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Journal
Developmental Biology, 118(2)

ISSN
0012-1606

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Publication Date
1986-12-01

DOI
10.1016/0012-1606(86)90020-5

DEVELOPMENTAL BIOLOGY 118, 488–493 (1986)

The Migration of Dermal Cells during Blastema Formation in Axolotls

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Received June 2, 1986; accepted in revised form July 31, 1986

Using the diploid/triploid cell marker in the axolotl (*Ambystoma mexicanum*) we have examined the movement of cells from the dermis into the early limb blastema. Cells of dermal origin begin to migrate beneath the wound epithelium at about 5 days postamputation, and by 10 days they are widely distributed across the amputation surface. By 15 days, a dense accumulation of blastema cells is present beneath the apical cap, and these cells are preferentially oriented in a circumferential direction. These results are discussed in relation to previous studies showing that the progeny of dermal cells become widely distributed during regeneration, and that cells of dermal origin are a major source of blastema cells. The results are also discussed in relation to ideas about how growth and patterning of the new appendage occur.

INTRODUCTION

When an amphibian regenerates a limb, it does so from a blastema consisting of cells from the mesodermally derived tissues adjacent to the wound site (Butler and O'Brien, 1942; Hay and Fischman, 1961; O'Steen and Walker, 1961). A recent analysis using marked cells has shown that cells of the dermis contribute extensively to the blastema, and that in proportion to their relative availability in the stump, they overcontribute by greater than twofold (Muneoka *et al.*, 1986). This finding is significant because it identifies a particular tissue type (dermis) and most likely a particular cell type (fibroblast) as being important for the regeneration process. In addition, these data provide an explanation for previous observations showing that the dermis has a major influence on the patterning of the regenerate: stumps in which small pieces of skin (or dermis alone) have been added to an abnormal location around the circumference (e.g., dorsal to ventral, anterior to posterior) form supernumerary limb parts during regeneration (Tank, 1981; Rollman-Dinsmore and Bryant, 1982). Such observations suggest that cells of the dermis possess the ability to interact in a position-specific manner to stimulate additional growth during the regeneration process.

In addition to being a major source of blastema cells, progeny of dermal cells have also been shown to become displaced from their original circumferential position during regeneration (Rollman-Dinsmore and Bryant, 1984). For example, progeny of anterior dermal cells may be found at all circumferential positions of the regenerated limb. Furthermore, in limb stumps which consist of half diploid and half triploid cells, about 25% of the cells were found to become displaced from their side of origin during normal regeneration (Muneoka *et al.*, 1985; Tank *et al.*, 1985). Such cellular displacements do not occur, however, when supernumerary limbs are formed as a result of blastema or limb bud grafting (Muneoka and Bryant, 1984a,b). In these studies blastemas or limb buds were grafted to a contralateral limb stump of different ploidy so as to appose anterior and posterior tissues. Supernumerary limbs develop where anterior and posterior cells have been confronted, and they contain discrete boundaries between the tissues originating from stump and graft. We previously suggested that the cellular displacements occurring in normal regeneration are related to the fact that this process involves the healing of a major, open wound (Bryant *et al.*, 1981; Rollman-Dinsmore and Bryant, 1984; Muneoka *et al.*, 1985); whereas, when supernumerary limbs develop, open wound healing is minimal, and thus little if any cellular displacement occurs.

In this paper we have studied the behavior of marked dermal cells during the early stages of regeneration. We have found that cells from the dermis migrate from the periphery toward the center of the amputation surface as the blastema is forming. These results provide new insights into the cellular mechanisms of growth and patterning of regenerating limbs.

MATERIAL AND METHODS

All experiments were performed on axolotls (*Ambystoma mexicanum*) spawned at the University of California, Irvine. They were maintained individually at room temperature (20°C) in 1 liter plastic boxes in 25% Holtfreter's solution, and were changed to fresh saline and fed tubifex worms three times a week. The animals used in this study ranged in length from 135 to 192 mm snout to tailtip. Triploid animals were produced using

0012-1606/86 $3.00

the protocol of Gillespie and Armstrong (1979), and screened for triploidy using the protocol of Muneoka *et al.* (1984). Triploid and diploid sibling larvae were maintained separately but under identical conditions.

