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The role of retinoic acid in embryonic and post-embryonic development

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Retinoic acid (RA) is the bioactive metabolite of vitamin A (retinol) which acts on cells to establish or change the pattern of gene activity. Retinol is converted to RA by the action of two types of enzyme, retinol dehydrogenases and retinal dehydrogenases. In the nucleus RA acts as a ligand to activate two families of transcription factors, the RA receptors (RAR) and the retinoid X receptors (RXR) which heterodimerize and bind to the upstream sequences of RA-responsive genes. Thus, in addition to the well-established experimental paradigm of depriving animals of vitamin A to determine the role of RA in embryonic and post-embryonic development, molecular biology has provided us with two additional methodologies: knockout the enzymes or the RAR and RXR in the mouse embryo. The distribution of the enzymes and receptors, and recent experiments to determine the endogenous distribution of RA in the embryo are described here, as well as the effects on the embryo of knocking out the enzymes and receptors. In addition, recent studies using the classical vitamin A-deprivation technique are described, as they have provided novel insights into the regions of the embryo which crucially require RA, and the gene pathways involved in their development. Finally, the post-embryonic or regenerating systems in which RA plays a part are described, i.e. the regenerating limb, lung regeneration, hair cell regeneration in the ear and spinal cord regeneration in the adult.

Retinoic acid: Retinoids: Development: Regeneration

Retinoic acid (RA) is derived from vitamin A in the diet by two enzymic conversions from retinol (see p. 66). Thus, by the simple expedient of feeding animals a vitamin A-free diet the role of RA in the maintenance of the differentiated state in the adult can be examined, and by mating these deprived adults the role of RA in embryonic development can be examined. Both these types of study have a long history.

From studies dating back to the 1920s it has become clear that in the absence of RA adult animals become sterile, the mucous epithelium (such as the trachea) transforms into keratinized epithelium and the immune function is severely compromised (Wolbach & Howe, 1925; Underwood, 1984; Ross & Hammerling, 1994). In embryos which develop under conditions of vitamin A deficiency a wide range of abnormalities have been described since the first observation of Hale (1933) of a litter of pigs, all of whom had anophthalmia. These abnormalities, seen in rats, rabbits, cattle, sheep and human subjects (Kalter & Warkany, 1959), include defects of the central nervous system (CNS; hydrocephalus and spina bifida), eye (anophthalmia and microphthalmia), face (harelip and cleft palate), dentition, ear (accessory ear and otosclerosis), limb, urogenital system (cryptorchidism, ectopic ovaries, pseudohermaphroditism and renal defects), lungs (hypoplasia) and heart (incomplete ventricular septation, spongy myocardium, aortic arch defects, aorticopulmonary septal defects and valvulus communis). An additional defect seen in chick and quail embryos is in the haematopoetic system, with the failure of the vitelline veins to form (Thompson *et al*. 1969; Dersch & Zile, 1993).

In recent years many of these defects have been described in more detail (see p. 69), and a molecular understanding of the defects has been obtained now that we know a lot more about the cellular mechanisms of how RA acts in cells. This information has led to the phenocopying of the

Abbreviations: ADH, alcohol dehydrogenase; CNS, central nervous system; RA, retinoic acid; RALDH, retinal dehydrogenases; RAR, retinoic acid receptors; ROLDH, retinol dehydrogenases; RXR, retinoid X receptors.

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RA-deprivation effects by knockouts of retinoid receptors and RA-synthesizing enzymes in the mouse embryo, as described on pp. 66–68.

It is important to point out that the effects of excess RA applied to the embryo, including human subjects, are remarkably similar to the effects of deprivation, i.e. defects in the CNS, eye, face, dentition, ear, limb, urogenital system, cardiovascular system and the vertebrae and ribs (Knudsen, 1966; Shenfelt, 1972; Kochhar, 1973; Fantel *et al*. 1977; Lammer *et al*. 1985; Rosa *et al*. 1986). Thus, we may reasonably conclude that since either too much or too little RA is harmful to the embryo, embryonic cells must strictly regulate the levels of endogenous RA so that the correct balance is obtained. How this balance of RA is generated and the cellular machinery on which it operates has been revealed primarily in the last decade.

