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Cellular Contribution from Dermis and Cartilage to the Regenerating Limb Blastema in Axolotls

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Using the triploid/diploid cell marker in the axolotl, *Ambystoma mexicanum*, we have analyzed the extent to which cells derived from the dermis and the skeleton contribute to the regenerating limb blastema. We found that dermal cells contribute 43% of the blastemal cell population whereas cells derived from skeletal tissue contribute only 2%. When compared to the availability of cells at the plane of amputation, dermal cells overcontribute by greater than twofold whereas skeletal cells undercontribute by several-fold. These data correlate with the effects that these two tissues have on the formation of the limb pattern during regeneration; dermis has a dramatic influence on pattern and skeletal tissue has virtually no effect. It is suggested that the fibroblasts present in the dermis and in other parts of the limb form virtually all of the mesodermal tissues in the regenerate with the exception of the muscle. © 1986 Academic Press, Inc.

INTRODUCTION

The blastema which forms on an amputated amphibian limb stump is derived from mesodermal tissues present at the wound site (Butler and O'Brien, 1942; Hay and Fischman, 1961; O'Steen and Walker, 1961). These tissues lose their specialized characteristics (dedifferentiate), accumulate beneath the wound epidermis at the tip of the stump, and form the blastema from which the regenerated structures are elaborated. Thirty years ago, Chalkley (1954) studied the mitotic indices of various stump and regenerating tissues in an effort to estimate the relative contributions of different tissues to the blastema. He found that mitotic figures were present in all major mesodermal tissues immediately adjacent to the amputation surface and concluded that these tissues provide cells to the blastema in the same proportion in which they are present in the stump. However, no direct measurements of the contributions of different tissues to the blastema have been made, in part due to the lack of a suitable cellular marker. Recent improvements in the triploid/diploid cell marker in axolotls now make such analyses possible (Muneoka *et al.*, 1984).

A variety of different grafting experiments have led to the conclusion that some limb tissues play a larger role than others in the patterning processes occurring during regeneration (see Tank and Holder, 1981). The two tissues we have analyzed in this study are two which have quite divergent effects on patterning. Dermis has a dramatic effect, such that even small implants of skin strips or dermis can stimulate the regeneration of supernumerary limb structures (Tank, 1981; Rollman-Dinsmore and Bryant, 1982). Skeletal tissue, on the other

hand, appears not to have an influence over the pattern at all. When the skeleton of the limb stump was rotated through 180° (Carlson, 1975), or when a limb stump was supplied with an extra skeletal element (Goss, 1956), no major effect on the pattern of the subsequent regenerate was observed. One obvious route by which tissues might exert differential effects on limb patterning is by differential contribution of cells to the blastema. In this study we analyze the contribution of dermal and cartilage cells to medium bud blastemas in the axolotl and find that dermis overcontributes to the blastema in relation to its availability in the stump, whereas cartilage undercontributes.

MATERIALS AND METHODS

All experiments were performed on axolotls (*Ambystoma mexicanum*) spawned at the University of California, Irvine. They were maintained individually at 20°C in 1-liter plastic boxes in 25% Holtfreter's solution and were changed and fed tubifex worms three times a week. Animals used for studies of dermal contribution measured 10 to 12 cm, and those used for studies of skeletal tissue contributions measured 12 to 15 cm, snout to tail tip. Triploid animals were produced using the protocol of Gillespie and Armstrong (1979). Treated animals were screened for triploidy following the protocol outlined in Muneoka *et al.* (1984). Triploid and diploid larvae from the same spawn were maintained separately but under identical conditions.

Sibling axolotls (donor triploid, host diploid) were matched for similarity of size, and anesthetized in MS222 (Eastman) diluted 1:1000. For dermal grafts, a cuff of

skin was removed from the entire upper arm of the host. A similar piece of skin was removed from the donor upper arm, transplanted to the host maintaining normal orientation, and sutured in place. For skeleton grafts, host limbs were first amputated distal to the elbow or knee. The soft tissues were pushed proximally to enable removal of most of the skeleton; muscle attachments were freed with scissors. Skeletons were removed from donor limbs in the same way, and grafted to the empty host sockets maintaining normal orientation. The ends of the limbs were sutured closed. Grafted animals were kept at 4°C for 1–2 days and then at room temperature until a week after grafting, at which time limbs were amputated through the distal half of the upper arm or leg. All amputated limbs were trimmed to ensure a flat plane of amputation.

Regenerates were removed at the stage of medium bud (Tank *et al.*, 1976), fixed in Carnoy's fixative, and processed immediately for histology. Serial cross sections were cut at 10 μ m and stained with bismuth (Muneoka *et al.*, 1984).