To create limb stumps in which skin and internal tissues differed in ploidy, diploid and triploid siblings were matched for size. A cuff of skin extending from the knee to hip was removed from both thighs of each diploid and triploid pair following a middorsal incision, then proximal and distal circumferential incisions. The cuffs were transplanted reciprocally between equivalent hind limbs to create diploid limbs with triploid skin cuffs and vice versa. The skin was transplanted so as to maintain its normal orientation, and was sutured in place. Animals were kept at 4°C for 1 day and then at room temperature. One week after grafting, limbs were amputated through the graft, and the limb stumps were trimmed flat leaving a minimum of 1.6 mm of grafted skin proximal to the amputation plane. At 5, 10, and 15 days after amputation, limbs were removed and fixed in Carnoy's fixative. Whole mount preparations of the wound area (epidermis and underlying cells) were made by peeling the skin from the limb in a proximal to distal direction, after making several longitudinal incisions up to the edge of the wound area. The residual (skin-free) limb stumps were embedded in paraffin and sectioned transversely at 10 μm. Both whole mount preparations and sections were stained with bismuth for visualization of nucleoli (Muneoka *et al.*, 1984).

Maps of the distribution of subepidermal, trinucleolate cells in whole mounts of the wound area, and in sections of the skin-free stumps were made utilizing a digitized microscope stage (Minnesota Datametrics Corporation) interfaced with an Apple IIe microcomputer. For whole mounts, the entire wound area and adjacent skin were scanned and each plotted point represents a minimum of one trinucleolate cell in a region measuring 35 × 125 μm. Cells which were not clearly identifiable as mesodermal cells (e.g., epidermis and blood) were not mapped. The interface between dense, fibrous connective tissue and wound epidermis was designated as the boundary between mature skin and wound area. For sections of the skin-free stumps, individual transverse sections of the distal stump were mapped as described above and compiled to give a qualitative three-dimensional image of the position of marked cells.

RESULTS

The technique of whole mounting the skin and wound area used in this study is well suited for visualizing and analyzing the distribution of blastema cells immediately adjacent to the wound epidermis. In Fig. 1, the edge of the wound area of a whole mount preparation, fixed 10 days after amputation, is shown in section. The most distal few layers of blastema-forming cells detach with the wound epidermis and are present in the whole mount preparations. It is these early blastema cells whose distribution we have analyzed in this paper by direct examination of whole mounts, such as the one illustrated in Fig. 3a.

FIG. 1. Light micrograph of a section of a whole mount preparation at the junction between mature dermis (D) and wound surface. The epidermis (E) is to the right, and the arrow indicates the cut edge of the mature dermis, marked by the edge of the dense fibrous connective tissue and by a dermal gland. Hematoxylin and eosin staining, ×140.

Triploid onto Diploid

A total of 26 limbs were analyzed in which triploid skin cuffs had been grafted onto diploid limbs. The limbs were fixed at either 5, 10, or 15 days postamputation, at stages equivalent to wound healing, dedifferentiation, and early bud (Tank *et al.*, 1976). Whole mount preparations of the wound area were analyzed to map the distribution of subepidermal, trinucleolate cells. Some skin-free stumps were analyzed to determine the nature of patches which appeared to be cell free in the whole mount preparations.

Five days postamputation. The distribution of subepidermal, trinucleolate cells was mapped in 10 whole mount preparations from limbs fixed 5 days postamputation. As can be seen in the example shown in Fig. 2a, trinucleolate cells are abundantly present in the dermis bordering the wound area. Some trinucleolate cells can be seen to extend a few cell diameters beyond the original wound margin, but in none of the preparations were any trinucleolate cells seen within the central region of the amputation surface. The entire area beneath the wound epidermis is mostly cell free with scattered binucleolate cells, presumably of stump origin, enmeshed in cellular debris. Examination of serial cross sections of three residual stumps confirms that these cell-free areas correspond to stump regions occupied by blood

