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RETINOIDS AS TERATOGENS

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ABSTRACT

Vitamin A is a necessary nutrient in the diet. However, excessive doses of retinoids by pregnant women result in teratogenesis. In this chapter, we initially discuss the occurrence and characteristics of fetal malformations associated with maternal ingestion of natural and synthetic retinoids in both experimental animals and humans. We then turn to an examination of the pharmacology of teratogenic retinoids, focusing on structure-function relationships and pharmacokinetics. Finally, we review the current literature on the molecular mechanism of action of teratogenic doses of retinoids and the role of the retinoic acid receptors and other target genes in this process.

INTRODUCTION

Vitamin A is a necessary nutrient in the diet for growth (116, 117), tissue differentiation (116, 117), reproduction (107), and vision (108) (for review,

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see 35). Dietary deficiency of vitamin A results in numerous and diverse pathological alterations in the body. Retinoic acid (RA) can prevent all symptoms of vitamin A deficiency except those associated with vision and male reproduction. On the other hand, excess intake of vitamin A can be toxic (for review, see 4). A single large dose of vitamin A can be lethal, and even chronic intoxication with lower doses of vitamin A can have adverse effects on many tissues and organ systems.

Numerous studies have documented the effects of vitamin A deficiency on reproduction in both male and female experimental animals. In the male, retinol is required for normal spermatogenesis, whereas in the female, vitamin A is necessary for both conception and normal development of the fetus. A discussion of the role of vitamin A in normal development is beyond the scope of this article. However, several recent reviews have been written on this subject (see for example 42). Although vitamin A is essential for normal reproduction, excess doses are teratogenic. This is of particular concern in humans since pharmacological doses of both RA and synthetic derivatives of RA have been found to be useful in treating several diseases of the skin (for review, see 85) and a variety of cancers, including promyelocytic leukemia, breast cancer, carcinomas of the respiratory tract, and ovarian cancer (for review, see 41, 43). In this chapter, we initially discuss the occurrence and characteristics of fetal malformations associated with maternal ingestion of natural and synthetic retinoids in both experimental animals and humans. We then turn to an examination of the pharmacology of teratogenic retinoids, focusing on structure-function relationships and pharmacokinetics. Finally, we review the current literature on the molecular mechanism of action of teratogenic doses of retinoids and the role of the retinoic acid receptors (RARs) and other target genes in this process.

RETINOIDS AND ABNORMAL DEVELOPMENT

The term vitamin A refers to all compounds (except carotenoids) that qualitatively exhibit the biological activity of retinol. These include retinol, retinal, and RA. The more general term, retinoids, includes both naturally occurring compounds with vitamin A activity and synthetic analogues of retinol, irrespective of whether they have biological activity. Many thousands of new retinoids have been synthesized by organic chemists for potential pharmacological applications. To date, retinoids with high efficacy have been synthesized with considerable success; however, these compounds have also exhibited high intrinsic teratogenic activity, resulting in little gain in therapeutic ratio (5). Our discussion focuses on the teratogenic properties of vitamin A and those retinoids that are presently prescribed or that potentially will be

