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Stability of positional identity of axolotl blastema cells in vitro

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Summary. Previous grafting experiments have demonstrated that cells from non-contiguous positions within developing and regenerating limbs differ in a property referred to as positional identity. The goal of this study was to determine how long the positional identity of axolotl limb blastema cells is stable during culture in vitro. We have developed an assay for posterior positional properties such that blastema cells can be cultured and then grafted into anterior positions in host blastemas, to determine if they can stimulate supernumerary digit formation. We report that posterior blastema cells are able to maintain their positional identities for at least a week in culture. In addition, we observed that blastema cells are able to rapidly degrade collagenous substrates in vitro, a property that apparently distinguishes them from limb cells of other vertebrates. These results provide information regarding the time boundaries within which the positional properties of blastema cells can be studied and manipulated in vitro.

Key words: Axolotl – Cell culture – Regeneration – Positional information

Introduction

Developing and regenerating vertebrate limbs are useful experimental systems for studying pattern formation because of the informative ways in which these structures respond to experimental manipulation. A characteristic regulative response of both developing and regenerating limbs is their ability to generate extra structures through interactions between cells with different positional information (Bryant et al. 1981; 1987). For example, grafts of limb buds or blastemas that confront cells from anterior and posterior positions result in the stimulation of growth and the intercalation of cells with positional values that are intermediate between the anterior and

posterior boundaries (Muneoka and Bryant 1984). This ability to undergo pattern regulation in response to positional confrontations is particularly evident in the urodele amphibians in which pattern regulation occurs along all three limb axes (anterior-posterior, dorsal-ventral, and proximal-distal). In addition, urodeles are capable of redeveloping (regenerating) their limbs after the period of initial limb development has passed. Thus, urodele limbs represent a unique opportunity to study the cellular interactions involved in limb pattern formation, since their limb cells can reinitiate pattern formation at any time during the life of the organism.

Studies at both the cellular and tissue levels have provided abundant evidence indicating that interactions between cells with different positional values are critical for limb outgrowth and pattern formation. At present, the molecular basis of positional information and pattern formation in limbs is beginning to be uncovered. For example, retinoids have dramatic effects on limb pattern formation (Tickle et al. 1982), and their various nuclear and cytoplasmic receptors are being actively studied; TGF- β affects the pattern of specific skeletal elements in the developing chick limb (Hayamizu et al. 1991); and a variety of homeobox-containing genes appear to be involved in the specification and regulation of limb pattern (Dollé et al. 1989; Izpisua-Belmonte et al. 1991; Nohno et al. 1991; Yokouchi et al. 1991). In order to be able to assess the possible function(s) of candidate pattern formation genes, it will be necessary to experimentally alter the expression of such genes so as to observe effects on limb pattern formation. One approach to this in amphibians will be through the use of cultures of pattern formation-competent cells for transfection or other genetic manipulation, followed by a functional test of their ability to participate in pattern formation.

A first step in such an investigation is the establishment of an appropriate culture and assay system for pattern formation-competent cells. In this paper we report the development of techniques for dissociating axolotl blastemal tissue so as to obtain viable, pattern for-

mation-competent cells that can be used in a functional assay for cells with posterior positional information. Previously, primary cultures of blastema cells have been established from cells that migrate out of explants or minced blastemas rather than by dissociation of blastemal tissue (Conn et al. 1979; Jabaily et al. 1982; Ferretti and Brockes 1988). This approach, while useful for some types of studies, is not particularly well suited to the study of positional stability because of the long periods of time required to obtain reasonably large numbers of cells, and the variability in both cell number and viability associated with explant cultures. The assay we describe involves the grafting of dissociated and cultured cells into an anterior location beneath the apical cap of a medium bud blastema. Grafts of cells with anterior positional properties have no effect on the pattern of the regenerate, whereas grafts of posterior cells result in the formation of supernumerary skeletal elements. Using these procedures, we have been able to document the stability of posterior positional identity in axolotl blastemal cells that have been cultured for varying periods of time.

Materials and methods

Preparation of donor and host blastemas. All experiments were performed on axolotls (*Ambystoma mexicanum*) spawned at UCI and measuring between 9 and 20 cms, snout to tail tip. To generate both donor and host blastemas, animals were anesthetized in a 0.1% solution of MS222 (Sigma) prior to surgery, and both forelimbs were amputated through the mid-humeral region. The amputation surface was trimmed flat. The regenerates were staged using the system described by Tank et al. (1976).

