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Is retinoic acid an endogenous ligand during urodele limb regeneration?

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ABSTRACT The effects of retinoids on a regenerating urodele limb make them interesting candidates for endogenous ligands during regeneration. We review the evidence for considering this possibility. This includes analysis of retinoids and retinoic acid receptors in the regenerate, and studies on activation of retinoid reporter genes. Recent work has provided evidence that the wound epidermis is a source of 9-*cis* retinoic acid, and may be a favorable model for studying the synthesis and release of this modulator.

KEY WORDS: *blastema, epidermis, positional information, 9-cis retinoic acid, RAR*

Introduction

The ability of retinoic acid (RA) and precursor retinoids to respecify positional identity was originally noted in the context of anuran and urodele limb regeneration where treatment resulted in striking respecification along the proximodistal (PD) and anteroposterior (AP) axes (Niazi and Saxena, 1978; Maden, 1983a,b). The blastemata of both urodeles and anurans are proximalized after exposure to retinoids and give rise to serially duplicated structures in a dose-dependent manner. In addition, RA affects the anteroposterior (AP) axis of the anuran tadpole limb, where it induces mirror image duplications. Similar AP effects are not generally observed in the urodele where the action of RA along the AP axis is only revealed after surgical manipulation (Stocum and Thoms, 1984; Kim and Stocum, 1986a,b). The blastema of an RA treated anterior half limb will replace the missing posterior structures of the hand as well as generate serial duplications of the stump tissue along the PD axis. Furthermore, RA treated surgically constructed double anterior limb will produce twin mirror image regenerates with proximalized stump segments. RA is also capable of altering pattern along the dorsoventral axis in the urodele where it ventralizes both half and double dorsal limbs (Ludolph *et al.*, 1990). Positional respecification by RA is an unusual activity, yet there are several contexts in vertebrate development where it may act in this way.

In addition to its effect on positional identity, RA exerts a number of other interesting effects on the regenerating limb; three of these will be mentioned here (Maden, 1983a). First, the mitotic index in an RA-treated blastema is significantly decreased, a finding that is consistent with the general anti-mitotic effect on cells in a variety of contexts. Second, the extent of mesenchymal de-differentiation is apparently increased as assayed by histological analysis and acid phosphatase activity. Last, the wound epidermis of an RA-treated blastema shows a stimulation of mucopolysaccharide synthesis, and the precocious expression of an antigen called WE3 which is a marker of secretory differentiation (Goldhamer *et al.*, 1989; Tassava, 1992). This is reminiscent of the classical effects of RA in promoting the differentiation of secretory epithelia, and switching that of keratinizing epithelia to the secretory pathway (mucous metaplasia) (Darmon, 1991).

The actions of RA on the regenerating limb raise an important general question – to what extent are the effects indicative of a corresponding endogenous activity during regeneration? Do the normal controls exerted over cell division, de-differentiation, and development of the secretory phenotype of the wound epidermis include the local action of RA? Does the positional identity of the blastema reflect to any extent the graded action of endogenous RA? While acknowledging that these questions cannot be decisively answered at present, the aim of this review is to consider the available evidence. We begin by a brief consideration of the relation between exogenous and endogenous activities in the context of vertebrate development in general, and limb development in particular.

Abbreviations used in this paper: RA, retinoic acid; AP, anteroposterior; RAR, retinoic acid receptor; T3R, thyroid hormone receptor; RARE, retinoic acid response element; CAT*, normalized level of chloramphenicol acetyltransferase.