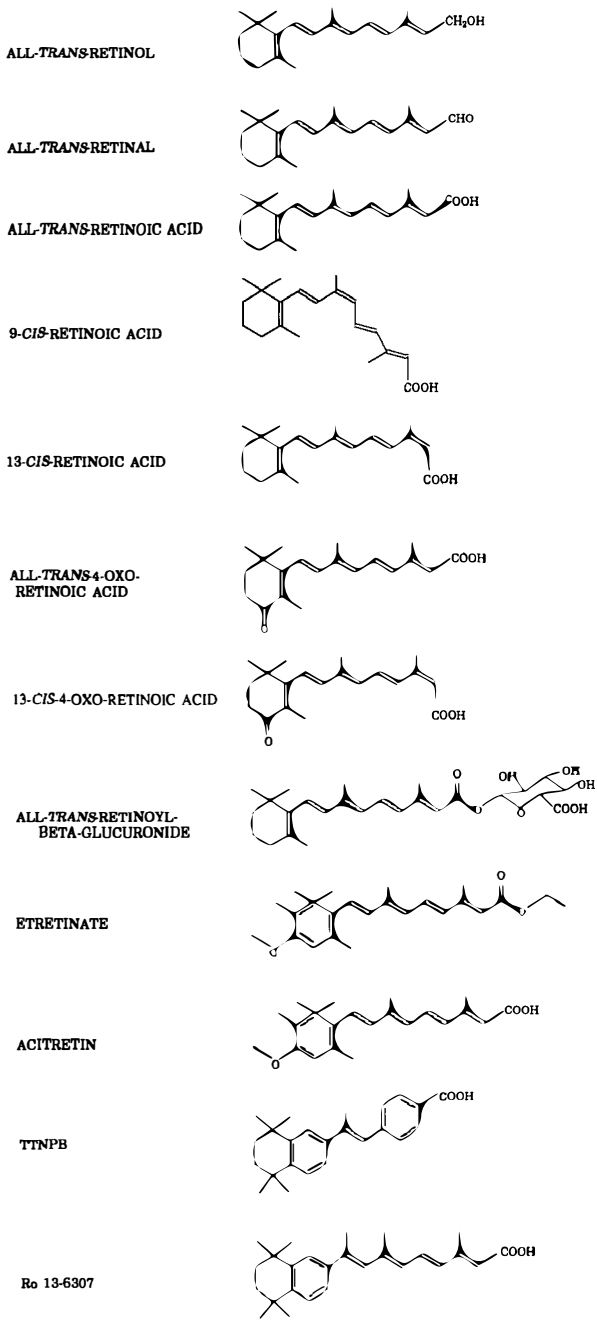

Figure 1 Structure of retinoids discussed in the text.

used for pharmacological applications in humans (see Figure 1 for retinoid structures).

Laboratory Animals

Cohlan (11, 12) first reported the induction of teratogenesis by high doses of vitamin A in pregnant rats more than 40 years ago. In this study, Cohlan demonstrated that oral feeding of pregnant female rats with 35,000 IU/day of a preparation of "natural vitamin A" (presumably esters of retinol) on gestation day (GD) 2–16 resulted in a large number of fetal anomalies. These included exencephaly, cleft lip and/or palate, brachygnathia, and various eye defects. Later studies showed that all-*trans*-RA was a much more potent teratogen (20–40 times greater) than retinol in several experimental animal species (55, 98). Furthermore, all-*trans*-RA resulted in a pattern of congenital abnormalities in experimental animals similar to that observed with retinol treatment. Three lines of evidence suggest that all-*trans*-RA is the derivative primarily responsible for vitamin A–induced teratogenesis: (*a*) Retinyl esters and retinol are readily oxidized to all-*trans*-RA in the body; (*b*) all-*trans*-RA is the most potent natural teratogenic vitamin A compound; and (*c*) all-*trans*-RA has been demonstrated to be a ligand for RARs.

Since these initial studies, more than 100 reports have described more than 70 types of anomalies affecting almost every organ system related to excess intake of retinoids (32). Similar anomalies have been described in embryos of nearly all species of experimental animals, including monkeys, rabbits, rats, mice, and hamsters (30, 51–53, 55, 57, 98). Among the congenital abnormalities that have been described most often in experimental animals are microcephaly, spina bifida, encephalocele, exencephaly, hydrocephaly, facial nerve palsy, cranial abnormalities, maxillary hypoplasia, mandibular hypoplasia, cleft palate, cleft lip, micrognathia, absent or deformed ear, exophthalmus, microphthalmus, coloboma, cardiovascular transposition, hypoplastic aorta, shortened limbs, ectrodactyly, syndactyly, anal atresia, thymic hypoplasia, amastia, and esophageal atresia, as well as defects of the uterus, kidney, thyroid, and pituitary.

Some generalizations have emerged from the many studies utilizing experimental animals. These include the following:

1. The anomalies observed in experimental animals are developmentally stage dependent, and the period of active organogenesis in the embryo is extremely sensitive to exposure to teratogenic levels of retinoids (32, 33, 51, 53, 56). For example, treatment of pregnant mice with a teratogenic dose of a retinoid before the embryos have implanted or after GD 14 results in few, if any, adverse effects. In contrast, treatment of pregnant mice on GD 7–10 with a teratogenic dose of a retinoid results in a large number of

resorbed embryos, and surviving embryos generally have anomalies of the head, sensory organs (particularly the ears), and cardiovascular system (aortic arches). Finally, treatment on GD 11–13 results principally in limb, craniofacial, and genitourinary defects.

2. The available literature on the minimal teratogenic dose of several retinoids clearly demonstrates that some retinoids are more potent than others (54, 56, 58–60). For example, in mice, 13-*cis*-RA and retinol are approximately 20 and 4 times less potent teratogens than all-*trans*-RA, respectively. Conversely, retinoids such as etretinate, Ro 13-6307, and Ro 13-7410 (TTNPB) are approximately 2, 40, and 700 times more potent teratogens than all-*trans*-RA, respectively. This wide range in the teratogenic potency of retinoids can be attributed to several factors, including the structure of the retinoid, pharmacokinetic parameters of the retinoid, and the ability of the retinoid to induce RAR-β2 mRNA levels. These issues are discussed in more detail in later sections.

3. For each retinoid, the teratogenic response is dose dependent. Embryonic defects are more frequent and severe with increasing doses of retinoid, eventually resulting in embryolethality and, ultimately, death of the mother. During the early stages of organogenesis, embryonic defects result from a relatively low dose of retinoids compared with the dose required for embryonic anomalies during the later stages of organogenesis.

Humans

A human embryo can be exposed to teratogenic doses of vitamin A or retinoids in three ways: (*a*) A pregnant woman eats a large excess of foods that contain vitamin A; (*b*) a pregnant woman consumes a massive dose of vitamin A as a nutrient supplement; or (*c*) a pregnant woman uses prescription drugs that contain retinoids. Although few adverse effects resulting from maternal hypervitaminosis A have been documented in human embryos, unequivocal evidence shows that retinoids are teratogenic in humans who have been inadvertently exposed to prescription drugs containing 13-*cis*-RA or etretinate.