Grafting of blastema tissue fragments. All tissues were obtained from medium bud blastemas. The donor limbs were marked at the anterior margin with subcutaneous implants of Nile red crystals. The limbs were removed from the animal and the mature skin (dermis plus epidermis) along with the apical wound epidermis was removed with watchmaker forceps. The mesenchymal portion of the blastema was divided into three equally sized pieces along the anterior-posterior axis of the blastema with iridectomy scissors. The anterior and posterior thirds were removed to separate dishes of culture medium for subsequent grafting, whereas the middle third was discarded. Prior to grafting, the thirds were lightly stained with a dilute solution of Nile blue (1:1800 in 60% L15 culture medium) for visibility, and cut into smaller pieces (size and number as reported in the Results) with iridectomy scissors.

The host site was prepared by making a small cut with iridectomy scissors through the skin (dermis and epidermis) just proximal to the base of the host blastema. The stage of the host blastema was varied as described in the Results. A tunnel was made distal to the cut, between the wound epidermis and the blastema mesenchyme, using a blunt-ended glass probe. The pieces of graft tissue were inserted into the tunnel with a small-bore pipette, and the tunnel opening was packed with Gelfoam (Upjohn) to keep the grafts in place.

Camera lucida drawings were made of the position of the grafted fragments within the host blastema, and the animals were then transferred to individual plastic boxes and examined 24 h later for graft retention. Animals in which the graft had not been retained were not analyzed further. The animals were fed and changed three times a week until regeneration was complete (approximately one month from the time of grafting). The limbs were then removed, preserved in Bouin's fixative and stained with Victoria blue for analysis of skeletal patterns (Bryant and Iten 1974).

Dissociation and culture of blastemal cells. Medium bud donor limbs were marked along their anterior margins and were removed from

the animal as described above. Tissues were sterilized in a 1% sodium hypochlorite solution for 90 sec prior to removal of the epidermis. Anterior and posterior thirds of the blastema mesenchymal tissue were collected as described above and rinsed in several changes of sterilizing medium (60% L15 with 1 mM glutamine, 50 µg/ml gentamycin sulfate, 200 U/ml penicillin, 200 µg/ml streptomycin sulfate and 0.5 µg/ml amphotericin B as fungizone (Gibco)). They were then treated sequentially with collagenase (0.2% collagenase (Sigma) in 60% L15 for 20 min at 4° C and then one hour at 26° C) and trypsin (0.05 trypsin, 0.53 mM EDTA in Ca++/Mg++ free Hanks (Gibco) for 20 min at 4° C and 40 min at 26° C). Fetal bovine serum (FBS) was added to 20% to neutralize the trypsin, the tissue pieces were gently pelleted by centrifugation, and resuspended in 1 ml of culture medium (60% L15 with 1 mM glutamine, 50 µg/ml gentamycin sulfate, and 5% FBS). The fragments were repeatedly pipetted to dissociate them and the resulting cell suspension was filtered through a double layer of Nitex 70 µm mesh. Cell counts and trypan blue viability were determined using a hemacytometer. The cells were then spun into a pellet (5 min at 250 × g) and resuspended to micromass density (1.6×10^7 viable cells/ml) in a fibrinogen solution (Sigma, 0.3% in L15 sterilizing medium).

Blastema cells were cultured and grafted as fibrin clots that were generated by adding the cell/fibrinogen mixture in 2.5 µl aliquots (approximately 4×10^4 cells) on top of an equal volume of reconstituted thrombin (Sigma). We initially observed that these micromasses did not remain attached to the culture dishes, and hence we embedded the clots in collagen gels (1 part rat tail collagen (Collaborative Research) to 2 parts culture medium) to keep them from floating away. Cultures were fed with fresh culture medium one hour after being embedded in the collagenous matrix. The clots were transferred with watchmakers forceps into fresh collagen gels and fed with fresh culture medium every three days.

After a variable number of days in culture, the cell pellets were removed from the gels and lightly stained with Nile blue as de-

Fig. 1. Supernumerary response to a graft of posterior blastemal tissue into an anterior site of a host blastema. The above limb received a graft of the posterior one third of a blastema that had been dissected into eight fragments. This represents the maximal response observed for such grafts ($\times 11$)

scribed above. They were then subdivided into 8–10 pieces with iridectomy scissors and grafted into host blastemas in the same manner as blastema fragments.

Evaluation of regenerates. As discussed below, the frequency of formation of supernumerary skeletal elements can be interpreted to indicate the presence or absence of differences in positional identity between graft and host tissues. In this study we characterize a supernumerary response as the presence of at least two extra skeletal elements associated with the host carpals or metacarpals and the presence of an externally evident extra digit morphology (Fig. 1). Isolated cartilaginous nodules, skin tags, and minor extra cartilage elements without a corresponding external digit morphology were not scored as a supernumerary response. In general, the supernumerary responses were characterized by the presence of one or two extra digits.

