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Retinoic acid and limb regeneration

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Summary

A key problem in the study of vertebrate development is to determine the molecular basis of positional value along a developmental axis. In amphibian regeneration, retinoic acid is able to respecify positional value in a graded fashion that is dependent on its concentration. In view of the fact that retinoic acid is a naturally occurring metabolite of vitamin A, this raises the possibility that it is deployed *in vivo* as an endogenous morphogen. Furthermore, the recent evidence that its effects are mediated by nuclear receptors of the steroid/thyroid hormone superfamily suggests the possibility of understanding the mechanism of its graded effects on morphogenesis. Such insights would be of crucial importance for our understanding of vertebrate patterning along an axis.

Introduction

In vertebrate development there are many contexts where a field of cells undergoes differentiation in a way that generates a pattern of tissues. This process is usually analysed in terms of the specification of the axes of the developing structure by the acquisition of some continually graded variable referred to as positional value (Wolpert, 1989). The validity of this general framework has been supported by the analysis of axial specification in the *Drosophila* embryo, using the methods of molecular genetics (Nusslein-Volhard *et al.* 1987). In vertebrates the molecular basis of positional value remains wholly unclear and is a major problem of current investigation. The progress in *Drosophila* has largely reflected the availability of mutants in axis formation, and this has confirmed the notion that axes are specified independently. Although such mutants have led to a detailed understanding of the genetic events that establish positional value, they have not so far led to any major insights into how such specification is implemented in terms of cell behaviour or cell–cell interactions. A major impetus in the study of pattern formation in vertebrates has come from the recognition that retinoids, and in particular retinoic acid, are able to re-specify positional value in a dose-dependent way. This has led to efforts to evaluate the possibility that these molecules are used as endogenous morphogens, as well as an interest in their mechanism of action.

The two key systems that have led to the present interest in the morphogenetic effects of retinoids are avian limb development (Tickle *et al.* 1982) and amphibian limb regeneration (Niazi and Saxena, 1978). The limb is often referred to as a secondary field, in contrast to the primary field of the whole embryo, and limb

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morphogenesis has long been the pre-eminent context for study of pattern formation in such a field. This paper focusses on limb regeneration as a context for evaluating retinoid action. There are some differences from the effects on avian limb development and I have reviewed these issues elsewhere (Brockes, 1989).

Amphibian limb regeneration

The vertebrates able to regenerate their limbs as adults are the urodele amphibians, and the principal laboratory species are the newt and the axolotl (Wallace, 1981). If the limb is amputated at any level on the proximo-distal axis (shoulder to fingertip), the wound surface is rapidly covered by epithelial cells migrating from the circumference of the amputation plane. This is followed by the appearance of the progenitor cells of the regenerate, referred to as blastemal cells. It is still a matter of some uncertainty as to how the blastemal cells arise, but they clearly are locally derived from the mesenchymal tissues within about half a millimetre from the amputation plane (Wallace, 1981). This process can be followed by using monoclonal antibodies that distinguish blastemal cells from normal limb mesenchyme (Kintner and Brockes, 1985). The blastemal cells are mesenchymal progenitors that give rise to connective tissue, cartilage and muscle, that is the cells that are the substrate for tissue patterning. They divide initially under control of the nervous system, and form a mound of undifferentiated cells called the blastema which undergoes differentiation and morphogenesis to reconstruct the regenerate. The blastema has considerable morphogenetic autonomy and can be transplanted to a location such as the anterior chamber of the eye, or the fin tunnel, where it may give rise to a limb (Stocum, 1984). This account is particularly concerned with the molecular basis of position-dependent properties of the blastema.

Positional value on the proximo-distal axis is manifest in several different properties of the blastema, most notably in the phenomenon known as distal transformation. The blastema arising at any position on the axis does not form structures proximal to its point of origin; thus a wrist blastema gives a hand while a shoulder blastema forms an arm. It is precisely this property that is altered by retinoid treatment. If a wrist blastema is treated with an appropriate dose of retinoic acid, its proliferation is arrested temporarily so that it appears retarded in comparison to a control. Nonetheless, the blastema subsequently grows out to give an entire arm arising from the wrist level (Maden, 1982). This is sometimes referred to as a serial duplication, and the extent of duplication is dependent on the dose of retinoic acid to which the blastema is exposed. Retinoids are the only class of compounds able to provoke these effects. Although the proximal effector of such morphogenetic effects is presently considered to be retinoic acid (RA), derived by oxidative metabloism from retinol (vitamin A), there is already evidence for other morphogenetically active metabolites (Thaller and Eichele, 1988).