Approximately 20 cases (13 published and 7 unpublished) have described developmental abnormalities in infants and children of women exposed to doses of vitamin A exceeding their recommended daily allowance (8 times higher than 800 retinol equivalents) (92, 93). An analysis of the defects in these children demonstrated that many organ systems were affected without any specific pattern. Although the heart and cardiac malformations were similar to those observed in children exposed to 13-*cis*-RA (see below), many of the other anomalies were quite distinct. However, not all of the malformations described in these 20 cases can necessarily be attributed solely to hypervitaminosis A. Several other factors, including other vitamin supplements, environ-

mental factors, and the possibility of chance occurrence of malformations in the population, could have contributed to the observed phenomena. However, owing to the established teratogenicity of vitamin A in a large number of experimental animals, including monkeys, described above and the proven teratogenicity of 13-*cis*-RA and etretinate in humans (see below), it is reasonable to assume that maternal exposure to a high although as yet undefined dose of vitamin A presents a risk to the developing embryo.

At present, three retinoids are approved in many countries for the systemic treatment of dermatological conditions. 13-*cis*-RA (or isotretinoin), which is marketed as Accutane®, is highly effective in treating severe, recalcitrant cystic acne. Psoriasis has been effectively treated with etretinate (Tegison®, Tigason®) and acitretin (Neotigason®). Since both 13-*cis*-RA and etretinate are known to be teratogenic in experimental animals, these two drugs are marketed in the US with the contraindication that they are not to be used by pregnant women. Unfortunately, a considerable number of babies have been born with congenital malformations as a result of the inadvertent exposure of pregnant women to each of these drugs. Of the many malformations observed in infants born to women exposed to 13-*cis*-RA, defects of the CNS such as hydrocephalus, cerebellar hypoplasia, absence of vermis, and structural malformation of the cerebral cortex were among those reported most often (6, 20, 62, 91). In addition, craniofacial abnormalities, including cleft palate and either absent or reduced size of the external ears and canals; heart defects; and thymus abnormalities, are also commonly observed in these children (31, 72). Finally, some evidence suggests that children exposed in utero to 13-*cis*-RA may also have impaired intellectual performance in the absence of any identifiable structural anomalies of the brain or CNS (1, 2).

The anomalies in infants exposed to etretinate in utero were similar to those observed with 13-*cis*-RA (37, 89, 92). However, limb defects occurred more often, whereas ear, cardiac, and thymus defects were observed less often. Interestingly, a case of a malformed baby born to a mother who had terminated ingestion of etretinate 11 months prior to conception has been reported (61). This finding suggests that the risk for fetal dysmorphogenesis persists for a considerably longer period of time following termination of treatment with etretinate than it does following treatment with 13-*cis*-RA because of the extended presence of detectable amounts of etretinate in plasma. This increased risk is most likely due to the lipophilic nature of etretinate, which necessitates its storage in deep body compartments, especially adipose depots (90), and to its extremely long half-life (estimated to be 120 days) in the human body (77).

The final prescription retinoid is all-*trans*-RA, or tretinoin (Retin-A®), which is used for the topical treatment of acne. In addition, the topical application of this retinoid has also been proposed to be effective in reducing skin wrinkling. Although a few reports in the literature have indicated a potential

teratogenic outcome associated with the topical use of all-*trans*-RA, these cases appear to result from chance occurrence rather than from a clear association with topical application of all-*trans*-RA (3, 10, 92). Considerable work strongly suggests that there is a very low teratogenic risk associated with topical treatment with all-*trans*-RA because limited amounts of drug can be externally applied and because a small amount of the drug ultimately is found in maternal circulation (113, 118). However, other retinoids may become available in the future that are teratogenic in humans when applied topically.

Although 13-*cis*-RA was known to be teratogenic in laboratory animals, reports of human cases of retinoid embryopathy came as a surprise. These reports suggested that human embryos are extremely sensitive to exposure to this retinoid. It is now apparent that the relative sensitivity to the teratogenic effects of this retinoid differs considerably from one animal species to the next. For example, whereas humans are extremely sensitive (1 mg/kg per day) and monkeys and rabbits are moderately sensitive (5 mg/kg per day) to exposure to this drug, mice and rats are relatively insensitive (75 mg/kg per day) (82, 83). Most, if not all, of the major species differences associated with the teratogenic potency of 13-*cis*-RA can be explained by pharmacokinetics (see below).

PHARMACOLOGY OF TERATOGENIC RETINOIDS

The administered dose of a drug or vitamin often does not correlate well with the effects produced by the same compound. This observation holds true for retinoids and their teratogenic potency in embryos. When determining the teratogenic potency of retinoids in a given animal species, one must take into account various pharmacological aspects of the retinoid. These aspects include the structural features of the retinoid itself and the exposure of embryonic target tissues to the retinoid during the sensitive period of organogenesis. The quantitative amount and the duration of embryonic exposure to a particular retinoid are directly related to its pharmacokinetic properties in a given animal species.