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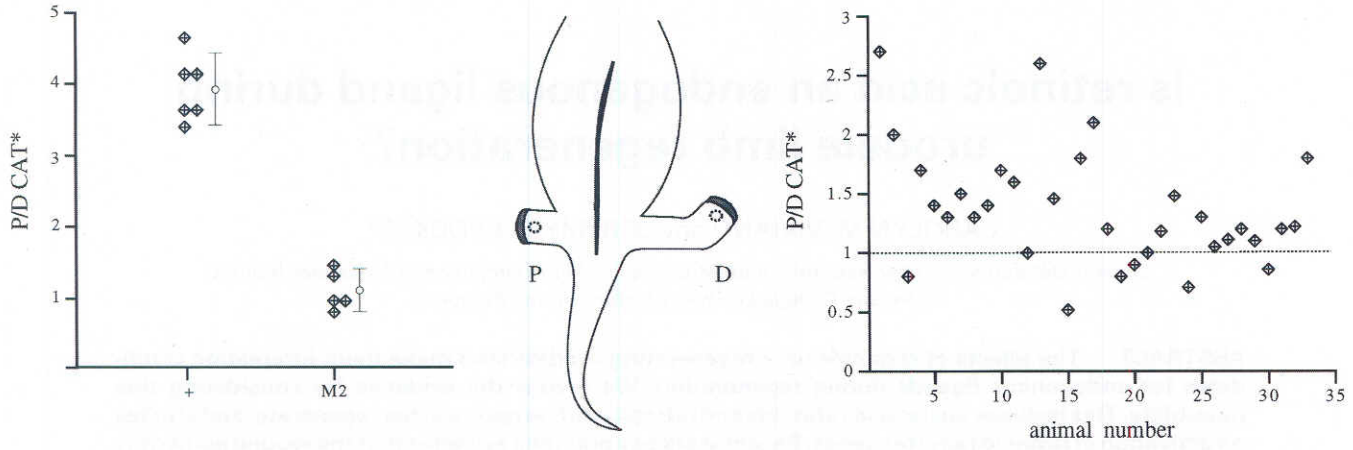

Fig. 1. A comparison of *in vivo* and *in vitro* reporter stimulation in proximal (P) versus distal (D) blastemas. (Middle) *Diagram of a newt with both proximal and distal hindlimb amputations. In in vivo experiments: a pellet of cells transfected with a RA-responsive reporter gene was implanted subjacent to the wound epidermis in both legs (broken circles). After 5 days, each blastema was assayed and CAT activity of P versus D implants from the same animal were compared* **(left).** *Each diamond represents a single animal which was implanted with cells containing either the RA-responsive reporter gene (+) or the control (M2) (see Brockes, 1992 for discussion). In in vitro experiments, wound epidermis explants from contralateral proximal and distal blastemas were removed and co-cultured with cells carrying a RA-responsive reporter gene. After 2 days, the cultures were assayed and CAT activity P versus D co-cultures were compared* **(right).** *Each diamond represents the P:D ratio of CAT activity from contralateral limbs of a single animal (n=33). This ratio was significantly greater than one (dotted line), P<0.01 (Viviano et al., 1995).*

Retinoids and mammalian development

Retinoids are essential for normal mammalian development as revealed initially by the multiple effects of maternal vitamin A deficiency. These include effects on eye development, cleft palate, cardiovascular, limb and urogenital development. The recent analysis of retinoic acid receptor (RAR) null mice, has confirmed and extended the earlier observations, leaving no doubt that RA is the active metabolite in the developmental roles of vitamin A (Lohnes *et al.*, 1993, 1994; Mendelsohn *et al.*, 1994). There is, in addition, an extensive literature on the teratogenic effects of applying exogenous RA during development, for example in relation to craniofacial abnormalities, neural crest, limb and vertebral column formation (for review see Morriss-Kay, 1993). At present one cannot necessarily conclude from a particular teratogenic phenotype that RA is normally involved in that aspect of development, although this is apparently true in many cases. If the teratogenic effects are mediated by receptors, the presence of receptors does not guarantee a role for the ligand during development. Furthermore there may well be tissues which are protected from prevailing concentrations of retinoids during development, and which give a teratogenic response when such protective mechanisms are exceeded.