Results

Assay for posterior positional information

The assay for the presence of posterior positional information is to graft blastema cells or tissue fragments beneath the apical epidermis on the anterior edge of a host blastema. For reasons presented in the Discussion, grafts containing cells with posterior positional information should stimulate the formation of supernumerary structures when grafted into a population of cells with anterior positional identity. In a preliminary experiment, we determined that the frequency of the supernumerary response from grafts of posterior blastema thirds was increased when the donor fragment was cut into small pieces (8–10 pieces) prior to grafting (frequency of 27% for whole thirds, $n=15$, versus 70% for fragmented thirds, $n=23$). In all subsequent experiments, grafts were fragmented into 8–10 pieces (when thirds were used) prior to grafting. The highest frequency of response we observed in any one experiment was 93% ($n=28$). In contrast, we never observed a supernumerary response when anterior cells or tissue fragments were grafted into anterior host sites; for example, grafts of anterior one third fragments from medium bud blastemas resulted in a supernumerary response of 0% ($n=28$).

Medium bud blastemas proved to be a more responsive host site as compared to later stages of regeneration. We examined the effect of the stage of the host blastema on the supernumerary response for three stages of regenerates: medium bud, in which the entire blastema is undifferentiated; palette/early digits, in which the anterior of the blastema is beginning to condense digits while the posterior is still undifferentiated; and late digits, in which all the digits are condensed. The highest frequency of response was observed for medium bud hosts (93%, $n=28$); whereas the frequency decreased progressively with increasing maturity of the host blastema (70%, $n=29$ for palette/early digit; 25%, $n=14$ for late digit).

The magnitude of the supernumerary response was proportional to the total number of cells grafted. Pieces of blastema ranging from a third down to a sixteenth were tested and all elicited responses. Unlike the thirds, which were fragmented into 8–10 pieces, quarters were cut into 8 pieces, fifths into 6 pieces, eighths into 4 pieces and sixteenths into 2 pieces. The frequency of supernu-

merary response was comparable for thirds, fourths and fifths (75–85%), lowest for sixteenths (25%), and intermediate for eighths (50%). Fractions of the blastema less than one sixteenth were not tested.

Assay for anterior positional information

To test for anterior positional information, we grafted anterior and posterior one third blastema fragments as described above into medium bud hosts, except that they were grafted into posterior rather than anterior host sites. For reasons presented in the Discussion, grafts containing cells with anterior positional information should stimulate the formation of supernumerary structures when grafted into a population of cells with posterior positional identity. Tissue fragments from both anterior and posterior regions of the blastema induced a supernumerary response when grafted into posterior host sites. Anterior grafts resulted in a slightly higher frequency of response (54%, $n=24$) as compared to posterior grafts (37%, $n=24$). Since both anterior and posterior regions of the blastema appear to contain cells with anterior positional information (see Discussion), we confined our further analyses of positional stability *in vitro* to posterior positional information, for which we could unambiguously assay.

*Stability of posterior positional identity *in vitro**

Utilizing the blastema dissociation procedures developed for this study, we can obtain reasonably large numbers of viable cells for primary culture. On average we obtain 10^5 cells/medium bud blastema ($SD=2 \times 10^4$ cells, $n=12$ independent experiments) with a mean trypan blue viability of 85% ($SD=6\%$). In our earlier studies, blastema cells were plated on plastic or other substrates (primaria, collagen, fibronectin, polylysine, and gelatin) at densities ranging from 10^5 to 3×10^5 cells/cm². The plating efficiency at 24 h ranged from 50% to 82%. Differences in substrate did not appear to affect plating efficiency (data not shown).

We observed that the total number of cells in the monolayer cultures declined for several days after plating even though the cells appeared healthy and mitoses could often be observed. This loss of cells seemed to be due to detachment since floating, viable cells could be found in the medium. In order to culture blastema cells for prolonged periods and still be able to harvest them for grafting into host blastemas, we modified the culture procedure to that described in the Materials and Methods. In these micromass cultures embedded in collagen gels, the blastema cells rapidly degraded the surrounding collagen matrix, and after a period of about three days would become free floating. The progression of matrix degradation by a micromass culture over a three day period is documented in Fig. 2. In order to maintain blastema cell cultures for longer periods, it was necessary to manually transfer the micromasses into fresh collagen gels every 3 days. In this way we have maintained blastema micromasses for periods up to two weeks.