The action of retinoids on limb morphogenesis must be considered against the

background of other effects mediated by this family. The classical vitamin requirements include the role of retinol and retinal in the visual pigments and reproductive function, and that of RA in epithelial differentiation and maintenance (Pitt, 1971). It has long been recognised that high doses of vitamin A or RA provoke a well defined and wide ranging set of teratological abnormalities when administered to vertebrate embryos (Kochhar, 1977). In addition, a wide variety of cell types are affected by RA in culture. The responses show dose dependence, and include positive and negative regulation of division and differentiation. Some of these effects, for example the ability to induce differentiation of F9 teratocarcinoma cells to parietal endoderm (Strickland and Mahdavi, 1978), have been studied intensively as paradigms for understanding the molecular basis for retinoid action. Lastly, there are the morphogenetic effects which concern the specification of axes in regenerating and developing limbs. It has been difficult to analyse such a diverse set of effects in terms of a unitary mechanism, and an important step forward was the identification of hormone nuclear receptors of the steroid/thyroid superfamily which interact with RA, and mediate its effects on gene expression (Petkovich *et al.* 1987; Giguere *et al.* 1987).

Retinoic acid receptors

Most of the current information about the receptors has been reviewed elsewhere (Ragsdale and Brockes, 1990). The receptor proteins have a structure that is usually represented from the N-terminus and comprises six regions A–F. Two of these regions have the status of functionally independent domains: the C region which binds to response elements associated with retinoid modulated genes, and the E region which binds RA. Recent evidence suggests that the receptor binds to DNA in the absence of RA (de The *et al.* 1990), but is stimulated to activate transcription in its presence. The molecule acts, therefore, as a ligand-dependent transcription factor. This can be directly demonstrated in the standard co-transfection assay by introducing a reporter gene along with an expression constuct for the RA receptor. Trans-activation of the reporter occurs in the nmol l^{-1} range of RA concentration but requires much higher concentrations of retinol for any effect (Giguere *et al.* 1987; Petkovich *et al.* 1987).

At present, three retinoic acid receptor subtypes have been identified in mouse and human, and they are termed a, b and c (Brand *et al.* 1988; Krust *et al.* 1989; Zelent *et al.* 1989). The subtypes show high conservation of amino acid sequence in the C and E regions, but marked divergence in the other regions. The cross-species identity within a subtype is much greater than the identity between subtypes within a species. There is evidence for other members of the superfamily that the spectrum of genes activated is dictated, at least in part, by the A/B region at the N-terminus of the molecule (Tora *et al.* 1988; Bocquel *et al.* 1989). It seems likely that a, b and c activate distinct gene sets in response to RA, and that receptor heterogeneity and regulation may underly at least some of the diversity in the response to RA (Ragsdale and Brockes, 1990).

The urodele blastema is important for our understanding of the morphogenetical effects of RA, thus it has been of interest to identify the receptors that are expressed in this context. To date, three different receptors of the newt limb blastema have been identified in cDNA libraries. These include the newt homologue of human and mouse alpha (Ragsdale *et al.* 1989) and a partial cDNA clone that is the probable homologue of beta (Giguere *et al.* 1989). The third newt receptor, termed delta (Ragsdale *et al.* 1989), is closest to mouse and human gamma but diverges markedly in the A/B and F regions, a divergence that is particularly striking considering the close relationship of newt and human alpha. Newt gamma is expressed at higher levels in a distal than a proximal blastema, and at higher levels in the blastema compared to normal limb. It is also differentially spliced at the A/B border (Ragsdale and Brockes, 1990) and seems at present to be an attractive candidate for mediating at least some of the effects of RA on the blastema.

Although it is by no means certain that all relevant retinoid receptor subtypes have been identified, it is already clear that there is significant receptor heterogeneity and that a major challenge for the future is to identify the roles of the different subtypes. As mentioned earlier, RA has several different effects on regenerating limbs, ranging from morphogenetic effects that can be observed on the transverse and proximodistal axes, to teratogenic effects that have been widely studied in vertebrate embryos. There is a need for more basic comparative information on the receptors, for example on their affinities and regulation, and such information will help to guide hypotheses about their possible roles. Ultimately it will be necessary to modify expression of the receptors in developing and regenerating systems, and to observe the functional consequences of such manipulations. This might be done in the context of transgenic mice, or in the urodele by implantation of blastemal cells that have been genetically modified in cell culture (Ferretti and Brockes, 1988).