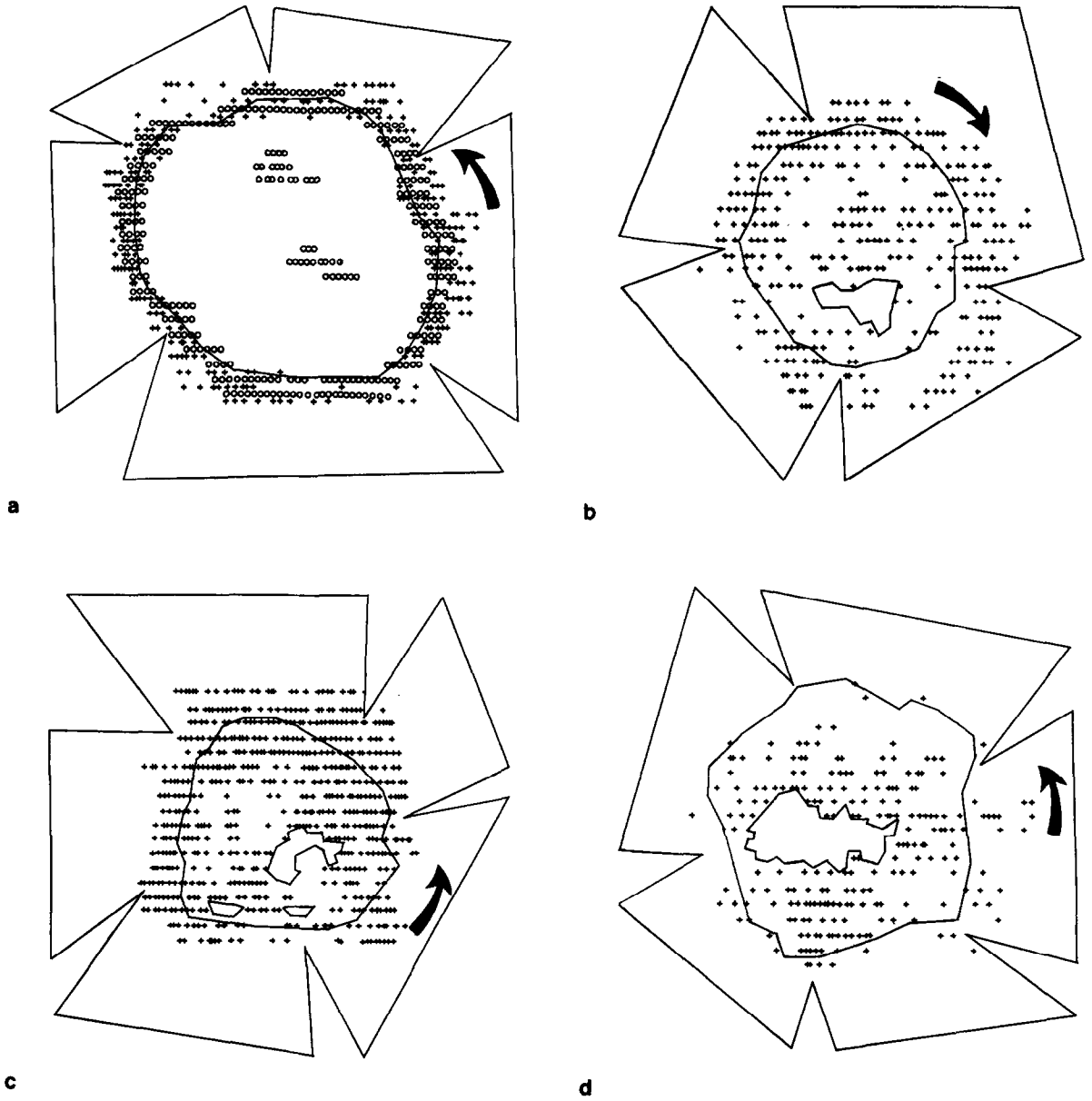

FIG. 2. Computer-assisted plots of the distribution of subepidermal cells in whole mount preparations. Each symbol (+) represents a minimum of one trinucleolate cell in a region measuring 35 × 125 μm. The edge of the wound area and cell-free regions within the wound area are outlined by solid lines. Middorsal is marked by an arrow which points towards the anterior. (a) Five days postamputation; triploid skin grafted onto a diploid stump. In addition to the distribution of trinucleolate cells (+), the distribution of binucleolate cells (○) is also indicated. (b) Ten days postamputation; triploid skin grafted onto a diploid stump. (c) Fifteen days postamputation; triploid skin grafted onto a diploid stump. (d) Ten days postamputation; diploid skin grafted onto a triploid stump. Note that the trinucleolate cells indicated here are of stump origin. ×17.

clot, cellular debris, and scattered binucleolate cells. Trinucleolate cells were not observed in the central regions of the sectioned limb stumps at this time point, instead they were localized around the periphery of the limb stump.

Ten days postamputation. The distribution of subepidermal, trinucleolate cells was mapped in eight whole mount preparations from limbs fixed at 10 days postamputation. A further two preparations were not analyzed in detail because mesodermal cells did not separate with the wound epidermis but remained attached to the limb stump. In contrast to the situation at 5 days, by 10 days, trinucleolate cells of dermal origin are widespread beneath the wound epidermis, and can be seen to occupy the central subepidermal area (Fig. 2b). The distribution of trinucleolate cells is somewhat uneven with interspersed areas of binucleolate cells. Most of the preparations show a small more or less centrally located cell-

free area. Such cell-free areas are considerably smaller than at 5 days. Examination of sections of the skin-free stumps of three limbs revealed that the cell-free areas correspond to small pockets of cellular debris.

Fifteen days postamputation. The distribution of subepidermal, trinucleolate cells was mapped in six whole mount preparations from limbs fixed at 15 days postamputation. Trinucleolate cells are widely distributed beneath the wound epidermis (Fig. 2c), and are present at much greater density than at previous time points. Some regions were unscorable due to the dense accumulation of cells. As was seen at 10 days, although abundant in some regions, trinucleolate cells can