Evidence that the nerve controls molecular identity of progenitor cells for limb regeneration

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Summary

Adult urodele amphibians can regenerate their limbs after amputation by a process that requires the presence of axons at the amputation plane. Paradoxically, if the limb develops in the near absence of nerves (the ‘aneurogenic’ limb) it can subsequently regenerate in a nerve-independent fashion. The growth zone (blastema) of regenerating limbs normally contains progenitor cells whose division is nerve-dependent. A monoclonal antibody that marks these nerve-dependent cells in the normal blastema does not stain the mesenchymal cells of developing limb buds and only stains the amputated limb bud when axons have reached the plane of amputation. This report shows that the blastemal cells of the regenerating aneurogenic limb also fail to react with the antibody in situ. These data suggest that the blastemal cells arising during normal regeneration have been altered by the nerve. This regulation may occur either at the time of amputation (when the antigen is expressed) or during development (when the limb is first innervated).

Key words: aneurogenic limb, limb regeneration, limb development, growth control, blastema, monoclonal antibody, Pleurodeles waltl.

Introduction

The regeneration of limbs in adult urodele amphibians proceeds by the formation of blastemal (progenitor) cells at the amputation plane, followed by their proliferation under the control of peripheral nerves (Todd, 1823; Singer & Craven, 1948; Singer, 1952; Tassava & Olsen, 1986). In limb development, however, there is substantial outgrowth before nerve fibres enter the bud, thus suggesting an alternative control of proliferation to that operating in regeneration. In a previous study we showed that in newts (Pleurodeles waltl and Notophthalmus viridescens) blastemal cells of the regenerating limb can be distinguished from mesenchymal cells of the developing limb bud by virtue of their reactivity with a monoclonal antibody called 22/18 (Fekete & Brockes, 1987a). This reagent was originally selected as specific for blastemal cells versus normal tissue of the limb, and was found to be a good marker for cells whose division is nerve-dependent (Kintner & Brockes, 1985). Its low level of expression in the embryonic limb bud is thus consistent with the preceding considerations.

In further experiments, we amputated the limb bud at various stages of its development, and analysed the resulting blastema for its reactivity with 22/18 (Fekete & Brockes, 1987a). At early stages, the bud regenerated with a blastema that was 22/18-negative, while at later stages amputation evoked the 22/18 response of the adult. The appearance of 22/18-positive blastemal cells was correlated in time with the arrival of axons (and probably accessory cells such as Schwann cells) at the amputation plane, and immunohistological analysis indicated that the first 22/18-positive cells were spatially associated with axons. We hypothesized that the appearance of 22/18 reactivity and hence the molecular identity of the blastemal cells was dependent on the presence of the nerve, although the results were purely circumstantial in supporting such a connection. It is a clear prediction of this hypothesis that if the nerve is prevented from entering the limb bud, then regeneration should proceed with a 22/18-negative blastema.

The preparation of urodele limbs that develop and regenerate in the virtual absence of innervation was described by Yntema (1959) who termed them aneurogenic. Aneurogenic limbs of axolotls develop
normally although their muscle later degenerates due to a failure of trophic support from the nerve (Tweedle et al. 1974; Popiela, 1976). Yntema (1959) showed that the aneurogenic limb will regenerate in the absence of the nerve—-a paradox that has provoked several attempts at explanation (Singer, 1974; reviewed by Wallace, 1981). One possibility is that in the absence of the nerve, the limb cells respond to a growth factor that supports their proliferation but, when the nerve is present, then the synthesis, availability or efficacy of this molecule is reduced, making the cells dependent on the nerve as a future source. Such an explanation makes no explicit predictions about the nature of the blastemal cells arising in the presence and absence of the nerve. In contrast, our earlier work proposes that the blastemal cells arising in the two cases are different (Fekete & Brockes, 1987a, b), and hence that this is a potential point of regulation for the acquisition of nerve dependence. The results reported in the present study demonstrate such a distinction in the blastemal cells.

Materials and methods

Breeding

*P. walti* embryos were obtained either from Xenopus Ltd (UK) or from breeding pairs maintained in the laboratory. In either case, females were induced to spawn by subcutaneous injections of 0.1 mg/ml luteinizing-hormone-releasing hormone ethylamide (Sigma L4513) at 0.1 mg kg\(^{-1}\) body weight. Up to 400 fertile eggs could be obtained from a single female during the breeding season (November-March).

Surgical procedures

Embryos were washed extensively in autoclaved tap water containing 35 μg ml\(^{-1}\) gentamycin sulphate (Sigma) and the gelatinous mass was dissolved by brief immersion in 2% cysteine- HCl, pH 8. Embryos were staged according to Gallien & Durocher (1957). Surgical procedures were similar to those described by Yntema (1959), except that operations of early embryos were performed in 60% L-15 media (Gibco) containing gentamycin sulphate. Embryos were held in place during the operations by placing them into shallow wells that were dug out of polymerized Sylgard (Dupont Corp.). Parabiosis was accomplished by removing a patch of ectoderm from the opposite flank of two embryos at the head process stage (stage 21–22) and pressing the wounds together so that repair occurs across the two animals. Several hours later (stage 24–26) the neural tube and neural crest were removed from one partner from the level of the hindbrain to the 12–14th somite. Tricaine (1:6000) was used as anaesthetic at these and later stages. At stage 28–30, the epibranchial and postotic placodes were removed to minimize development of the lateral line system. After wound healing, embryos were raised in autoclaved tap water.