The synthesis of retinoic acid

The vitamin A that is obtained from the diet is stored in the liver in the form of retinyl esters (Blomhoff, 1994). To release this stored form the esters are hydrolysed to retinol, which is released into the bloodstream for transport round the body bound to plasma retinol-binding protein. Cells which require RA take up retinol and convert it to RA through the action of two types of enzymes. The first type of enzyme, the retinol dehydrogenases (ROLDH), convert retinol to retinaldehyde which is used in the visual cycle, and the second type of enzyme, the retinal dehydrogenases (RALDH), convert retinaldehyde to RA (Duester, 1996; Napoli, 1996). In addition, there is a cytochrome P450 enzyme known as CYP26 which is thought to break down all-*trans*-RA to 4-*oxo*-RA, 4-hydroxyRA and 18-hydroxyRA (Abu-Abed *et al*. 1998; White *et al*. 1996, 1997) or 5,8-epoxy-RA (Fujii *et al*. 1997), and these breakdown products were thought to be inactive metabolites on their way to being excreted. However, 4-*oxo*-RA is a potent bioactive retinoid which respecifies the head-to-tail axis of the *Xenopus* embryo (Pijnappel *et al*. 1993), and the overexpression of CYP26 in embryonal carcinoma cells induces neuronal differentiation (Sonneveld *et al*. 1999).

There are several other isoforms of RA which are bioactive, i.e. all-*trans*-RA, 9-*cis*-RA and didehydoRA, in addition to 4-*oxo*-RA. It seems likely that there are specific enzyme pathways for each isoform, as the established ROLDH and RALDH generate all-*trans*-RA, 4-*oxo*-RA is generated by CYP26, and there have been recent descriptions of 9-*cis*-specific enzymes which can generate 9-*cis*-RA from 9-*cis*-retinol (Mertz *et al*. 1997; Romert *et al*. 1998). The didehydro retinoids are found most prevalently in the chick embryo (Maden *et al*. 1998*b*; Thaller & Eichele, 1990), and it is thought that there are parallel ROLDH and RALDH enzyme pathways which operate on didehydro-retinol to convert it to didehydroRA.

Clearly, a crucial indication as to which regions and cell types of the embryo require RA is to be obtained from studies on the distribution of these enzymes in the embryo.

Retinoid-synthesizing enzymes in the embryo and their knockouts

The expression of one particular ROLDH enzyme, alcohol dehydrogenase(ADH)-IV, begins early in the development of the mouse embryo, at day 7·5, during the primitive streak stage (Ang & Duester, 1997). It is detected in the posterior embryonic tissues (Fig. 1(A)) and in the mesoderm, and later becomes more widely expressed. No other ROLDH are expressed this early, as ADH-1 does not begin expression until day 10·5 of mouse development, and then it is localized to the mesonephros and limb buds (Vonesch *et al*. 1994). On subsequent days of development ADH-1 expression becomes more widespread in areas such as the mesenchyme of the head, in the developing vertebrae, the foetal gonads and in the epithelium of organs such as the lungs, the gut, the bladder and adrenal gland. The heart is a particularly intense region of expression of ROLDH, as is the myotome of the somites, the gut epithelium and the dorsal ectoderm of the limb buds (Bavik *et al*. 1997).

The initial expression of ADH-IV described earlier exactly overlaps with the expression of a RALDH enzyme, RALDH-2, in the early mouse and chick embryo (Niederreither *et al*. 1997; M Maden and P McCaffery, unpublished results). The expression of RALDH-2 also begins very early in embryogenesis, just after primitive streak formation, in a discrete domain in the mesoderm with a sharp border just behind the node and tailing off towards the posterior end (Fig. 1(B)). There is no expression at the anterior end of the embryo, in the midline, or in the node. As development continues the location of the sharp border becomes clearer, and it is at the level of the first somite. Surprisingly, the developing CNS at these early stages does not express RALDH-2, so some other enzyme must be responsible, as high levels of endogenous RA can be detected in the CNS (see pp. 68–69).