be scarce or absent in adjacent, albeit cellular, regions. Many of the subepidermal cells at 15 days can be seen to be oriented preferentially in a circumferential direction (Fig. 3b). Three residual stumps were analyzed in sections to determine the nature of small cell-free areas in the whole mounts. In all cases, these were found to be areas where blastema cells had remained attached to the stump. There was no evidence of blood clot or cellular debris beneath the epidermis at this time point.

Diploid onto Triploid

A total of three limbs in which diploid skin cuffs had been grafted onto triploid limbs were analyzed as whole mount preparations of the wound area 10 days postamputation. As can be seen from Fig. 2d, trinucleolate cells of stump origin also contribute to the early population of subepidermal blastema cells.

DISCUSSION

Our results show that an early event in the establishment of the blastema is the movement of dermal cells from their matrix at the limb periphery across the wound surface. This movement begins at 5 days, and by 10 days after amputation, cells of dermal origin are widely distributed beneath the wound epidermis. Our data also show that cells of stump origin enter the early population of subepidermal blastema cells at about the same time. Thus by 15 days, the blastema cells adjacent to the wound epidermis consist of a mixture of cells of peripheral and more central origin. These results are consistent with the previous finding that, on the average, cells of dermal origin make up 43% of the cells of the medium bud blastema, and with the suggestion that the remaining cells of the early blastema could be derived from other connective tissue components of the stump (Muneoka *et al.*, 1986). The centripetal displacement of periphally located cells can account for the previous observation that the progeny of dermal cells become redistributed to new locations in the limb circumference of the regenerate during outgrowth (Rollman-Dinsmore and Bryant, 1984). The data also are consistent with the finding that when regenerates are produced from limb stumps which are half diploid and half triploid, a fraction of the cells which originate on one side of the limb have progeny which are located on the opposite side at the end of the regeneration process (Muneoka *et al.*, 1985; Tank *et al.*, 1985). Finally our observations can also account for previous results in which the movement of peripherally located cells was inferred but not demonstrated (Bryant and Baca, 1978; Bryant *et al.*, 1981).