A second uncertainty in considering the developmental role is the possibility of local sources of RA. There are clearly many tissues able to take up circulating retinol and convert it to RA, which then plays a role in that tissue. Whether there are contexts where local autocrine or paracrine interactions operate is much less clear. In order to address this question, it is necessary to demonstrate that RA or other retinoids are not only synthesized but also released by tissue sources, and that the retinoids after release are competent to stimulate RA reporter gene expression. Evidence along these lines has been presented for the notochord, floorplate and Hensen's node, although direct analysis of products or their active release by these tissues has not yet been reported (Wagner *et al.*, 1990, 1992; Hogan *et al.*, 1992). The potential presence of retinoid signaling centers has figured prominently in hypotheses about limb development, a subject that exemplifies the uncertainties surrounding the role of exogenous RA.

Limb development and RA

Local implantation of a source of RA in the anterior margin of the chick limb bud evokes mirror image duplications of the anteroposterior axis thus mimicking the activity of the polarizing region (ZPA) (Tickle *et al.*, 1982). These results gave rise to the hypothesis that RA might be a morphogen produced by the ZPA. Subsequently, RA and retinol were detected in the chick limb bud at the relevant stages and the former molecule was found to be present at 2.5 fold higher concentration in posterior mesoderm, reinforcing the morphogen hypothesis and leading to the suggestion of an instructive gradient across the AP axis (Thaller and Eichele, 1987). This idea was brought into question when it was shown that RA induces polarizing activity in cells at the anterior margin of the chick limb bud (Noji *et al.*, 1991; Wanek *et al.*, 1991). These results led to the re-evaluation of the morphogen hypothesis in favor of the idea that a local source of RA induces the ZPA, which in turn determines the AP axis. A recent repetition of the earlier analysis of retinoid levels in the chick bud has found a smaller difference, 1.4 fold, and also favors the interpretation of a peripheral location for RA rather than an AP gradient across the limb bud (Scott *et al.*, 1994). The functional status of endogenous RA in the bud has also been questioned because of the lack of activity of an endogenous RA responsive gene after grafting of the polarizing region (Noji *et al.*, 1991), although it has been pointed out that the interpretation of this result is not clear cut (Tickle and Eichele, 1994). Finally the current interpretation of the local implantation experiments favors the view that RA induces a polarizing region by turning on the expression of

the *hedgehog* gene (Riddle *et al.*, 1993). *Hedgehog*, which is expressed by the polarizing region on the posterior margin, is thought to act on and in conjunction with a FGF signal from the apical ectodermal ridge to alter positional identity on the AP axis (reviewed in (Tickle and Eichele, 1994). Such a view would "relegate" RA to a possible role in inducing the polarizing region in the limb bud or flank mesenchyme rather than a subsequent instructive role in limb patterning. The phenotypes from those RAR knockout mice where limb development is altered are not obviously consistent with such an inducing role, and on the other hand it remains possible that RA is important for later events of limb morphogenesis. These issues should be clarified in future research.

Presence of RARs and retinoids in the urodele blastema

The effects of RA are thought to be mediated by two classes of nuclear receptors, the RARs and RXRs. RARs apparently act as heterodimers with RXRs (for review see Leid *et al.*, 1992). Ligands which bind to RARs and not to RXRs are capable of stimulating RAR/RXR heterodimers to activate gene expression at the appropriate RA response elements (RAREs). The benzoic acid retinoid derivative TTNPB is a potent RAR agonist, is largely inactive on RXRs, and yet is active at respecifying the urodele blastema (Kim and Stocum, 1986c). Therefore it seemed likely that the RARs would be expressed in the newt blastema. The five species that have been identified in the newt are termed α1, α2, δ1A, δ1B and δ2 (Ragsdale *et al.*, 1989, 1993). While the δ1 species are clearly the newt counterpart of γ1 in other vertebrates, the sequence of the N terminal A region has diverged significantly between δ1 and γ1 (see discussion in Ragsdale *et al.*, 1993). The δ1 RARs are the major receptors in the newt limb and blastema and are expressed at a sufficiently high level to be detected by isoform-specific antibodies (Hill *et al.*, 1993). The receptors are expressed in both epithelial and mesenchymal compartments of the limb, and it is possible, but not established, that individual blastemal cells express the five RARs.