Later on in development RALDH-2 becomes intensely expressed in the mesonephros and the somites, becomes quite widespread in the mesodermal tissues including limb regions such as the interdigital areas, and is very dynamically expressed in the heart (Moss *et al*. 1998). RALDH-2 makes its first appearance in the CNS in the developing motor neurons, but interestingly only in the motor neurons at the levels of the limbs (Zhao *et al*. 1996), which is correlated with the hot spots of RA synthesis which have been observed in mouse embryos (McCaffery & Drager, 1994). Most recent studies have revealed that RALDH-2 is not expressed in all motor neurons, but only in a subset of motor neurons known as the LMC, and that RA is involved in the generation of these particular neurons (Sockanathan & Jessell, 1998).

The 9-*cis*-generating enzyme, 9-*cis* retinol dehydrogenase, is predominantly expressed in the developing CNS, the developing ear and eye, the cranial and spinal ganglia, the gut epithelium and the myotomes (Romert *et al*. 1998).

The CYP26 enzyme is found in many regions of the embryo, and from an equally early stage as ADH-IV and RALDH-2. However, rather than being expressed at the posterior end of the embryo as ADH-IV and RALDH-2 are, CYP26 is expressed at the very anterior end of the mouse and *Xenopus* embryo in a fascinating reciprocal distribution

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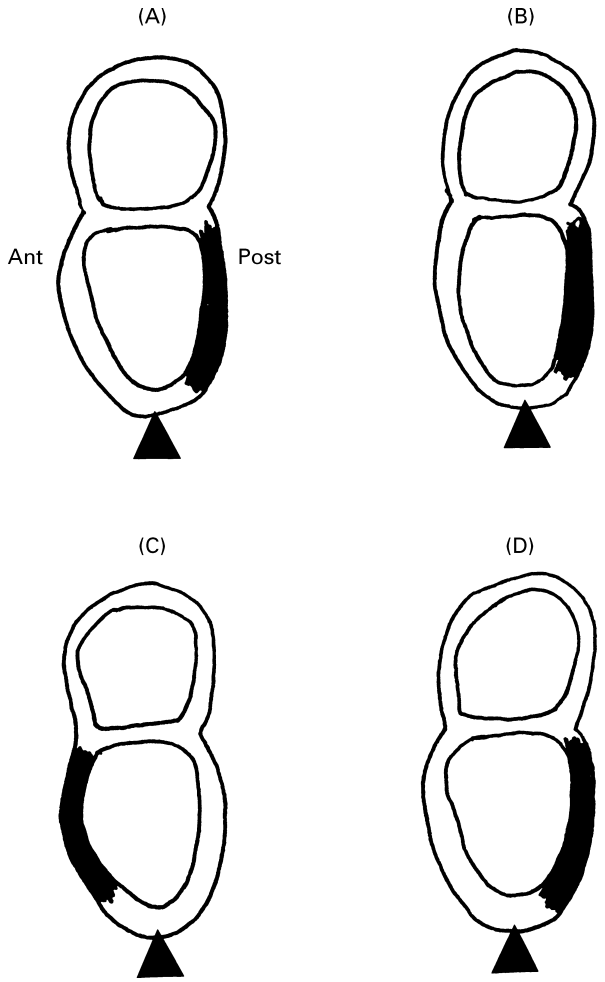