Structure-Function Relationship of Retinoids

Because many thousands of retinoids have been synthesized and a considerable number of these have been tested for their teratogenic potency, a large body of information on the structural features of retinoids and their teratogenic potency is available. Some studies have examined the importance of all three structural domains of retinoids, i.e. the hydrophobic cyclohexenyl ring, the polar terminal group, and the tetraene side chain (see Figure 1) (15, 45, 101, 109–112, 114, 115). In addition, several conformationally restricted retinoids with a specific, rigid, three-dimensional configuration have also been investi-

gated. From these studies, several conclusions can be drawn. Many of the features associated with a highly teratogenic retinoid are also associated with retinoids with high biological activity. Moreover, many of the critical features associated with teratogenic potency also appear to be key determinants for binding to the nuclear retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs).

TERMINAL GROUP The carboxylate group at the terminus of the retinoid is extremely important in determining the teratogenic potency of a retinoid (101, 112, 114). Although the carboxylate group confers the highest teratogenic potency to a retinoid, substitution to other acidic groups, such as phosphoric acid, sulfonic acid, sulfinic acid, or tetrazole, results in teratogenic retinoids with significant potency. In several teratogenic retinoids, the carboxylate group is derivatized, e.g. to an ester or amide. The teratogenic potency of these retinoids appears to be directly related to the rate of metabolism of the derivatized retinoid to the free acid form either in the pregnant woman or the embryo. This association may also exist for retinol and retinal. Therefore, a free acid, preferably carboxylic acid, appears to be required at the terminus of a retinoid for high teratogenic potency.

SIDE CHAIN Both the length of the side chain and the *cis-trans* configuration of the double bonds are important structural features of retinoids (109, 111). If the length of the side chain is greater or less than that of RA, the teratogenic potency of a retinoid is reduced. Hence, the distance from the ring structure to the terminal carboxylate group is critical. With respect to the *cis-trans* configuration of retinoids, the data are more difficult to interpret. 9-*cis*-RA has been reported to be equal in teratogenic potency to all-*trans*-RA (15), whereas 13-*cis*-RA has been estimated to be 8- to 20-fold lower in potency than all-*trans*-RA (54, 56, 58–60). These studies are difficult to interpret because a significant amount of isomerization of retinoids occurs within cells. Therefore, at this time the role of the configuration of the double bonds in the side chain is unclear.

HYDROPHOBIC RING A hydrophobic ring is required for high teratogenic potency (45). However, the effects of modification of this ring on the teratogenic activity of retinoids appear to be minimal compared with those of the side chain or the polar terminus. Modifications such as replacement of the cyclohexenyl ring with a phenyl or a cyclopentenyl ring result in retinoids with a teratogenic potency similar to or higher than that of all-*trans*-RA. Similarly, substitutions of the C_4 (hydroxylation or oxygenation) and C_5 (hydroxymethyl) positions result in retinoids with high teratogenic potency.

Pharmacokinetics

In vitro studies utilizing rat embryos have demonstrated that retinoids act directly on the embryo, causing abnormal patterns of development (80). Therefore, the amount of an active retinoid that will accumulate over time (concentration-time relationship) in the embryo during the sensitive stages of organogenesis is a major factor in determining the outcome of development. Upon oral administration of a retinoid to the pregnant woman, several factors can play an important role in determining the amount of active retinoid ultimately observed in the embryo. These include the rate of retinoid absorption by maternal intestine, maternal retinoid metabolism, the half-life of the retinoid in maternal plasma, and the placental transfer rate of the retinoid. At present, the extent of the role of embryonic metabolism in determining the teratogenic potency of any given retinoid is unclear because of the paucity of information on the identification and quantitation of retinoid metabolites in the limited quantities of sensitive embryonic tissues.

The pharmacokinetic features of a particular retinoid can vary considerably from species to species. This variation results in very different embryonic tissue retinoid levels and, consequently, in different effects on developmental events in the face of equivalent doses of the same retinoid. The most striking and clinically important example of this phenomenon is the vast difference in the teratogenic potency of 13-*cis*-RA among animal species. As stated above, humans are extremely sensitive, rabbits and monkeys are moderately sensitive, and mice and rats are relatively insensitive to the teratogenic effects of 13-*cis*-RA. Most, if not all, of the differences in potency between humans and rodents can be attributed to three species-specific pharmacokinetic features of 13-*cis*-RA: (*a*) limited placental transfer of 13-*cis*-RA by rodents, (*b*) the higher clearance rate of 13-*cis*-RA by rodents, and (*c*) differences in the metabolic products of 13-*cis*-RA.