Although the procedure was performed on 41 pairs of animals, 27 could not be evaluated due to abnormal development, fungal contamination, inadequate histology or a failure of the limbs to regenerate if amputated after the yolk supply was exhausted (in cases where the host larva did not feed). All of these methodological problems can occur in unoperated controls.

Results

In order to have normally innervated blastemas as 22/18-positive controls, we followed the procedure described by Yntema (1959) to create parabiotic pairs of newt embryos (*P. walti*) and then removed neural...
tissue from one of the pair (Fig. 1A). At the time of extirpation, all but the most posterior one to two segments are removed. During subsequent development, the body continues to elongate and the more posterior segments arise and continue to form normally. Consequently, by stage 41, only a relatively small section of neural tissue corresponding to the brachial region is missing from the operated animal. Crest-derived pigment cells can migrate into this initially crest-free area from adjacent regions, making it difficult to assess the true rostrocaudal extent of aneural tissue based on external cues. Nonetheless, in each case pigment cells were sparse and the crest-derived dorsal fin was absent from the operated animal in the region of the forelimb (Fig. 1B).

Previous work (Fekete & Brockes, 1987a) has established that the earliest 22/18-positive response to midbud amputation can be detected at the notch stage of forelimb development (stage 38). At later stages, more nerves entered the bud and the 22/18-positive response to amputation was enhanced. For this reason, it would have been preferable to wait until limb morphogenesis was complete (approximately stage 52) to assess the 22/18-positive response at its maximum. In the present experiments, however, we were limited to stages where the yolk was still abundant because most of the host animals did not feed, and forelimbs amputated after the yolk supply was exhausted failed to regenerate. This result was also obtained with control animals which were denied food after stage 42. Therefore, the successful cases were amputated through the proximal humerus (dashed lines) at the 2-digit stage. Drawing tube tracings of the resulting blastemas were taken through a dissecting microscope at the days indicated.

Although we were primarily interested in early stages of regeneration, two pairs that were feeding well were maintained for several weeks after amputation in order to confirm previous reports (Yntema,
1959; Thornton & Thornton, 1970) that both host and aneurogenic limbs can regenerate. Drawing tube tracings taken at various times before and after amputation show that the host and aneurogenic limbs develop and regenerate at similar rates (Fig. 2). The upper pair of Fig. 2 was processed histologically 25 days after amputation, sectioned and examined for the presence of nerves by immunofluorescent localization of antibody directed against neurofilament protein. The 'aneurogenic limb' was typical in that a few small-calibre axons were evident in the limb, but in numbers much reduced compared to the host side. The lower pair died following fungal contamination and was not examined histologically.

Because 22/18 immunoreactivity is primarily associated with the first two weeks of regeneration, most of the limbs were examined when the blastemas were at early stages. Several criteria were used to assess the extent of blastema formation. Gross morphological evidence of limb outgrowth following amputation was obtained with drawing tube tracings. In addition, at the 2-digit stage muscle differentiation is well underway at the level of the proximal humerus in unamputated limbs, as detected by immunostaining using a muscle-specific monoclonal antibody called 12/101 (Kintner & Brockes, 1984). Following amputation, muscle tissue is lost for some distance proximal to the plane of amputation, and this 12/101-negative zone is operationally defined as the blastema. Examination of sections using Nomarski differential interference contrast confirmed the absence of myofibrils. Note that the degeneration of muscle observed in long-term aneurogenic limbs does not occur until a later stage (Popiela, 1976), and should not interfere with this analysis. By these criteria, early blastemas were evident on both host and aneurogenic sides in 12 cases that were compared for 22/18 immunoreactivity. There was no systematic difference in the sizes of the two blastemas.

The majority of cases (n = 8) showed a clear difference in the 22/18 immunoreactivity of aneurogenic blastemas in comparison to the host side. The aneurogenic blastemas showed either no 22/18 response (n = 4) or very weak staining (n = 4) that approached the low levels observed in some unamputated limbs. An example is shown in Fig. 3. This example illustrates that at these stages strong 22/18 immunoreactivity of innervated blastemas is primarily associated with cell processes and does not involve all of the blastemal cells (see also Fekete & Brockes, 1987a). The fainter fluorescence seen in the aneurogenic limb of this case is not associated with blastemal cells. Rather, in Fig. 3E,F and J it is the acellular shell of the developing humerus that is detectable by immunofluorescence (arrowheads). This staining pattern is presumed to be an artifact as it was observed in both amputated and unamputated limbs with one particular batch of ascites only. Also, slight immunoreactivity is associated with the epidermis of these developing limbs, a feature previously shown to be unrelated to either amputation or regeneration (Fekete & Brockes, 1987a).