Fig. 1. Drawings of 7·5 d later primitive-streak mouse embryos to show the relative distribution of three enzymes involved in the retinoic acid (RA) pathway (A,B,C) and of RA itself (D). The upper part of each drawing represents the allantois and the lower part is the embryonic tissue with anterior (head end) to the left (Ant) and posterior (tail end) to the right (Post). (▲), The location of the node. (A) Expression of ADH-IV (a retinol dehydrogenase enzyme), the enzyme which converts retinol to retinal, is in the posterior part of the embryo (from Ang & Duester, 1997). (B) Expression of retinal dehydrogenase-2, the enzyme which converts retinal into RA, is in the posterior part of the embryo (from Niederreither *et al.* 1997, and equivalent chick stages from M Maden and P McCaffery, unpublished results). (C) Expression of CYP26 (a cytochrome P450 enzyme), the enzyme which converts all-*trans*-RA into 4-*oxo*-RA and other downstream metabolites, is in the anterior end of the embryo (from de Roos *et al.* 1999). (D) Generation of RA is in the posterior part of the embryo (from the transgenic reporter mouse line of Rossant *et al.* 1991 and equivalent chick stages of Maden *et al.* 1998*b*).

(Fujii *et al*. 1997; Hollemann *et al*. 1998; de Roos *et al*. 1999; Fig. 1(C)). During subsequent development CYP26 is expressed in the CNS, cephalic mesenchyme, developing ear and various epithelia of the nose, mouth and tongue, in the mesonephros and gut mesenchyme, and in the epithelium of the limb buds (Sonneveld *et al*. 1999*a*). This reciprocal distribution between the RA-generating enzymes and CYP26 (Fig. 1) suggests that the posterior end of the embryo makes RA and the future head metabolizes RA, with the head developing specifically in the absence of all-*trans*-RA.

The eye is one part of the embryo which is particularly sensitive to retinoid deprivation (for example, see Hale, 1933), and, it is another region which has a fascinating distribution of these enzymes. In the dorsal hemisphere an aldehyde dehydrogenase known as AHD-2 is specifically expressed (McCaffery *et al*. 1991, 1992, 1993; Godbout *et al*. 1996), and in the ventral hemisphere there is an enzyme known as V-1 (McCaffery *et al*. 1993). Between these two regions there is a stripe of expression of CYP26 (McCaffery *et al*. 1999), thereby generating territories of RA synthesis and metabolism.

Thus, there are intriguing relationships between these retinoid-synthesizing enzymes, with some degree of co-localization between the ROLDH and RALDH, and a non-overlapping distribution of the CYP26 enzyme which uses RA as a substrate.

Only one enzyme knockout has been reported so far and that is RALDH-2. From the distribution of the enzyme in the posterior of the embryo (Fig. 1(B)) we might expect it to be deficient at this end and perhaps have a fairly normal head. This situation is indeed what seems to be the case (Niederreither *et al*. 1999), as these embryos have a single dilated heart tube, lack the associated extraembryonic blood vessels, the somites are smaller resulting in a considerably shortened antero–posterior axis, the limb buds are reduced or absent, the posterior branchial arches are missing, there is a truncated fronto-nasal region and the otocysts are hypoplastic. The surprising result is that this one enzyme deficiency recapitulates virtually all the effects of totally depriving the embryo of retinoids, suggesting that this enzyme is the single RALDH enzyme responsible for generating most of the RA in the embryo. The only other enzyme activity which seems to play a role is AHD-2, which is present in the dorsal half of the eye, and this region is unaffected in the RALDH-2 knockout embryos.

The retinoid receptors and their knockouts

The retinoid receptors are ligand-activated transcription factors present within the nuclei of RA-sensitive cells. There are two classes of these transcription factors, the RA receptors (RAR) and the retinoid X receptors (RXR), and they form part of the gene superfamily including the steroid hormone receptors. In the human subject and the mouse three RAR have been identified and designated RARα, RARβ and RARγ (Kastner *et al*. 1994*a*). There are also three RXR: RXRα, RXRβ and RXRγ (Kliewer *et al*. 1994). Furthermore, there are several isoforms of each of these six genes, formed by differential promoter usage. The ligand for the RXR is 9-*cis*-RA, whereas the RAR bind both 9-*cis*-RA and all-*trans*-RA. These receptors act as ligand-dependent transcription factors by recognizing consensus sequences known as RA response elements which are present in the upstream promoter sequences of RA-responsive genes. The RAR and RXR do not act alone, but as heterodimers, and the RXR can also heterodimerize with a variety of other related receptors such as the thyroid hormone receptors, vitamin D receptors, peroxisomal proliferator-activated receptor and

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several other orphan receptors. These analyses thus reveal how retinoids can elicit such a diversity of biological responses involving other hormone pathways.

