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Some Current Problems in Amphibian Limb Regeneration

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Some current problems in amphibian limb regeneration

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SUMMARY

Limb regeneration in adult urodele amphibians proceeds by formation of a blastema at the amputation plane. This paper discusses how the blastema forms, and how its positional identity on the proximodistal axis is manifest. Retinoic acid is able to reset axial specification and there is particular interest in determining how it acts. Although limb regeneration is restricted among vertebrates to the urodeles, its mechanism poses fundamental questions in development biology.

INTRODUCTION

The urodele amphibians are the only adult vertebrates able to regenerate their limbs. Limb regeneration proceeds by formation of a growth zone or blastema at the plane of amputation, and is therefore an example of epimorphic regeneration (Morgan 1901). The blastema gives rise to the structures lost on amputation, a process that reconstructs the complex mesenchymal architecture of the vertebrate limb (reviewed in Wallace (1981)). The aim of much research on neural regeneration is to achieve restoration of neuronal connectivity, either by promoting regrowth of severed axons or by transplanting an exogenous source of neurons. It is not yet a feasible possibility to consider regenerating a cerebellum in the way that a urodele is able to regenerate a limb. Thus although in one sense the fields of nerve and limb regeneration are quite different, there are nonetheless some similarities. For example, in both cases the system has to solve the problems of 'staging' developmental events in the context of an adult tissue.

After amputation of a urodele limb at any level on the proximodistal (PD) axis from shoulder to fingertips, the wound surface is rapidly covered by migrating epidermal cells which form the wound epidermis. The blastemal cells arise underneath the wound epidermis within 200–500 μm of the amputation plane. They proliferate under the influence of the nerve supply and the wound epidermis, both of which seem to be necessary for this phase of blastemal development (Wallace 1981). After two to three weeks, the blastemal cells differentiate in a proximal to distal direction to form the cartilage, muscle and connective tissue of the regenerate. The blastema has considerable morphogenetic autonomy, and will give rise to a limb if transplanted to a neutral location in the animal where it can be vascularised and innervated (Stocum 1984). The limb is reconstructed on a much larger scale in regeneration as compared to development, and it is therefore possible to obtain significantly more material from the blastema than the limb bud (Ferretti & Brockes 1991). One of the major opportunities for molecular studies of limb regeneration is to investigate the properties of blastemal progenitors at a stage before the appearance of overt differentiation. In this account I shall focus on the origin of the blastema and the specification of position on the PD axis.

ORIGIN OF THE BLASTEMA

The relation of development to regeneration is a central issue for our understanding of the mechanisms involved in adult regeneration. It seems quite unlikely that there are two fundamentally different solutions to the problems of specifying and constructing the pentadactyl limb. None the less, in one case the structure develops from the flank of the embryo, whereas in the other it arises from a stump of adult tissue. The classical histological descriptions of the blastema have emphasized that the differentiated tissues lose their integrity in the vicinity of the amputation plane, and have postulated that cartilage and muscle may dissociate and give rise to blastemal cells by a process of de-differentiation (Chalkey 1954; Hay 1959; Hay & Fishman 1961). On the other hand there is good evidence for a contribution to the blastema from connective tissue fibroblasts, particularly of the dermis which seems to be most important in relation to tissue patterning (Gardiner *et al.* 1986). There is no reason at present to invoke de-differentiation for the transition of this latter population to blastemal cells, yet the term persists as an overall description of the events leading to formation of the blastemal cells.

One contribution to these issues has come from the identification of monoclonal antibodies that react with blastemal cells but not with the differentiated mesenchymal tissue of the limb. The two reagents that

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have proved most useful are 22/18 (Kintner & Brockes 1985), directed against a conformational determinant on intermediate filaments (Ferretti & Brockes 1990), and various antibodies that react with the K8/K18 cytokeratin pair (Ferretti *et al.* 1989). These antibodies show that there are not cells in the normal limb that are identical to blastemal cells, and that some change in properties must occur on amputation (Ferretti & Brockes 1991). The molecular events leading to the formation of blastemal cells are not understood, but they are clearly pivotal in the process of regeneration since they mediate the transition between adult tissue and the essentially embryonic behaviour of the blastema.