TRANSPLACENTAL TRANSFER The transplacental transfer of 13-*cis*-RA in rodents is much less efficient than that of all-*trans*-RA (16). Similar results have also been reported for 13-*cis*-4-oxo-RA and 13-*cis*-acetretin compared with their respective all-*trans* isomers (17, 69). The reason for this difference between the transplacental distribution of 13-*cis* isomers and that of all-*trans* isomers in rodents is not known. Because the two cellular RA-binding proteins [Type I (CRABP-I) and Type II (CRABP-II)] and the RARs have a lower affinity for 13-*cis*-RA than for all-*trans*-RA (19, 44), the levels of these proteins have been suggested as a determining factor. However, recent studies have demonstrated that transgenic mice in which the production of functional CRABP-I has been completely prevented are normal (34). Furthermore, these mutant mice lacking functional CRABP-I did not exhibit an increased sensi-

tivity to teratogenic doses of all-*trans*-RA. Thus, CRABP-I at least does not appear to be involved in transplacental transport of RA.

These observations in rodents do not necessarily hold true for nonrodent species. Evidence suggests that the placental transfer of 13-*cis*-RA is more extensive in monkeys than in laboratory rodents (27, 84). This transfer efficiency partially accounts for the species differences in teratogenic potency of this retinoid and may reflect differences in the placenta at the retinoid-sensitive stage of organogenesis. At the retinoid-sensitive stages of development in rodents, the placenta is principally the yolk sac, whereas at the comparable stage in monkeys and humans, the hemochorial placenta is already well developed.

CLEARANCE RATE The plasma half-life of 13-*cis*-RA in rodents is markedly different from that in humans. The plasma half-life of 13-*cis*-RA in mice (17, 49, 50), for example, is approximately 1 h compared with 10–20 h in humans (9, 81). Because the plasma half-life of 13-*cis*-RA is 10–20 times longer in humans than in rodents, the concentration-time relationship of 13-*cis*-RA in the human embryo should be 10–20 times higher than that in the rodent embryo.

METABOLISM All-*trans*-RA and 13-*cis*-RA can interconvert in tissues and coexist in the plasma of humans, monkeys, and mice (28, 60). In addition, a considerable portion of the administered dose of 13-*cis*-RA in rodents is glucuronidated to form 13-*cis*-retinoyl-β-glucuronide, whereas in monkeys and humans, a significant percentage of this dose is converted to 13-*cis*-4-oxo-RA (14, 18). The 13-*cis*-4-oxo-RA formed in humans may itself be teratogenic; if not, it can easily be isomerized to all-*trans*-4-oxo-RA, which is highly teratogenic and similar in potency to all-*trans*-RA (97). On the other hand, the glucuronidated retinoids formed in the rodent are very poorly transferred across the placenta to the embryo and hence are not teratogenic (36). Formation of 13-*cis*-retinoyl-β-glucuronide in the rodent can be considered a detoxification pathway with respect to teratogenesis since this more water-soluble transport form of the retinoid would remain in the maternal tissues and not be available to the embryo. The reason for the species differences in the metabolites (13-*cis*-retinoyl-β-glucuronide in rodents compared with 13-*cis*-4-oxo-RA in humans), which in turn contribute to the species differences in the teratogenic potency of 13-*cis*-RA, is unknown but merits further consideration.

MOLECULAR MECHANISM OF ACTION OF TERATOGENIC RETINOIDS

Endogenous RA has been implicated in the regulation of expression of developmentally important genes during normal organogenesis (for review, see 104).

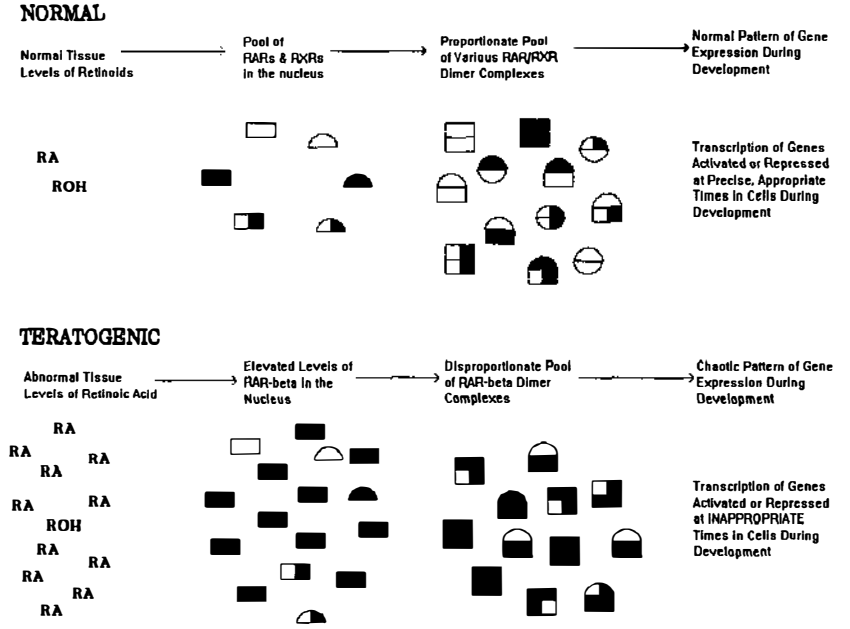

Figure 2 Proposed molecular mechanism of action of teratogenic retinoids.