The distribution of these receptors has been most intensively analysed in the mouse embryo. The RARα gene is considered to be ubiquitously expressed, whereas RARβ and RARγ are expressed in spatially- and temporally-restricted patterns (Dolle *et al*. 1990; Ruberte *et al*. 1990, 1991, 1993). For example, RARβ is expressed in the anterior facial mesenchyme, the mesonephros, the branchial epithelium, the epithelium of the digestive tract and peripheral mesenchyme, whereas RARγ is generally expressed in the precartilaginous mesenchymal condensations. In the developing CNS RARα is expressed in the neural tube up to a discrete level in the hindbrain, RARβ is expressed up to an equally discrete but slightly more posterior level in the hindbrain and RARγ is expressed in the open neural tube before the neural folds close. Slightly later, RARβ is expressed in the motor neurons of the spinal cord (Muto *et al*. 1991) and RARα is expressed in the neural crest and branchial arches. There are also differential patterns of expression of individual isoforms.

In order to assess the function of isoforms and genes, knockout mice embryos have been generated (Kastner *et al*. 1995). Mice deficient in individual RAR isoforms are normal, as are mice with knockouts of all the RARβ isoforms. Disruption of all isoforms of RARα resulted in early postnatal lethality and testis degeneration, but development was normal (Lufkin *et al*. 1993). Knockout of the RARγ gene resulted in early postnatal lethality, male sterility and one particular developmental abnormality, a change in phenotype of certain vertebrae (homeotic transformations; Lohnes *et al*. 1993). Thus, there is virtually no alteration to development in the absence of one of these RAR genes. However, RAR double mutants show severely disrupted development, and almost all the abnormalities of vitamin A deprivation are recapitulated by the different combinations of RAR double mutants, i.e. respiratory tract defects, spongy myocardium, heart outflow and aortic arch derivative abnormalities, diaphragmatic hernia, ureter abnormalities, genital tract abnormalities and particular ocular abnormalities (Kastner *et al*. 1995).

With regard to the RXR, RXRβ is ubiquitously expressed, RXRα is ubiquitously expressed early on in development and then becomes more highly expressed in the epidermis and other squamous epithelia, and RXRγ is more restricted to the myogenic lineage, the developing ear, the retina and the pituitary and thyroid glands (Mangelsdorf *et al*. 1992; Dolle *et al*. 1994). Within the CNS RXRγ is expressed in the developing diencephalon, the striatum and in the ventral horns of the spinal cord, and in the latter location is co-expressed with RARβ.

RXRα null mutant mice display ocular and cardiac malformations and die from cardiac failure at about day 15 of gestation, suggesting a vital role for this receptor in heart development (Kastner *et al*. 1994*b*; Sucov *et al*. 1994). RXRβ null mutant mice are developmentally normal and the adult males are sterile (Kastner *et al*. 1996).

Finally, compound mutants of RAR and RXR have revealed that it is the RAR–RXR heterodimer which is the functional unit transducing the RA signal *in vivo*. This conclusion was reached because the severity of the defect in the eye with, for example, RXRα null mutants increases with successive removal of the two alleles of either RARβ2 or RARγ. Similarly, the inactivation of only one RARα allele from a RARγ null background can cause eye defects identical to those observed in RXRα null mutants. Furthermore, the defects seen with compound RAR mutants can be recapitulated in specific RXR–RAR compound mutants (Kastner *et al*. 1997). This work also revealed that the RXRα receptor is the main RXR receptor implicated in the developmental functions of these receptor heterodimers (Mascrez *et al*. 1998).