It might have been argued that the presence of the response machinery is strong evidence for an endogenous role of RA, but the experience from RAR negative mice cautions against too strong a viewpoint. Our emphasis therefore, has been on assessing their role in the different responses to RA that were discussed earlier. The five RARs have been converted to RA/thyroid hormone (T3) chimeras by replacing the RA binding E and F regions with the EF regions from the *Xenopus* T3R-α (Schilthuis *et al.*, 1993). When a plasmid encoding a chimaeric receptor is transfected into newt limb cells, the equivalent of a single RAR can be activated by T3. When limb blastemal cells are exposed to RA in culture the growth rate decreases as described earlier for the blastema *in situ*, but T3 has no effect. If the RARα1/TR chimera (referred to as $\chi\alpha$1) is transfected into the cultured cells, T3 is now able to mimic quantitatively the effect of RA (Schilthuis *et al.*, 1993). The other four chimeras show little or no activity in this assay and in particular $\chi\alpha$2 is inactive.

These results implicate RARα1 as mediating the effect on cell growth, while comparable experiments have implicated RARδ1 in mediating the effect on secretory differentiation in the wound epidermis (Pecorino *et al.*, 1994). It may be possible to use the chimaeric receptor approach to determine whether the positional identity of transfected mesenchymal cells can be respecified with T3, and if so which RARs mediate the effect. Such a result would indicate that the mechanism of action on the blastema was different from that on the limb bud. While these experiments do not provide direct evidence for an endogenous role of RA, they would nonetheless be informative in the context of such a role.