This role of RA is most likely mediated by the nuclear RARs and/or RXRs. Therefore, an excess of RA may well result in maldevelopment owing to an uncontrolled chaotic expression of genes at sensitive stages of development that is mediated by RARs and/or RXRs. In this section, we first examine the role of RARs and RXRs during both normal and teratogenic development and then focus on homeobox genes as one group of target genes (see Figure 2).

RARs and RXRs

The discovery of the nuclear RARs and RXRs independently by Pierre Chambon and Ronald Evans and their respective colleagues has greatly advanced our understanding of the mechanism of action of RA (for review, see 74, 86, 102). These nuclear receptors, which belong to the multigene family of the steroid and thyroid hormone superfamily, are RA-dependent transcriptional regulatory factors. Three subtypes of each receptor (RAR-α, RAR-β, and RAR-γ and RXR-α, RXR-β and RXR-γ) have been described. Each subtype is encoded by an unique gene, and many subtypes have several isoforms that are the products of alternative promoter usage and differential splicing. RARs and RXRs form homodimers or heterodimers and transduce the RA signal at the level of gene expression via retinoic acid response elements (RAREs) and

retinoid X response elements (RXREs), which are DNA sequences located in the regulatory regions of target genes. The natural ligand for the RXRs is 9-*cis*-RA, whereas the RARs have two natural ligands: all-*trans*-RA and 9-*cis*-RA (40, 67). In addition, several other RA metabolites and synthetic retinoids bind RARs and/or RXRs with high affinity and regulate gene transcription in addition to displaying some receptor specificity (7, 19, 65, 66, 71).

NORMAL DEVELOPMENT Using in situ hybridization, Dolle et al and Ruberte et al (22–24, 94–96) examined the expression of the different RAR mRNA subtypes and RXR mRNA subtypes during normal murine development. From these studies, they concluded that the relative expression of each receptor subtype mRNA varies from tissue to tissue and from developmental stage to developmental stage. In general, RAR-α expression is ubiquitously distributed, whereas RAR-β and RAR-γ expression is more restricted and shows a unique pattern of localization. For example, RAR-γ is not expressed in the CNS. In contrast, both RAR-α and RAR-β are expressed in the CNS, but RAR-α is expressed ubiquitously, whereas RAR-β exhibits a very specific pattern of expression. Expression of RXR-β mRNA is diffuse and appears to be ubiquitous over an extended period of development. Like that of RXR-β, expression of RXR-α is diffuse at early developmental stages. However, enhanced levels of RXR-α mRNA are detected in the epidermis and in several other squamous epithelial cells during later stages of development. Finally, expression of RXR-γ is restricted in cells of the myogenic lineage (myotomes and later differentiating muscles) as well as in the optic epithelium, retina, pituitary, thyroid gland, and discrete areas of the CNS. Because each RAR and RXR subtype exhibits a unique pattern of expression, these subtypes may regulate at least some specific, nonoverlapping functions in the developing embryo.

Several reports have begun to address this possibility. They describe studies in which a specific nuclear receptor subtype or one of its isoforms has been functionally inactivated in mice by disrupting the specific gene of interest utilizing gene targeting technology. Homozygous RAR-α1 mutant mice were viable, with no apparent phenotypic abnormalities (68, 73). However, homozygous RAR-α mutant mice, which express no RAR-α isoform transcripts, exhibited early postnatal lethality and testis degeneration (73). Similar studies have also been performed with RAR-γ (70). Homozygous RAR-γ2 mutant mice also had no apparent phenotypic abnormalities. Interestingly, RAR-γ mutant mice, which also express no RAR-γ isoform transcripts, exhibited several abnormalities previously associated with vitamin A deficiency, including growth deficiency, early lethality, and male sterility. Finally, homozygous RXR-α mutant mice died between GD 13.5 and 16.5 (103). This embryonic lethality in the RXR-α-deficient mice was due to hypoplastic development of

the ventricular chambers of the heart, which resulted in a very thin ventricular wall and defects in ventricular septation. This study links RXR-α function with important events associated with cardiac morphogenesis. To date, no RAR-β, RXR-β, or RXR-γ mutant mice have been reported. Although some specific aberrations have been found to be associated with the lack of certain nuclear receptor subtypes, these studies seem to suggest a high degree of functional redundancy among these receptors.