Thus, the only single receptor gene knockout which gives a significant developmental defect is the RXRα gene. This surprising lack of abnormalities in other single knockouts suggested that the different receptors could be functionally redundant. For example, it could be that the cell only requires a threshold level of RAR and RXR which could be achieved through any combination of isoforms. However, the striking sequence conservation of these isoforms across the vertebrates would suggest an individual function. Such individual functions have indeed been identified in the newt limb-regeneration system using chimeric receptors (see pp. 69–70). It is therefore possible that the lack of phenotype in the knockout mouse studies is either due to the existence of some subtle phenotype which has not yet been detected, or that other isoforms can indeed substitute for the missing one in the uniquely abnormal knockout situation and at a lower efficiency, but at an efficiency which nevertheless does not result in defective gene functioning.

Endogenous retinoic acid

The detection and measurement of endogenous RA levels in the embryo obviously gives us an important insight into which systems of the embryo are likely to be affected by RA deprivation. Such measurements have only been performed recently either by direct HPLC detection or by reporter methodologies.

HPLC has been used to detect all-*trans*-RA and all-*trans*-retinol in the mouse embryo (Satre & Kochhar, 1989; Scott *et al*. 1994; Horton & Maden, 1995), and in the chick embryo the didehydroretinoids have also been detected (Thaller & Eichele, 1990; Scott *et al*. 1994; Dong & Zile, 1995; Maden *et al*. 1998*b*). *Xenopus* and zebrafish (*Danio rario*) embryos also contain a variety of retinoids (Durston *et al*. 1989; Pijnappel *et al*. 1993; Creech-Kraft *et al*. 1994; Costaridis *et al*. 1996). Chick and mouse embryos have been divided into eight regions, and all parts of the embryo were found to contain RA, but at varying levels (Horton & Maden, 1995; Maden *et al*. 1998*b*). In both cases the highest levels of RA were detected in the neural tube, and decreasing levels found in the somites, eye, tail bud, fronto-nasal mass, branchial arches, limb buds and heart. Interestingly, the levels of RA were not uniform within the neural tube. The region which will form the spinal cord has the highest levels, whereas the forebrain and midbrain have virtually undetectable levels; the hindbrain has intermediate levels of RA. It is possible therefore that the spinal cord is the source of RA which diffuses into the developing brain (or is broken down there by the enzyme CYP26, p. 66) and

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is present in the form of concentration gradient spanning the developing hindbrain. The hindbrain is the region of the embryo which is exquisitely sensitive to altered levels of RA or disturbances in the signalling machinery, making this possibility more likely. The embryonic eye is a site of intense RA production, since there are two different RALDH enzymes present in discrete regions (McCaffery *et al*. 1992, 1993).

Reporter methodologies have been used to detect endogenous bioactive retinoids. The reporter used is a combination of the upstream sequence from the RARβ gene, containing a RA response element which is linked to a promoter and the *lacZ* gene. In one set of experiments four different groups have used this reporter to create transgenic mice strains (Reynolds *et al*. 1991; Rossant *et al*. 1991; Balkan *et al*. 1992; Shen *et al*. 1992). When the embryos are fixed at particular stages and the *lacZ* gene histochemistry performed, the regions of the embryo which contain RA turn blue. In this way it has been shown that RA appears at the later primitive streak–head fold stage, and only in the posterior half of the embryo (Fig. 1(D)) and in all three germ layers including the heart. The head remains RA free at the early stages, except for the eye and in some cases the maxillary region.