Comparison of the whole mount preparations with their accompanying sectioned limb stumps indicates that the cell-free areas in the whole mount preparations at 5 and 10 days postamputation are occupied by blood clot and cellular debris. Initially this material is extensive

FIG. 3. Light micrographs of a whole mount preparation 15 days postamputation. (a) Low-power photograph of whole mount preparation to show lighter, central wound area. The dark, crescent-shaped region within the wound area is the tip of the blastema which folded over during sample preparation. ×16. (b) Higher magnification of the upper right region of the wound area in (a). The nuclei of blastema cells in the wound area (light area) near the boundary with the mature dermis (D) are oriented parallel to the wound edge (i.e., in an arc from left to right). A patch of epidermal cells (*) is visible in the lower right hand corner of the micrograph. ×170.

and later it appears to be confined to smaller, more central regions of the wound area. By 15 days, no such regions of debris can be seen, and the small cell-free areas seen in whole mounts are in fact regions where cells failed to detach with the wound epidermis. The disappearance of cellular debris is coincident with the inward migration of cells, and it is possible that the two are causally connected, with the peripheral cells migrating into the space created as debris disappears. The mechanisms involved in the removal of debris are not fully understood although previous authors have suggested that the epidermis plays an active role in the transport of such materials to the exterior (see Singer and Salpeter, 1961).

The migration of peripheral cells and the closure of the epidermis are temporally distinct. The epidermis is closed between 8 and 12 hr after amputation, whereas migration of cells from the dermis does not begin until 5 days. We have no information at this time as to whether epidermal closure is a necessary prerequisite for this migration. However, Thornton and Thornton's (1965) observations that blastema cells accumulate beneath an eccentrically positioned apical epidermal cap, suggest a possible role for the wound epithelium in this migration process. At this time we have no information about the substrate on which peripheral cells are moving. However, previous studies have shown that the early regenerate is characterized by the presence of extracellular matrix molecules known to favor cell migration, and by the absence of those which might interfere with it. For example, Toole and Gross (1971) demonstrated a peak in the synthesis of hyaluronate at about 10 days postamputation in newt limbs. Grillo, *et al.* (1968) showed collagenolytic activity was highest at these same early times, suggesting that collagen fibers, which might impede movement, are being actively removed at this time. Furthermore, the presence of fibronectin, a molecule which is implicated in cell attachment and motility, is present between the cells of the very early blastema (Repesh *et al.*, 1982; Gulati *et al.*, 1983).

Numerous previous studies of limb patterning have pointed to the central role played by the connective tissue of the limb, and in particular by the dermis (see Bryant, 1978; Tank, 1979). In the Polar Coordinate Model (PCM), it has been proposed that the means by which the dermis acts in pattern regulation is that its cells possess information about their proximal-distal and circumferential positions. During wound healing or after grafting, these cells are confronted with new neighbors with normally nonadjacent positional values (Bryant *et al.*, 1981). The data presented in this paper provide direct evidence that dermal cells do in fact acquire new neighbors during the early phases of blastema formation, and hence they provide important corroboration for the plausibility of this aspect of the PCM at the level of individual cells. A further postulate of the PCM is that when positional disparities are created, cell division is stimulated. Growth is proposed to continue until positional disparities between cells have been eliminated; this process is known as intercalation. As described in Bryant *et al.* (1981), formation of the new limb can be seen as a consequence of such position-dependent growth stimulation. Although we have no direct data on the distribution and frequency of mitotic cells in the limbs we have examined, reports in the literature indicate that the mitotic index begins to increase at 4–6 days after amputation (Kelly and Tassava, 1973; Tassava *et al.*, 1974; Maden, 1978). Hence, the onset of dermal cell redistribution and the onset of mitosis are temporally and conceivably, causally linked. This intriguing relationship will be explored further in future studies.

We thank Kirby Black, Rosemary Burton, Laurie Iten, and Stan Sessions for critically evaluating the manuscript; and Warren Fox and Sharyl Yoshimura for technical assistance and manuscript preparation. This work was supported by PHS Grant HD06082, and a gift from the Monsanto Company.

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