A second important contribution of these reagents has been to show that the blastemal cells are not identical to the mesenchymal cells of the developing limb bud (Ferretti & Brockes 1991). Neither 22/18 nor the anti-keratin monoclonal antibodies react detectably with the latter cells (Fekete & Brockes 1987; Ferretti *et al.* 1989). In the case of 22/18 this distinction has been traced to the relation between the limb and the nerve supply (Fekete & Brockes 1988), a relationship that is different for development and regeneration. It is interesting that two different reagents, selected for their ability to distinguish blastemal cells from normal limb mesenchyme, should also distinguish blastemal cells from limb bud cells.

POSITIONAL IDENTITY OF BLASTEMAL CELLS

Limb morphogenesis is one of the key systems in vertebrate developmental biology for understanding positional specification and pattern formation (Brockes 1990). The blastema and adult urodele limb are accessible to a variety of grafting operations and other manipulations. This has given rise to an extensive literature of both experimental and theoretical attempts to understand positional specification of the blastema (French *et al.* 1976; Stocum 1984). The molecular basis of this phenomenon is not understood, and in general remains a major problem of current investigation in limb development and other vertebrate systems. I shall focus on specification along the PD axis, partly because of the experimental opportunities offered by the effects of retinoic acid. It is a fundamental property of a blastema that it only gives rise to structures distal to its point of origin, a property classically referred to as distal transformation (Wallace 1981). Thus a wrist blastema gives rise to a hand, whereas a shoulder blastema gives an entire arm. If a wrist blastema is transplanted to a shoulder level stump then the resulting graft produces a normal arm, but the contribution of the wrist blastema to the regenerate is restricted to the hand (Pescitelli & Stocum 1980). Thus the experimental conjunction of shoulder level stump and wrist blastema mobilises the stump to contribute the regenerate up to the wrist level, a phenomenon called PD intercalation. Intercalation provides a second assay for specification along this axis, and both assays lead to the conclusion that such specification appears to be encoded as some continuous variable.

RETINOIDS AND LIMB REGENERATION

The only molecules that are able to reset this variable are the retinoids and in particular retinoic acid (RA). RA has long been recognized as a requirement for epithelial differentiation and maintenance *in vivo* (Thompson *et al.* 1964), and it effects proliferation and differentiation in a variety of cell culture systems. If a distal limb blastema in an axolotl or newt is exposed to RA or its precursor retinoids, it is 'proximalized' in a dose-dependent fashion (Maden 1982; Thoms & Stocum 1984). The resulting blastema now gives rise to structures that are normal but arise from an inappropriately distal location, an effect sometimes referred to as a serial duplication.

The effect is graded in that within the critical range of RA concentration, the distal blastema is reset to progressively more proximal levels. The effect is exerted on the blastemal mesenchyme but it could involve some participation from the wound epidermis. A treated blastema can be transplanted to a distant location, such as the anterior chamber of the eye, and will give rise to serial duplications.

Such effects are very rare to date in developmental biology, and it has heightened interest that RA exerts a similar effect on the anteroposterior axis of the chick limb bud (Tickle *et al.* 1982). Although there are differences in the effects, which I have reviewed elsewhere (Brockes 1990), there has been great interest in the possibility that RA and other retinoid metabolites might be used as endogenous morphogens. This possibility has gained particular momentum in the chick case because of analytical data indicating that RA is not uniformly distributed across the anteroposterior axis (Thaller & Eichele 1987). In the urodele there is no evidence at present that RA is used as an endogenous morphogen, but it may well play a role in limb regeneration. An equally important focus of current effort is to investigate the mechanism by which RA is able to proximalize the blastema, since this may offer an important clue to understanding the molecular basis of axial specification. Although it has long been recognized that RA is able to affect gene expression, these studies received a major stimulus from the discovery that RA interacts with nuclear receptors of the steroid/thyroid hormone superfamily (Petkovitch *et al.* 1987; Giguere *et al.* 1987).