Another technique has used this reporter construct, but transfected it into embryonal carcinoma cells. When pieces of embryo are placed onto a lawn of these cells and cultured for several hours the cells turn blue around the pieces of embryo that contain and release RA, and there are no blue cells around pieces of embryo that do not contain and release RA. In this way various areas of the embryo have been found to release RA, such as the limb bud and spinal cord, but not the forebrain (Wagner *et al*. 1992); the spinal cord has been shown to have regions of high RA generation at the cervical and lumbar enlargements where the limb buds will form (Colbert *et al*. 1993), i.e. the 'hot spots' of RA synthesis (McCaffery & Drager, 1994); the development of the olfactory region is dependent on RA generation by the olfactory cranial mesenchyme (LaMantia *et al*. 1993); the ventral part of the developing eye generates more RA than the dorsal part (McCaffery *et al*. 1992). We have used this technique to investigate when RA production begins in the early embryo (Maden *et al*. 1998*b*). It transpires that the chick embryo begins to make RA soon after gastrulation begins, at stage 4–5, with a distribution which exactly coincides with the distribution of RALDH-2, and with the same distribution as the reporter mice (Fig. 1(D)). This distribution reveals a sharp on–off border of RA synthesis behind the node, which later is at the level of the first somite. Clearly, the anterior end of the embryo initially develops in the absence of RA (perhaps due to the presence of CYP26), and RA is required for the development of the trunk.

Deprivation studies

As mentioned earlier, vitamin A-deprivation studies in the embryo have a long history, beginning in the 1930s. More recently, interest in this experimental paradigm has re-awakened, and a rat and mouse model (Morriss-Kay & Sokolova, 1996; Dickman *et al*. 1997; Antipatis *et al*. 1998; White *et al*. 1998) as well as a chick and quail model (Thompson *et al*. 1969; Heine *et al*. 1985; Dersch & Zile, 1993) have been developed. In the former system embryos can be deprived at chosen stages of development to investigate the dependency of particular organ systems, and in the latter systems the embryos are deprived from the start of development.

The quail embryos which are deprived from the very beginning of development have virtually identical phenotypes to the RALDH-2 knockout embryos described earlier. They are antero-posteriorly shortened, with much smaller somites, the heart fails to loop correctly and is a single distended tube, the vitelline veins fail to form, the limbs are stunted, the neural crest cells die, the posterior hindbrain fails to develop, the neural tube fails to extend neurites into the periphery and the posterior branchial arches are lost (Maden *et al*. 1996, 1998*a*). Rat embryos which are deprived at later stages have an underdeveloped hindbrain, loss of posterior cranial nerves and posterior branchial arches, microphthalmia, narrow limb buds, neural crest cell death, failure or hypoplasia of lung development, failure of septation of the trachea and oesophagus, and lack of differentiation of neuronal populations in the brain (Dickman *et al*. 1997; Antipatis *et al*. 1998; White *et al*. 1998). These studies have confirmed the role that RA plays in many systems of the embryo and at many times throughout development rather than at any one stage of development.

Post-embryonic development

Post-embryonic development, or regeneration, has been one of the foci of RA research since the discoveries concerning limb regeneration. Most amphibians can regenerate their limbs before metamorphosis, but only the tailed amphibians (newts) can regenerate their limbs into adulthood. Limb regeneration involves the perfect replacement of the structures that are amputated, so if the hand or foot is amputated it is replaced, and if the whole of the arm or leg is amputated then the whole structure is replaced. However, it was discovered that if the animals are treated with RA then extra structures are regenerated. For example, after amputation through the hand, instead of regenerating a hand as the controls would, the RA-treated limb regenerates a complete limb or often a complete pair of limbs from the amputation plane (Niazi & Saxena, 1978; Maden, 1982, 1983; Niazi & Ratnasamy, 1984). Most amazingly of all, a tadpole tail can be induced to regenerate not a new tail but a number of hindlimbs from the amputation plane after RA treatment (Mohanty-Hejmadi *et al*. 1992; Maden, 1993), showing a complete homeotic transformation of tissue type.

As in the developmental studies mentioned earlier, the profound effects of excess RA give an indication as to where RA is required, and this situation is true for regeneration also. There are three sets of experiments which demonstrate that RA is required for normal limb regeneration. First, the RAR that are involved in transducing the RA signal have been identified in the regenerating limb, and individual functions have been ascribed to several of them. There are at least five RAR in newt limbs (Ragsdale *et al*. 1989, 1992*a*,*b*), and the precise function of three of them have

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been determined, thanks to the construction of chimeric receptors. These chimeric receptors have the ligand-binding domain of the thyroid hormone receptor and the DNA-binding domain of the RAR. When transfected into the regenerating limb the receptor concerned now becomes responsive to thyroid hormone, but activates RA-responsive genes. In this way it has been shown that the α_1 isoform mediates the inhibition of blastemal cell division by RA, the δ_1 isoform induces an antigenic change in the wound epithelium and the δ_2 isoform mediates the proximodistal change in identity induced by RA (Schilthuis *et al*. 1993; Pecorino *et al*. 1994, 1996).