TERATOGENIC DEVELOPMENT One fruitful approach to unraveling the sequence of molecular events that mediate RA-induced dysmorphogenesis is to examine the pattern of expression of RARs in murine embryos exposed to teratogenic doses of all-*trans*-RA. In initial studies, Harnish et al demonstrated that RAR-β mRNA levels in susceptible embryos were elevated as early as 3 h following dosing of the pregnant dams. These levels remained elevated for 9 h, returning to normal within 12 h of treatment (38). Embryonic RAR-α and RAR-γ mRNA levels were unaffected by this treatment. Later studies in GD 11 mouse embryos demonstrated that mRNA levels of the RAR-β2 isoform were elevated significantly (6- to 8-fold), whereas mRNA levels of RAR-α2 isoform were only modestly elevated (2-fold) after exposure to teratogenic doses of all-*trans*-RA (39). Furthermore, only the elevation in RAR-β2 mRNA levels correlated with those regions of the GD 11 mouse embryo that were most susceptible to the teratogenic effects of all-*trans*-RA (39). For example, RAR-β2 mRNA levels were elevated approximately 12-fold in the limb buds (highly sensitive), 8-fold in the head (moderately sensitive), and only 3-fold in the remainder of the body (relatively insensitive) following treatment of the pregnant dams with a teratogenic dose of all-*trans*-RA. In contrast, RAR-α2 mRNA levels were elevated 2-fold in each of the embryonic regions examined (39). The elevation in RAR-β2 mRNA additionally resulted in a comparable elevation in RAR-β protein levels in each embryonic region (100). Finally, on GD 14, when the mouse embryo is no longer sensitive to the teratogenic effects of retinoids, the elevation in RAR-β2 mRNA levels in all embryonic tissues was only 2- to 3-fold, similar to the fold elevations observed in adult tissues (39). These results are consistent with the possibility that elevation in the mRNA level of specific isoforms of RARs, in particular RAR-β2, helps mediate abnormal development in RA-treated embryos.

Evidence supporting this hypothesis has come from follow-up studies utilizing a large number of other teratogenic and nonteratogenic retinoids and dosing schedules. Studies utilizing nonteratogenic dosing regimes of all-*trans*-RA and 13-*cis*-RA have demonstrated a rapid elevation in RAR-β2 mRNA levels. However, RAR-β 2 mRNA levels do not remain elevated for the entire 9-h period, as they do following treatment with teratogenic doses of all-*trans*-RA (100). Pharmacokinetic analysis of all-*trans*-RA levels in embryos at

various times after exposure showed a strong correlation between the extent of elevation in all-*trans*-RA levels and the time course of RAR-β2 expression. In addition, a strong correlation has been demonstrated between those retinoid treatments that are teratogenic and the elevation of RAR-β2 mRNA levels in embryonic regions. Nonteratogenic dosing regimes (including a RXR-specific retinoid), on the other hand, resulted in little or no elevation in RAR-β2 mRNA levels (47). Most recently, studies have been performed to determine whether the elevation in RAR-β2 mRNA levels is a necessary event in the pathway resulting in RA-induced dysmorphogenesis. In these studies, micromass limb-bud cell cultures were treated with teratogenic doses of all-*trans*-RA with or without antisense oligonucleotide directed to the 5′ end of RAR-β2 mRNA. Treatment with the antisense oligonucleotide reversed the inhibition of chondrogenesis typically observed following treatment of these cells with teratogenic doses of retinoids (H Jiang, DR Soprano, S-W Li, KJ Soprano, JD Penner, M Gyda & DM Kochhar, submitted for publication). This outcome further suggests that elevations in RAR-β2 mRNA levels and in protein are events in the pathway leading to RA-induced dysmorphogenesis.

Finally, the role of elevation in RAR-β2 mRNA and RAR-β protein in the events resulting in RA-induced dysmorphogenesis invites speculation (see Figure 2). Heterodimerization of RARs and RXRs has been shown to play an important role in the control of gene expression by RA. In addition, RXRs form heterodimers with other members of the steroid and thyroid hormone superfamily. Any alteration in the level of any of these receptors, including RAR-β2, will result in an imbalance in the relative concentrations of heterodimers formed among the various members of the RARs, RXRs, and other hormone receptors that may be present in the embryos. Because each of these receptors may mediate the expression of a specific group of genes, an alteration in the relative amounts of one receptor could have a cascade-like effect and ultimately result in wide-ranging effects on the expression of a variety of genes. These effects could in turn cause either the transcriptional activation or the repression of genes important for development to occur at an inappropriate time (see Figure 2).

Homeobox Genes

Homeobox (Hox) genes were initially discovered in the fruit fly, *Drosophilia melanogaster,* and were demonstrated to play an important role in pattern formation. Each homeobox gene contains a 183-bp DNA sequence encoding the homeodomain or homeobox. The homeodomain of the Hox proteins binds DNA through a helix-turn-helix motif. These genes are believed to exert their role in pattern formation through the regulation of transcription of developmentally important genes. These genes have also been found in vertebrates and have been well characterized in both humans and mice. Thirty-eight human

Hox genes have been identified. These are distributed among four homologous clusters (HOX A-D), with each cluster located on a separate chromosome in the genome. During normal development, the genes located at the 3′ end of each cluster are transcriptionally active both earlier in development and more anteriorly in the embryo than the genes on the 5′ end of the cluster (for review, see 21, 78).