Adjacent sections were stained with antiserum directed against the low molecular weight subunit of neurofilament protein (NF-70) to assess the success of neural tube removal and the extent of innervation of 'aneurogenic' limbs. In seven of these cases, the 'aneurogenic' limb was found to be sparsely innervated, with 5-20 small-calibre nerve fibres discernible by immunostaining. In one 22/18-negative 'aneurogenic' blastema, approximately 30 nerve fibres were visible in cross sections through the limb. The axons were mostly confined to the proximal stump and the epidermis, and usually originated from more rostral areas of the operated partner although some sprouting of host spinal nerves is occasionally observed. It should be emphasized that at this stage the axons of host limbs are too numerous to count precisely using immunofluorescence, particularly as they tend to travel in bundles through the core of the limb.
Nonetheless, we estimate that the average number of axons in the host limb exceeds the number in sparsely- innervated limbs by approximately a factor of ten, in agreement with previous estimates using silver stains of limbs from slightly later stages in axolotl (Edgar et al. 1973).

In two cases, innervation of the 'aneurogenic' limb approached normal levels, and likewise the 22/18 immunoreactivity of the blastemas was similar to the hosts. These cases indicate that the absence of 22/18 expression in other operated larvae is not due simply to the trauma of surgery, but appears to be related to the extent to which nerves have been reduced or eliminated. Two cases where both host and aneurogenic limbs failed to express 22/18 in response to amputation were difficult to interpret due to the lack of internal positive controls.

Discussion

Urodele limbs can develop, differentiate and regenerate in the near absence of innervation after surgical removal of the embryonic spinal cord (Yntema, 1959). Once nerves are allowed to enter the aneurogenic limb (by homotypic transplant of the limb to a normal host), then further regeneration becomes nerve-dependent (Thornton & Thornton, 1970). That is, exposure to nerves, even at much later stages than normal, imposes nerve-dependence on regeneration.

This study provides the first demonstration of a molecular difference between the blastemal cells of aneurogenic and normal limbs. The aneurogenic limb resembles the preinnervated limb bud (Fekete & Brockes, 1987a) in that it responds to amputation by forming a 22/18-negative blastema that can regenerate to form the missing parts of the limb. Like the normal larval bud (Butler & Schotte, 1941), the aneurogenic limb acquires nerve dependence once it is innervated (Thornton & Thornton, 1970), and we predict that such a limb would respond to amputation by forming a 22/18-positive blastema (Brockes, 1984; Fekete & Brockes, 1987b). It is not clear if the change from nerve-independent to nerve-dependent growth control occurs at the time of innervation (Singer, 1974), or whether something occurs at the time of amputation in the presence of the nervous system to change the responsiveness of the blastemal cells. The 22/18 antibody has identified at least one change that occurs at the time of amputation in the presence but not the absence of nerves.

Although the antigen recognized by 22/18 has not been fully characterized, it appears to be a cytoskeletal component that may be intermediate filament-associated based on overlapping immunofluorescent staining patterns of 22/18 and anti-intermediate filament antibody in cultured newt cells (Fekete et al. 1988). On Western blots of a cytoskeletal fraction from the newt blastema, the antibody reacts with two bands with relative molecular masses of 42000 and 51000 (P. Ferretti, personal communication). It will be interesting to determine if the change in the cytoskeleton detected by 22/18 is a cause or a consequence of the difference in growth control.

It is worthwhile to consider at a cellular level how nerves might control the appearance of 22/18 in the blastema. Previous hypotheses concerning the mechanism of acquisition of nerve-dependent proliferation have mostly stressed the role of axons (Singer, 1974; reviewed by Wallace, 1981). They have proposed a long-term effect of axons either in suppressing the production of putative growth factor(s) derived from non-neural sources (such as the limb cells themselves) or in altering the limb cells so that they can only respond to nerve-derived growth factors. Our results support the latter hypothesis but do not rule out a further regulation of growth factor availability. Ingrowing nerves alter the cellular composition of the limb not only by the invasion of axons, but also by the addition of Schwann cells. We propose that the establishment of nerve-dependent regeneration may involve an interaction between axons, Schwann cells and precursors of blastemal cells that occurs at the time of amputation (Brockes, 1984). Schwann cells are known to express 22/18 in response to injury or amputation (Kintner & Brockes, 1985; Gordon & Brockes, 1988) and to contribute some cells to the blastema (Maden, 1977; Kintner & Brockes, 1985). In addition, rat Schwann cells have specific growth factor requirements in vitro that can be met by an activity present in innervated but not denervated blastemas of newts (Brockes & Kintner, 1986). We plan to test the specific role of Schwann cells in controlling the formation of a 22/18-positive nerve-dependent blastema by amputating limbs that have axons but lack Schwann cells due to early neural crest removal (Harrison, 1924). The mechanism by which one system of growth control supplants another is likely to be of general significance in development, and merits further investigation.

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References


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