The nuclear receptors are ligand dependent transcription factors that bind to response elements in the regulatory sequences flanking genes. The RA receptors (RAR) apparently bind to DNA in the absence of RA (de The *et al.* 1990), but only in its presence are they competent to activate the transcription machinery. The activation function is thought to be encoded, at least in part, by the A region at the N terminus of the molecule. Three classes of RAR have been identified in mouse and human, and termed alpha, beta and gamma (reviewed by Ragsdale & Brockes (1990)). They differ most markedly in the A region and C terminal F region, while the DNA binding C region

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and ligand binding E region are relatively conserved. The function of these molecules can be demonstrated by expressing them in cultured cells in the presence of plasmids encoding a reporter gene with an upstream response element. The expression of the reporter is stimulated by RA in the nanomolar concentration range. In the newt limb and limb blastema a homologue of alpha (Ragsdale *et al.* 1989), and probably one of beta (Giguere *et al.* 1989) have been identified from cDNA cloning and shown to be expressed. The major form, however, has been called delta, because it has diverged significantly from gamma which is its closest relative (Ragsdale *et al.* 1989). Delta is apparently present as at least two isoforms because of the substitution of different A regions (Ragsdale & Brockes 1990). The major isoform, delta 1, varies markedly in its expression in different tissues and is present at high levels in the limb blastema. The major challenge is to identify which RAR or combination of RARs are responsible for mediating the morphogenetic effects of RA.

Although RA effects gene expression in many systems, only a few of these have been shown to result from the direct action of the activated RAR. It will clearly be a formidable problem to identify which of the blastemal genes whose expression is affected is causally implicated in proximalization. One approach to this problem is to analyse the effects of RA in relation to expression in a proximal and distal blastema. This is exemplified by the newt type II keratin gene NKII (Ferretti *et al.* 1990). The keratins are excellent markers for the effects of RA on epithelia, but as mentioned earlier a subset of these molecules are expressed by the mesenchymal cells of the blastema (Ferretti *et al.* 1989). When distal blastemas are proximalised with RA, the expression of NKII is strongly decreased as assayed by ribonuclease protection analysis (Ferretti *et al.* 1991). Furthermore, NKII is expressed at higher levels in a distal than a proximal blastema. It is therefore regulated in a consistent way both by position and retinoic acid, and seems an interesting candidate for further study. There will probably be other genes whose expression is higher proximally and is stimulated by RA. The use of the two criteria in conjunction appears to be a more stringent method for selecting relevant genes than either alone.

In assessing the functional role of the RARs and other molecules it seems important to have a system whereby gene expression can be altered in the blastemal cells, and the consequences for morphogenesis observed. One approach to such a system is to establish blastemal cells in dissociated culture. The cells display certain characteristic blastemal cell markers, such as the 22/18 antigen, and can be maintained as dividing populations for long periods (Ferretti & Brockes 1988). It is possible to microinject the cells with plasmids expressing constructions that modify gene expression, and subsequently to introduce these appropriately marked populations back into the regenerating limb. The cells are recruited into the blastema and contribute to the regenerate. The prospects seem quite favourable for using this method to evaluate the role of various genes in limb morphogenesis, and for other issues in limb regeneration such as the origin of the blastema.

In conclusion, I should like to return to the phenomenon of limb regeneration. Although restricted in its occurrence in vertebrates, it remains a basic phenomenon whose mechanisms we must seek to understand. In this brief account I have tried to underline that these mechanisms involve issues of positional specification and the stability of the differentiated state that are of general significance. I think that only when we have a relatively detailed understanding of these mechanisms can we begin to evaluate the possibility of provoking epimorphic regeneration in higher vertebrates.

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