Considerable evidence linking the expression of the human and murine homeobox genes with RA comes from studies utilizing both human and mouse teratocarcinoma cells. Exposure of these pluripotent stem cells to RA results in their differentiation. Accompanying this differentiation process are a number of alterations in gene expression that are mediated by RA treatment. The transcription of Hox a–1 (formerly murine Hox 1.6) was demonstrated several years ago to be rapidly elevated in F9 teratocarcinoma cells after RA treatment (64). Furthermore, using the human teratorcarcinoma cell line (NT2-D1), Simeone et al (8, 99) demonstrated that the induction of mRNA expression from the genes in the Hox B (formerly murine Hox 2) cluster was related to RA concentration. Low concentrations of RA (10^{-8} M) were sufficient to induce the genes located on the 3′ end of the cluster, whereas higher concentrations of RA (10^{-5} M) were required to activate the expression of the genes located at the 5′ end of the cluster. This finding suggests that the concentration of RA to which a cell is exposed can dramatically affect which genes will be transcriptionally activated. In addition, using NT2-D1 cells, these same investigators demonstrated that the homeobox genes of each of the four clusters are transcriptionally activated following RA treatment in a sequential order colinear with their 3′ to 5′ arrangement (8, 99). The genes on the 3′ end of the cluster are transcriptionally activated initially following RA treatment, whereas the genes on the 5′ end are transcriptionally activated progressively later. These observations were recently extended to show that treatment of human teratocarcinoma cells with antisense oligonucleotides directed to the 3′ Hox B genes affects the transcriptional activation of the 5′ genes in the cluster (29). This association suggests a cascade in which the transcriptional activation of the 3′ Hox B genes is required for the subsequent transcriptional activation of the 5′ genes.

To explore the basis for the RA-regulated expression of homeobox genes, investigators have cloned and examined the promoters of several genes for DNA sequences that mediate RA-dependent transcriptional activation. Analysis of the promoters of three such genes, Hox b–1 (formerly murine Hox 2.9) (76), Hox a–1 (63), and Hox d–4 (formerly murine Hox 4.2) (87), has revealed a RARE within each promoter that functions to mediate the RA response. Finally, the altered expression of specific homeobox genes has been associated with RA-dependent abnormal development in several different organs. Alteration in Hox b–1 expression has been linked to defects in the hindbrain in

embryos treated with teratogenic doses of RA (13, 75). In the limb bud, activation of the Hox D (formerly murine Hox 4) cluster has been associated with RA-dependent abnormal development (46, 79). In the future, additional homeobox genes may be identified that are regulated by RA. Taken together, several studies have demonstrated a direct link between RA, RARs/RXRs, and the transcriptional regulation of specific homeobox genes.

FUTURE CONSIDERATIONS

One of the important goals for the future is to develop retinoids with high therapeutic value and low teratogenic potency for either dermatological applications or cancer chemotherapeutic and chemopreventive applications. At present, the vast majority of retinoids with high therapeutic value have been found to be extremely potent teratogens in humans. Whether these two activities of retinoids can be separated and specific retinoids designed directed only at the pharmacological application of interest remains to be determined. At least two potential avenues can be pursued to accomplish this goal. First, three RAR subtypes and three RXR subtypes have been described, each of which appears to have a somewhat unique pattern of expression in embryonic and adult tissues. As more information becomes available on the exact function of each receptor within specific tissues and during development (i.e. a battery of genes that are transcriptionally regulated), a particular receptor(s) may be associated with a pharmacological application but not with teratogenesis. It may be possible to design retinoids that specifically bind only one receptor subtype and that elicit the transcriptional effects of that specific receptor. Some progress has already been made in designing receptor-specific retinoids (7, 19, 26, 65, 66, 71) and in understanding the nature of the RA binding site of these receptors (105; N Tairis, JL Gabriel, KJ Soprano, DR Soprano, submitted for publication). As we learn more about the RA binding site of the RARs and RXRs, additional receptor specific retinoids may become available that are of pharmacological value but that are not associated with the teratogenic process.

A second approach to this problem is to develop retinoids with substitutions that prevent their accumulation in the embryo while retaining biological activity within the mother. β-glucuronidation or the formation of other glycoconjugates may be useful. Retinoyl-β-glucuronide has already been demonstrated to be inefficiently transported to the embryo and thus exhibits low teratogenic potency while remaining biologically active within the mother (36). This biological activity presumably results from slow hydrolysis of retinoyl-β-glucuronide to RA in maternal tissues.

A second important future goal is to understand, at the molecular level, the mechanism of action of teratogenic retinoids in the developing embryo. The available data strongly suggest a cascade of events that is initiated by terato-

genic doses of RA. Clearly, transcriptional activation of RAR-β2 and many of the homeobox genes are part of this cascade of events, which ultimately leads to RA-induced dysmorphogensis. Additional work is necessary to identify other target genes directly regulated by RA. Furthermore, because the homeobox genes and RAR-β2 are transcription factors, the target genes that are regulated as a result of their transcriptional activation must be identified and characterized. Recently, considerable attention has been focused on the RA-regulated expression of sonic hedgehog and its potential role in normal and teratogenic development (25, 88). Research directed toward these goals will provide invaluable information not only on teratology but also potentially on the role of retinoids during normal development.

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