Counts of trinucleolate cells were made in control triploid, control diploid, and experimental blastemas at the stage of medium bud. In all cases counts were made on the 5 sections which were between 50 and 100 μ m from the apical tip of the blastema (distal counts) and on the 2 sections which were between 300 and 320 μ m from the tip (proximal counts). The trinucleolate cell frequency was determined as the number of cells with 3 nucleoli divided by the total number of cells with 2 and 3 nucleoli. Cells with 0 or 1 nucleolus were not scored; neither were cells which were obviously not blastema cells (i.e., epidermis, blood cells). The trinucleolate frequency was then converted to a real triploid frequency (observed frequency/control frequency), using data from matched control triploid blastemas.

The proportion of cells grafted relative to the number of cells present in the stump was estimated for both dermis and cartilage. For the dermis, skin was removed from limbs as if for grafting, but replaced inside out on the same limb to facilitate accurate counting of grafted cells. Cell counts were made on cross sections of the mid-upper arm to determine both the number of dermal cells included in grafts and the total number of stump cells. For the cartilage, the skeletal element was removed as if for grafting, and cell counts were made on cross sections. To estimate the total number of ungrafted stump cells, mature limbs from animals covering the size range used in this experiment were processed for histology and total cell counts were made on cross sections through the mid-stylopod. Total cell counts and cross-sectional area were found to correlate linearly ($r = 0.96$) (Fig. 1). Total cell number in each experimental limb was read

FIG. 1. The relationship between the cross-sectional area and the total number of cells in cross sections of axolotl upper arms and legs. Cell counts and area measurements are exclusive of epidermis. Correlation coefficient (r) = 0.96.

from Fig. 1 using the cross-sectional area of the limb stump proximal to the blastema.

RESULTS

Control Cell Counts

To control for the "background" frequency of apparent trinucleolate cells in diploid tissues, control diploid medium bud blastemas from four different animals were counted. The frequency of apparent trinucleolate cells in diploid blastema tissue is about 0.005.

In a previous study (Muneoka *et al.*, 1984) we showed that there is considerable variation from one triploid animal to another in the frequency of trinucleolate cells, although the frequency within animals is fairly uniform. In addition, sectioning introduces frequency artifacts which may vary from place to place in the tissue depending on cell density, shape and size. To control for these factors, a control medium bud blastema was harvested from each triploid donor animal and cell counts were made exactly as for experimental blastemas. The data are displayed alongside the experimental counts in Tables 1 and 2. The control trinucleolate frequencies of different animals varied from 16–39%.

Contribution from the Dermis

A total of 9 medium bud blastemas were harvested from chimeric limb stumps composed of triploid skin and diploid internal tissues. Since epidermis does not contribute cells to the blastema (Riddiford, 1960; Hay and Fischman, 1961; O'Steen and Walker, 1961), we con-

TABLE 1
DERMAL CONTRIBUTION TO BLASTEMA

Limb	Total cells	3N frequency	3N control frequency	Real 3N frequency
(A) Distal counts				
18R	264	0.18	0.39	0.46
18L	114	0.14		0.36
19R	337	0.19	0.33	0.58
19L	411	0.23		0.70
22L	154	0.08	0.27	0.30
26R	190	0.16	0.35	0.46
26L	407	0.10		0.29
28R	119	0.10	0.27	0.37
28L	283	0.09		0.33
(B) Proximal counts				
18R	295	0.10	0.26	0.38
18L	248	0.14		0.54
19R	357	0.11	0.23	0.48
19L	323	0.18		0.78
22L	698	0.12	0.19	0.63
26R	400	0.07	0.25	0.28
26L	597	0.07		0.28
28R	463	0.03	0.13	0.19
28L	549	0.03		0.19

sider these skin grafts to be dermal grafts for the purposes of this analysis. The data from counts of trinucleolate cells in distal and proximal regions of the blastema are displayed in Table 1. For each counting level and for each specimen, the number of trinucleolate cells and the total number of trinucleolate plus binucleolate cells were used to calculate the trinucleolate frequency. The real triploid frequency is then calculated (observed frequency/control frequency). As can be seen in Table 1, the proportion of blastema cells which were triploid (i.e., derived from dermis) ranged from 29 to 70% ($\bar{x} = 0.44$, $SD = 0.14$) in the distal part of the blastema, and from 19 to 78% ($\bar{x} = 0.42$, $SD = 0.20$) in the proximal part.

To assess the proportion of grafted dermal cells in the stump relative to all other mesodermal tissues, total counts were made on a single cross section of each of 5 limbs prepared as described under Materials and Methods. The results indicate that the grafted dermal cells represent 19% ($\bar{x} = 0.19$, $SD = 0.03$) of all mesodermal tissues. This value is similar to that reported previously by Tank and Holder (1979).