Regulation of gene expression by RA

In view of the complexity of the effects that RA exerts on cultured cells, it is likely that there are effects on gene expression that reflect a chain of events, for example the appearance of other transcriptional regulators. An important recent development is the identification of genes directly regulated by RA receptors. When human hepatoma cells are treated with RA, there is a rapid increase in RAR beta mRNA, whereas RAR alpha mRNA is not increased (de The *et al.* 1989). This effect occurs at the level of transcription and is mediated by a response element in the beta receptor promoter (de The *et al.* 1990). This element binds to RA receptors *in vitro* and confers RA responsiveness on a heterologous promoter. It is not clear if this is the only natural RA response element or if there are others. It will clearly be important to identify further examples of genes that are directly regulated.

A major goal of future research will be to identify the target genes that mediate

the morphogenetic effects of RA. Homeobox genes show dramatic spatial and temporal regulation in vertebrate development, and they have attracted considerable attention as potential targets for RA. For example, the recent elegant analysis of the distribution of transcripts for the five contiguous members of the *Hox*-5 complex in the mouse limb bud has revealed a position-dependent pattern of activation that reflects the order of genes on the chromosome (Dolle *et al.* 1989). The importance of this order, already recognised in terms of patterns of expression, has been emphasised in a recent study of F9 cells showing that genes at the 3′ end are more responsive to RA treatment than those at the 5′ end (Papalopulu *et al.* 1990). Thus far it has not been possible to identify a homeobox gene that is regulated by RA in limb morphogenesis, although there is no shortage of examples of regulation by RA of homeobox genes in culture (Mavilio *et al.* 1988). In limb regeneration, the one example of a homeobox gene that varies on the proximo-distal axis does not show a change in expression after treatment of a distal blastema with RA (Savard *et al.* 1988). It will nevertheless be important to try to find such a gene, because it would be an excellent candidate for one that is on the 'pathway' of positional specification.

Retinoids as endogenous morphogens

Much of the current interest in RA as an endogenous morphogen has come from studies on the chick limb bud that employ HPLC analysis of solvent-extracted retinoids. RA is apparently present in the chick limb bud, and is present at somewhat higher levels in the posterior segment than in an anterior (Thaller and Eichele, 1987). These observations are consistent with the hypothesis that RA is released from the polarising region, but much remains to be done to establish if this is true. In the case of the chick limb, the grafting experiments that led to the idea of a polarising region were instrumental in providing a framework for thought about retinoid action. In the developing and regenerating urodele, such grafts do not provoke duplications in the antero-posterior axis, and the models of morphogenesis stress the importance of local cell interactions rather than the action of signalling regions.

The presence of the RARs as well as the cytoplasmic binding protein for RA (CRABP) (McCormick *et al.* 1988) in the blastema is certainly suggestive that RA plays some role in limb regeneration, but what role? Are there mechanisms that ensure a varying response along one or more axes? It seems highly unlikely that there is a gradient of retinoid in the dimensions of the adult limb, but it is possible that blastemas arising at different locations have different concentrations. It is also possible that the responsiveness of the cells is regulated, for example at the level of the receptors or the binding proteins for RA (Maden *et al.* 1988). Some evidence is needed about the concentration of RA that actually impinges on the blastemal cells, and whether this level varies with position. There are several possibilities for sources of RA. It has been suggested that the epidermis or its associated pigment cells (Baranowitz, 1989) may release retinoid precursors or

RA that are instrumental in provoking the formation of blastemal cells. Alternatively, RA may be synthesised from precursor retinol by the blastemal mesenchyme cells, as it is by the mesenchymal cells of the chick polarising region (Thaller and Eichele, 1988).

An important contribution towards solving these problems may come from the recent advances in the basic molecular biology of retinoid action. It is possible to construct plasmids in which expression of a reporter enzyme comes under control of a RA response element (de The *et al.* 1990), and hence reflects the external concentration of RA. If such plasmids can be introduced into cells *via* the transgenic troute, or by implantation of genetically modified cultured cells, then the level of the reporter may give an indication of the local concentration of RA. It is not clear if this approach will give quantitative estimates for the concentration *in vivo*, partly because of uncertainties about other cell properties that can modify the response, but the questions at present are sufficiently broad that it may give useful information.

Conclusions

It is clear that the identification of RARs has given significant impetus to studies on the morphogenetic effects of RA. In future it will clearly be important to determine functional roles for the different subtypes, and to identify the target genes that are regulated when axial specification is altered. This latter point is a particularly challenging issue, but it is one of the rare possibilities for tackling the problem of positional value in vertebrates. I have indicated how many gaps exist in our present understanding of the role of retinoids in development, and that much remains to be determined before the case for an endogenous, morphogenetic role can be considered convincing.

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