Second, the wound epithelium which covers the regenerating limb has been shown to synthesize 9-*cis*-RA (Viviano *et al*. 1995). Third, the inhibition of RA synthesis inhibits the normal process of limb regeneration, which has been demonstrated using disulphiram which inhibits the retinaldehyde dehydrogenase enzymes. When disulphiram is applied to the amputated limb, regeneration is inhibited for the duration of application (Maden, 1998*a*).

Another two dramatic examples of the involvement of RA in the regeneration process in the adult are alveolar regeneration in the lung and the regeneration of hair cells in the ear. It has recently been demonstrated, using a rat model of human lung emphysema, that RA induces the regeneration of alveoli (Massaro & Massaro, 1997). If this process also occurs in human subjects then it opens up the amazing possibility of a treatment for emphysema, for which there is currently no treatment apart from lung transplantation. The involvement of retinoids in adult lungs is most likely to be, as in all the other cases cited here, a recapitulation of a developmental process involving retinoids. It comes as less of a surprise, therefore, but still a very important finding, that the risk of chronic lung disease and sepsis is reduced in extremely-low-birth-weight premature infants by the administration of vitamin A (Tyson *et al*. 1999). In the ototoxic poisoned organ of Corti from the rat, treatment with RA stimulates the regeneration of auditory hair cells, a result which must provide hope for a recovery of hearing function in human subjects (Lefebvre *et al*. 1993).

A final example of the involvement of RA in regeneration is the spinal cord which, as described earlier, has the highest levels of endogenous RA in the embryo. It has been known for many years that RA stimulates neurite outgrowth in a whole range of cells: embryonal carcinoma cells; neuroblastoma cells; primary neuronal cultures and explants of embryonic CNS; dorsal root ganglia (Maden, 1998*b*). Both neurite number and neurite length can be dramatically increased by RA treatment. However, there is a stark contrast between embryonic spinal cords which can extend neurites *in vitro* in response to RA and adult spinal cords which cannot. We have discovered that the response to neurite outgrowth both in the embryonic spinal cord and dorsal root ganglia is to up regulate one particular RAR, i.e. RARβ (J Corcoran and M Maden, unpublished results). The non-responsive adult spinal cord does not up regulate RARβ. To demonstrate whether this response was crucial, we have transfected adult mouse spinal cords *in vitro* with the RARβ gene using the Herpes Simplex virus. The result of this procedure is that the adult spinal cord now extends neurites into the culture dish, confirming the role of RA and its transduction machinery in the regeneration of neurites in adults.

Conclusion

RA is crucially involved in the developing embryo from very early stages, beginning soon after gastrulation when it is synthesized in the posterior part of the embryo and is absent from the anterior part of the embryo. As development proceeds RA is then found in most parts of the embryo at different concentrations, with the highest levels in the developing spinal cord. The absence of RA or the disruption of the transduction machinery in the RA pathway results in multiple defects in the embryo; the CNS, craniofacial region, limb, urogenital system, lungs and heart are all affected. The gene pathways involved in the generation of the defects in some of these systems are gradually being identified. In post-embryonic development, or regeneration, RA is again crucially involved. These systems include the regenerating limb in amphibians, alveolar development and regeneration in the lung, regeneration of hair cells in the ear and neurite regeneration in the CNS. From the human point of view, in the developing world many congenital defects may be caused by a maternal diet deficient in vitamin A, the source of RA for the embryo. However, it is also possible that genetic defects in the transduction machinery of RA are responsible for some developmental abnormalities and degenerative diseases in the developed world.

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