Fig. 2a–c. Degradation of a collagen matrix over time by micro-mass cultures of blastemal cells. **a–c:** Three day time-course of degradation on the same culture ($\times 100$). **a** = 1 day; **b** = 2 days; **c** = 3 days. On the third day of culture, the micro-mass began to float up out of the surrounding matrix. **d, e** = Overview of a single culture at the end of the three day time-course ($\times 11$)

Fig. 3. Stability in vitro of the ability of posterior blastemal cells to stimulate a supernumerary response when grafted to the anterior of a host blastema in vivo. Posterior blastemal thirds were dissociated into a single-cell suspension, pelleted and cultured as micro-masses in collagen gels for 0 to 14 days. The pellets were moved to fresh collagen gels every three days as the matrix was degraded. After culture for the specified period, the micro-masses were grafted into the anterior of medium bud host blastemas. Each graft was subdivided into 8–10 fragments prior to grafting, and the resultant response frequency was determined by analysis of Victoria blue-stained preparations

Posterior blastema cells maintain posterior positional identity for about one week when cultured under the conditions used in this study (Fig. 3). Dissociated and reaggregated blastema cells that are grafted into host sites without a period of culture stimulate a supernumerary response (Fig. 3; 92%, $n = 12$) that is equivalent to grafts of blastema fragments that have not been dissociated (93%, $n = 28$). Blastema cells that have been cultured stimulate a supernumerary response at a lower frequency that remains stable at 35–45% over a period of about one week. After that time, cultured blastema cells no longer stimulated a response (Fig. 3). Anterior blastema cells cultured over the same time periods did not stimulate a supernumerary response when grafted into the anterior of host blastemas ($n = 42$).

Discussion

We report here the development of a functional assay for posterior positional information in axolotl blastema cells. Unlike previous studies of positional information in amphibian limbs which have involved the grafting of intact limb buds or blastemas, this assay involves the grafting of small fragments or pellets of posterior cells into an anterior location. Such grafts are equivalent to the ZPA-type grafts commonly performed in studies of chick wing bud development (MacCabe et al. 1973; Tickle et al. 1975). Such an assay, in conjunction with the blastema dissociation and culture techniques reported here, allows for the experimental manipulation of blastema cells in vitro followed by a functional assay for alterations in positional information.

Using this assay, we found that posterior blastema fragments containing as few as 6000 cells can stimulate

a supernumerary response when grafted into the anterior of a host blastema. This result is comparable to results from an equivalent experiment in chicks, in which pellets containing between 300 and 6000 pattern formation-competent posterior cells were able to stimulate a supernumerary response when grafted to an anterior host site (Tickle 1981).

We observed that at least four parameters influence the frequency of the supernumerary response: positional origin of the grafted tissue, total number of grafted cells, size of the grafted fragments and the stage of the host blastema. Each of these is interpretable in the context of current views of the mechanism of pattern formation during limb regeneration. Briefly, growth and pattern formation are thought to occur as a result of short range cell-cell interactions such that interactions between cells with different positional identities stimulate growth and the intercalation of cells with positional identities that are intermediate between the initially interacting cells. Such interactions continue to occur until positional confrontations are resolved (Bryant et al. 1981; Bryant et al. 1987). In contrast, interactions between cells with the same positional information do not stimulate growth and pattern formation, since no positional confrontations exist. In this context, we conclude that grafts of anterior blastema fragments into anterior host sites do not stimulate a supernumerary response because no positional confrontations are generated between graft and host cells. In the situation in which positional confrontations were created by grafting posterior cells into anterior host sites, the frequency of response was proportional to the number of grafted cells. This was observed when the total number of grafted cells was increased by grafting in more fragments. In addition, the frequency of the response increased when the total number of cells remained constant but the grafts were dissected into smaller pieces prior to grafting. Since only cells on the surface of a fragment can interact with adjacent host cells, dissection of the graft into smaller fragments results in an increase in the number of peripheral cells available for interactions with host cells. An equivalent interpretation has been presented for results from grafts of variable numbers of pattern formation-competent cells in the chick limb bud (Tickle 1981). Finally, the decreased response to grafts into later stage regenerates may reflect a decrease in the number of undifferentiated, pattern formation-competent host cells coincident with the progression of redifferentiation of limb tissues. Consistent with this interpretation is the observation that increased positional disparities are required to stimulate a pattern formation response from late stage developing *Xenopus* limbs (Muneoka et al. 1986) or mature axolotl limbs (Bryant and Iten 1977).