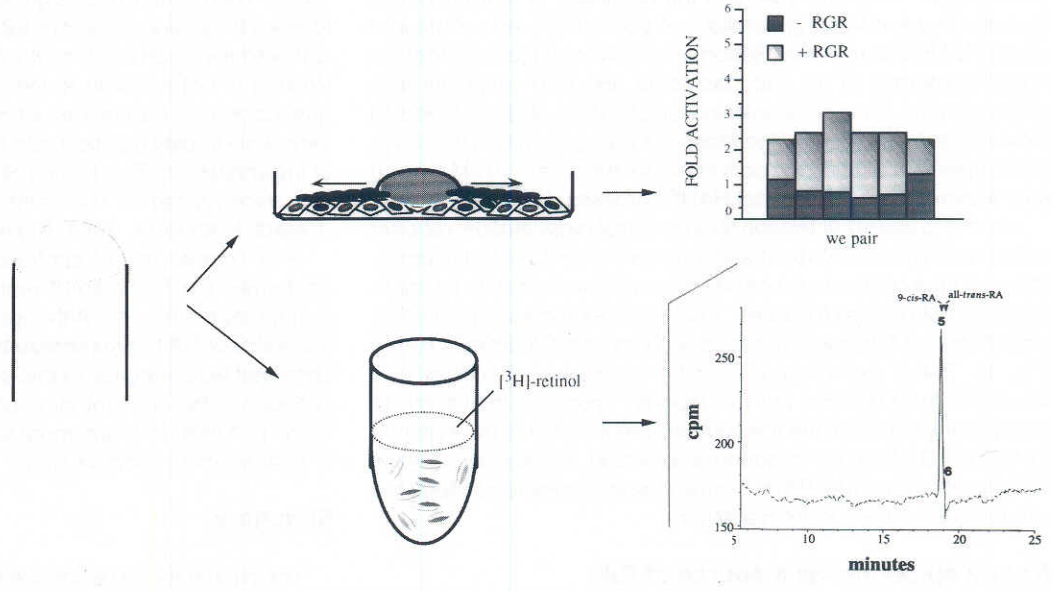

Fig. 2. Diagram of the *in vitro* analyses used to confirm the wound epidermis as a source of RA. (Left) *Schematic of the newt blastema 6 days after amputation. The gray line represents the wound epidermis. top left, reporter experiments: the wound epidermis is co-cultured with blastemal cells that have been transfected with a RA-responsive CAT reporter gene, and the cultures are assayed after 2 days.* **(Top right)** *Results from a typical experiment. The y-axis represents relative activation of the CAT reporter gene. Each bar on the x-axis represents the results from experimental (light gray) and control (dark gray) cultures which were done using wound epidermis tissue from the contralateral limbs of a single animal; the results have been superimposed for ease of comparison. bottom left, metabolic studies: several wound epidermis explants are cultured together in medium containing [^{3}H]-labeled retinol. After 24 h both tissue and media samples were analyzed by HPLC.* **(Bottom right)** *A typical chromatogram illustrating the metabolic conversion of [^{3}H]-labeled all-trans-retinol into [^{3}H]-labeled 9-cis retinoic acid by wound epidermis tissue. Retention times of internal standards are indicated by arrows. Profiles were reproducible and comparable for both media and tissue samples (see Viviano et al., 1995 for discussion).*

Direct evidence for the presence of retinoids in the axolotl blastema

The presence of several different RARs in the urodele blastema is the first piece of circumstantial evidence supporting an endogenous role for RA during limb regeneration. A second criteria that must be met is that RA should be present in the normal blastema. HPLC analysis of tissue extracts from the axolotl has shown retinoids to be present in significant quantities in both the epidermal and mesodermal compartments (Scadding and Maden, 1994). In addition the concentration of RA was reported as five times higher in the posterior versus anterior compartments and 2.5 times higher in distal versus proximal compartments. These results may seem confusing when considered together with the ability of exogenous RA to proximalize the blastema and when compared to the approximately 3-fold greater stimulation of a reporter gene proximally than distally (as discussed below). It is possible that direct biochemical measurements of total retinoid content are not an accurate reflection of the concentration of retinoid experienced by a cell or levels significant for effects on gene expression. Nevertheless the presence of retinoids in the blastema is an important observation in the context of establishing an endogenous role for retinoic acid during limb regeneration.

Reporter gene activation in the blastema

The analytical data show that retinoids and RA are present in the wound epidermis and mesenchymal blastema, but it is not clear if they are in a form that is competent to activate gene expression. Some or all may be complexed with proteins or other components such that the nuclear receptors are inaccessible. For this reason it is essential to complement chemical analysis with assay by reporter gene activation. In initial experiments to investigate reporter gene activation in the blastema, cultured blastemal cells were transfected with a RA-responsive reporter gene (Brockes, 1992). The reporter had a RARE in its promoter and the specificity of activation was controlled by transfecting parallel cell populations with a reporter whose RARE was mutated. Reporter cells were implanted under the wound epidermis of an early blastema and their response was evaluated after intraperitoneal injection of a dose of RA sufficient to proximalize the blastema. The response was generally in the range of that given by culturing the cells in the presence of 1-10 nM RA and was dependent on a functional RARE (Brockes, 1992).

In order to assess activation during normal regeneration, reporter cells were implanted into contralateral proximal and distal blastemas, allowing a the comparison of axial dependent activation to be made within a single animal (Brockes, 1992). This experiment gave a P/D ratio of about 3.5-fold which was dependent on the functional RARE (Fig. 1). These experiments raise the possibility of endogenous retinoid in the blastema but the reporter approach is indirect. In principle any aspect of the transduction pathway could be responsible for the PD difference in reporter expression, for example a kinase that phosphorylated the RARs or an accessory protein that interacted with the basic transcription machinery.

