonvulsant drug that has been demonstrated to be efficacious in a wide spectrum of convulsive disorders. Although it is considered to be relatively safe, VPA has been linked to a rare but serious hepatotoxicity, as well as to specific developmental malformations (139–142). In humans, in utero VPA exposure has been associated with neural, craniofacial, cardiovascular, and skeletal defects (143–145). The developing nervous system appears to be particularly sensitive to the teratogenic effects of this anticonvulsant drug because it has been estimated that 1% to 2% of the infants exposed to VPA in utero will develop a neural tube defect (NTD), specifically spina bifida, which is 10 to 20 times the prevalence rate of this defect within the general population (145, 146).

Humans are not unique in their response to VPA; in fact, this drug has also been shown to induce various neural, renal, cardiac, urogenital, and/or skeletal anomalies in numerous animal species, including rodents (147–151), rabbits (152) and nonhuman primates (153). Despite the occurrence of growth retardation, skeletal and soft tissue anomalies, and an increased incidence of embryo lethality in these laboratory animals, murine embryos remain the only animal model that is susceptibile, in vivo, to VPA-induced neural tube defects. Despite the fact that in utero VPA exposure has been associated with an increased risk for NTDs in both humans and the laboratory mouse, a clear understanding of the mechanism(s) of how VPA initiates the cascade of molecular and biochemical events that ultimately lead to aberrant neurulation still evades us.

The process of neurulation involves a series of morphogenetic events that are initiated with the formation of the neural groove and proceed through the elevation, apposition, and eventual fusion of the lateral edges of the neural folds to form the neural tube. The essential role that cellular proliferation plays in these morphogenetic events was initially realized over a century ago. Since then, numerous experimental systems have demonstrated that alterations in the normal cellular proliferative rate of the tissues involved with neural tube closure (NTC) can result in an embryo with an NTD (154–157). The hypothesis that NTC is dependent upon the neuroepithelia maintaining a normal proliferative rate is further supported by

studies performed on cultured rat embryos that were exposed to elevated temperatures during neurulation. Not only did these treatments produce NTDs, but they also decreased the rate of cellular proliferation in affected embryos by delaying the progression of the neuroepithelia cells through the G1/S and G2/M interphases of the cell cycle (158). Collectively, these data illustrate the necessity for continued normal proliferation of the developing neuroepithelia in order for neural tube closure to be completed.

Because cellular proliferation is so fundamental to normal neural tube development, the neuroepithelium needs to be continuously exposed to neurotrophic and growth factors. While studies are limited that investigate the direct effects of specific growth factors upon the in vivo development of the mammalian neural tube, numerous studies have shown growth and/or trophic factors are indispensable for the survival of central, as well as peripheral, nerves in vitro (159–162). Growth and trophic factors, such as nerve growth factor (*ngf*), transforming growth factor beta (*tgf*β), basic fibroblast growth factor (*bfgf*), cilliary neurotrophic factor (*cntf*), and brain-derived neurotrophic factor (*bdnf*) have all been shown to regulate the maintenance of neural viability and protect motoneurons from apoptosis (163). Additionally, VPA exposure inhibits the proliferation of neuronal cells in culture. At concentrations reportedly teratogenic to either humans or mice, VPA caused a 50% reduction in the proliferation rate of C6 glioma cells (164, 165). Specifically, VPA impeded the cell cycle during the G1 phase. If exposure of C6 glioma cells to VPA occurred after this specific cell cycle restriction point, the proliferation of these cells was not adversely affected (165). Furthermore, agents that inhibited cell proliferation in the C6 glial cell line within two times their therapeutic dosage were consistently associated with major neural tube defects (166). Therefore, it is clear that without the continued influence of these trophic and/or growth factors the development and the integrity of the developing neural tube would deteriorate.

Years of experimentation have resulted in the identification of inbred mouse strains that are either highly sensitive or highly resistant to valproic acid teratogenicity (150). The relative sensitivity of the different strains of laboratory mice to the development of NTDs in the offspring exposed to VPA is markedly different. The susceptibility to VPA–induced birth defects in humans is also thought to involve a genetic component; therefore, these laboratory mouse strains provide a useful system to model the genetics of susceptibility to VPA–induced birth defects.

As previously outlined, the process of neurulation is complex and requires elevation, apposition, and fusion of the neural folds to form the neural tube. This process involves many genes and their corresponding proteins. To ascertain if changes in the expression of genes might be playing a role in determining the susceptibility to VPA exposure, the transcriptional activity of genes known to encode proteins involved in neural tube closure was analyzed in embryos exposed to VPA. With the advent of newer molecular biological approaches such as in situ transcription and antisense aRNA amplification, it was possible to examine gene expression directly in the neural tubes of developing embryos (167). The experimental protocols for the gene expression studies have been previously described in some detail (168–170). Briefly, neural tube closure stage embryos from control or VPA-treated dams

were harvested at selected timepoints, generally gestational days (GD) 8.5, 9.0, and 9.5. The gene expression patterns in the embryos were analyzed by univariate and multivariate statistical approaches. In general, it appeared that teratogenic concentrations of VPA elicited strain-dependent effects on the expression of several genes that are important to normal embryonic development. These genes included cell cycle and apoptosis genes such as bcl-2 and p53. Strain-dependent changes were also observed in a number of growth factor genes including brain-derived neurotrophic factor (bdnf), nerve growth factor (ngf), and its receptor (*ngf-R*). Folate pathway genes including the folbp-1 and -2 genes as well as the *MTHFR* gene were examined (169–172). The gene expression data collected to date suggest that subtle collective changes in several molecules, each of which by itself may be developmentally harmless, together produce the adverse phenotypic changes that may result in the observed NTDs. Clearly cell cycle and growth factor genes are involved, and these changes may well be folate responsive.

CONCLUSIONS

Exposure to selected compounds through pharmacological and environmental sources such as agricultural chemicals and petrochemicals results in significant numbers of congenital malformations in the human population. A thorough understanding of the molecular mechanisms involved in the development of these birth defects is necessary in order to prevent them. Recent advances in the understanding of these birth defects have been accomplished by merging more traditional teratological methodologies with those of modern molecular biology and genetics. This synergistic approach allows for a more detailed elucidation of the molecular mechanisms responsible for birth defects due to exposure to environmental and pharmaceutical teratogens. Although we are beginning to gain new insight into the mechanisms of malformations due to exposure to compounds such as retinoic acid, thalidomide, and valproic acid, new technologies will likely provide this critical information more quickly in the near future. For example, genetic microarray technologies will provide insight into global changes in gene expression occurring in response to teratogenic insult. In addition, the identification and classification of all human and mouse genes by the human and mouse genome projects will allow for a more comprehensive and rapid identification of genes involved in birth defects. Although the discipline has made major advances in recent years, the future holds even greater promise for understanding the molecular mechanisms responsible for environmentally induced birth defects.

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