In experiments that are the reciprocal of the assay for posterior positional information, we grafted fragments of blastema tissue into a posterior host site. We observed that grafts of both anterior and posterior fragments stimulated a supernumerary response. Hence the posterior third of a medium bud blastema behaves as if it contains cells with anterior (as well as posterior) positional information. Although the distribution of positional information within the blastema has not been systematically mapped, this information is available for

the mature limb (Gardiner and Bryant 1989). These studies showed the presence of cells with anterior and ventral positional values in the limb center, leading to the conclusion that the posterior half of the limb contains not only posterior positional information, but also anterior information. Investigations of the patterns of contribution of stump cells to the blastema indicate that whereas there is some cell rearrangement, it is relatively limited, and particular regions of the stump contribute a greater proportion of cells to nearby regions of the blastema than to more distant regions (Muneoka et al. 1985; Tank et al. 1985). It is therefore likely that the distribution of positional information in the blastema reflects that of the stump, leading to the conclusion that posterior parts of the blastema not only contain cells with posterior identity, but also cells with anterior identity. Since the anterior cells are expected to be located deep within the blastema, and it is known that positional interactions only occur immediately beneath the permissive epidermis, the presence of anterior cells in the posterior part of the blastema is not likely to affect normal regeneration from an amputation stump. However, when posterior blastema thirds are fragmented and placed into a posterior location (as in the studies reported here), it is likely that some of the internal cells with anterior identity will end up under the permissive epidermis, and adjacent to posterior cells with which they can interact. Hence in the absence of a negative control (posterior into posterior) we were unable to develop an assay for anterior positional identity. It is possible that pretreatment of posterior graft fragments with retinoic acid would eliminate anterior cells from posterior fragments, and thereby make them suitable as controls for grafts of anterior cells in future studies.

We have used the assay for posterior positional information to develop dissociation and culture conditions that allow for the maintenance of pattern formation-competent cells in vitro. The dissociation and reaggregation procedures did not affect the ability of posterior cells to stimulate a supernumerary response since micro-masses that were assayed without having been cultured (zero days in culture, Fig. 3) stimulated a supernumerary response at the same frequency as blastema fragments that had not been enzymatically dissociated. This ability was reduced in cultured cells but remained stable for at least one week, after which it was lost.

Equivalent results have been reported for cultures of mouse and chick limb bud cells, except that the period of positional stability in vitro is shorter (36 h for chick and 24 h for mouse, see Hayamizu and Bryant 1992). The longer period of stability of blastema cells likely is related to the lower culture temperature (26° C vs 37° C for mouse and chick cells) as has been suggested previously (Honig 1983).

Observation of blastema micromasses embedded in collagen gels provides a dramatic illustration of the previously documented ability of whole blastemas to degrade collagenous substrates (Grillo et al. 1968; Dresden and Gross 1970). Under our micromass culture conditions, a clear area around the cells was evident after one day, and the circular zone of cleared collagen continued to increase in diameter with time. As the circle became larger, the micromass detached from the dish and

floated into the medium. Previous reports of collagenolytic activity associated with blastemas involved explants of entire blastemas. Since the cultures in the present study did not contain epidermal cells, it is evident that this activity is a property of blastema mesenchymal cells. Further, it is these blastemal mesenchyme cells that have been shown to be pattern-formation competent.

At the present time, we assume that the detachment of cells in monolayer cultures is a consequence of the matrix degrading activity observed in micromass cultures. We have observed that monolayer cultures of axolotl limb bud cells exhibit this same behavior (unpublished data). In contrast, cultures of limb bud cells from other vertebrate species exhibit either no decline in cell number prior to growth of the attached population (mouse: Gardiner et al. 1992; Shi and Muneoka 1992) or an initial period of decline followed by growth (*Xenopus*: Gardiner, unpublished). Since urodeles are unique in their ability to regenerate their limbs at any time during their life, this possibly unique property of urodele cells in culture may prove to be of functional significance in terms of mobilizing fibroblasts from the extracellular matrix of mature limb tissues (Gardiner et al. 1986), thus initiating the regeneration process. Studies to further characterize the matrix degrading properties of limb cells both in vitro and in vivo are in progress.

In this study we have provided further information about the properties of pattern formation-competent blastema cells, both in vitro and in vivo. Blastemas can be dissociated, and blastema cells can be cultured for at least one week and still maintain the ability to participate in cell-cell interactions leading to pattern formation. During this period, cultured blastema cells continue to exhibit a diagnostic and potent matrix degrading property that is characteristic of the intact blastema. This provides us both with further insight into the unique nature of blastema cells and thus their ability to undergo regenerative processes, as well as defining the window during which we can reliably manipulate such information in vitro in an attempt to alter the patterning ability of such cells.

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