Wound epidermis as a source of RA

The wound epidermis of the limb blastema has the ultrastructural, biochemical and antigenic properties of a secretory epithelium (Singer and Salpeter, 1961; Chapron, 1974; Ferretti *et al.,* 1989; Goldhamer *et al.,* 1989). Since this is a characteristic target tissue of RA, it seemed possible that it might also act as a source of retinoid in the blastema. In order to investigate this possibility we established the newt wound epidermis (5-6 days after amputation) as an explant in culture, essentially free from mesenchymal contamination (Viviano *et al.,* 1995). Under these conditions the keratinocytes migrate rapidly from the edge of the explant, and retain expression and RA-inducibility of the marker antigens WE6 and WE3 described by Tassava and colleagues (Tassava *et al.,* 1986; Tassava, 1992). When the explant is co-cultured over 48 hours with blastemal cells transfected with RA-reporter plasmids, the reporter is reproducibly activated in a range corresponding to 0.1-1 nM RA (Fig. 2). Each wound epidermis was assessed relative to its contralateral partner which was confronted with reporter cells carrying mutated plasmids. The stimulation observed in the co-cultures was substantially independent of cell contact since media conditioned by the wound epidermis activated the reporter cells (Viviano *et al.,* 1995).

The approach of reporter activation has been complemented by direct analysis of retinoids in tissue and medium after incubation of the isolated wound epidermis with tritiated all-*trans* retinol (Fig. 2). Radioactivity was detected in didehydroretinol and 9-*cis* RA after HPLC of tissue extracts. Although radioactive all-*trans* RA was detected the principle acidic metabolite was 9-*cis* RA. Samples of medium were analyzed after incubation with tissue, as well as after pulsing the wound epidermis with radioactive retinol and chasing with cold retinol. The medium contained radioactive retinol, 9-*cis* RA and other unidentified metabolites. The fractional release of cell-associated radioactivity was significant since over 50% of the cytosolic pool was released into the medium over 24 hours (Viviano *et al.,* 1995). In summary, the wound epidermis actively synthesizes and releases RA but the primary acidic metabolite is the 9-*cis* rather than the all trans isomer. 9-*cis* is the distinctive natural ligand for the RXRs although it also activates RARs effectively (Leid *et al.,* 1992). There is currently significant interest in the ability of RXRs to give a 9-*cis* dependent stimulation of transcription as heterodimers with various "orphan" receptors (Perlmann and Jansson, 1995). While it is not possible to identify a particular functional consequence of 9-*cis* (rather than all-*trans*) for the wound epidermis, it is very likely to play a critical role in differentiation and maintenance of the epithelium. This finding aligns newts with *Xenopus* embryos and mice as recent examples where 9-*cis* has been detected (Creech Kraft *et al.,* 1994; Kojima *et al.,* 1994).

In a comparison of contralateral proximal and distal wound epidermis, the P/D ratio of reporter stimulation was significantly greater than 1 (Fig. 1). Although these results are consistent with the ability of RA to proximalize the regenerate, the P/D difference observed was marginal in this system as it is rather insensitive to changes in the concentration of RA at the low end of its working range (0.1 nM). In future, more sensitive methods must be brought to bear on this important issue.

Summary

The circumstantial evidence in favor of a role for RA in urodele limb regeneration is quite strong. The combination of reporter assays and direct chemical analysis has provided evidence that retinoids are present in amounts that are sufficient to activate

nuclear receptors and stimulate gene expression. Nonetheless vertebrate developmental biology provides examples, such as mesodermal induction in *Xenopus*, where circumstantial evidence in favor of a particular molecule has been shown to be inadequate. Clearly it remains to investigate the consequences for regeneration of blocking RA synthesis, or inhibiting the activity of its receptors by dominant negative constructs or specific antagonists.

On the other hand, we find the evidence that the wound epidermis is a local source to be quite compelling. In fact the evidence seems stronger in this respect than for any other tissues examined to date. In comparing the wound epidermis with the other candidates, that is the notochord, floor plate and Hensen's node, there are similarities and differences (Fig. 3). For example, the notochord, Hensen's node and the wound epidermis are all transient structures, although the floor plate is not. We note that the wound epidermis, however, is an extremity and apposes a tissue, the mesenchymal blastema, which is known to be sensitive to the action of RA. It remains to be seen if this apposition is significant in respect of the release of RA by the wound epidermis, but this tissue may nonetheless be a good model for studying the cellular and molecular mechanisms underlying such activity.

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