Contribution from Skeleton

A total of 11 medium bud blastemas were harvested from chimeric limb stumps composed of triploid skeleton

and diploid skin and muscle. The data from counts of proximal and distal regions of the blastema are displayed in Table 2. The real triploid frequency for each counting level and each blastema was calculated as described above for the dermal contribution studies. As can be seen from Table 2, the proportion of blastema cells originating from grafted triploid cells range from 0 to 3% in both the distal and proximal parts of the blastema. This rather low contribution value cannot be attributed to poor graft survival since trinucleolate cells were found in the skeletal element proximal to the plane of amputation.

The proportion of stump cells represented by the skeletal element was determined from direct cell counts of explanted skeletons and estimates of total cell number derived from Fig. 1. From this, the proportion of the stump represented by the grafted skeletal element was found to be 6% ($\bar{x} = 0.06$, $SD = 0.02$).

DISCUSSION

In this paper, we report that two tissues which are known to have different effects on limb patterning also contribute differentially to the blastema. Skeletal tissue,

TABLE 2
SKELETAL TISSUE CONTRIBUTION TO BLASTEMA

Limb	Total cells	3N frequency	3N control frequency	Real 3N frequency
(A) Distal counts				
2R	231	0.00	0.32	0.00
2L	124	0.01		0.03
3RH	136	0.01	0.29	0.03
3LH	108	0.01		0.03
5R	137	0.00	0.39	0.00
5L	183	0.01		0.03
6RH	157	0.00	0.39	0.00
8R	367	0.01		0.03
8L	299	0.01	0.39	0.03
9RH	397	0.01		0.03
9LH	384	0.00		0.00
(B) Proximal counts				
2R	643	0.00	0.29	0.00
2L	607	0.00		0.00
3RH	499	0.00	0.27	0.00
3LH	496	0.00		0.00
5R	468	0.00	0.30	0.00
5L	609	0.00		0.00
6RH	879	0.00	0.30	0.00
8R	710	0.01		0.03
8L	738	0.00	0.30	0.00
9RH	999	0.00		0.00
9LH	865	0.00		0.00

which has been shown not to affect patterning is underrepresented in the blastema in proportion to its availability in the stump. Dermis, which is known to greatly influence limb patterning is overrepresented in the blastema by greater than twofold in comparison to its availability in the stump. In both cases, triploid cells were present in approximately equal frequencies in distal and proximal parts of the blastema. Since in both experiments viable triploid cells were observed in the limb stump proximal to the blastema, it is unlikely that differential contribution can be attributed to grafting artifacts influencing the availability of cells at the amputation plane.

This study is the first to directly compare the relative contribution of different limb tissues to the blastema. However, there are several earlier studies which are relevant to this issue. Chalkley (1954, 1956) conducted an extensive analysis of cell numbers and mitoses in the blastema, and in different tissues of the stump and the redifferentiating regenerate of *Notophthalmus viridescens*. From correlative evidence he concluded that regeneration could be largely tissue-specific, with different tissues in the stump contributing in a proportional way to the formation of equivalent tissues in the regenerate. Clearly, this conclusion is not supported by the evidence presented here using a heritable cell marker. In a more recent paper, Cameron and Hinterberger (1984) examined the percentage of triploid cells in different regions of early and medium bud blastemas derived from diploid stumps containing triploid cartilage grafts. The percentages of cartilage-derived cells that they observed in the central part of the blastema is higher than the percentages we found in our study. However, in their study, cartilage often protruded from the stump, and the necessary control cell counts (Muneoka *et al.*, 1984) required to accurately quantitate contribution were not performed, making a direct comparison between our study and theirs impossible.

The finding that skeletal tissues undercontribute to the blastema is consistent with a variety of lines of experimental evidence indicating that these tissues are without an effect on patterning processes. Goss (1956) showed that implanting an extra cartilage element into the forearm of *Notophthalmus viridescens* prior to amputation, had no major effect on the regenerated limb pattern. Normal limbs regenerate following rotation of the skeletal element with respect to the remaining tissue of the limb (Carlson, 1975), again indicating that skeletal tissues do not play an important role in limb patterning. The finding that dermal cells can contribute up to 78% of the cells in the blastema provides an explanation for a variety of experiments in which the role of the dermis in patterning has been shown to be dominant. Not only

are supernumerary outgrowths formed when skin is rotated 180° with respect to the remaining tissues (see Carlson, 1974) but small implants of skin or dermis are capable of stimulating supernumerary outgrowths when they are transplanted into new locations around the circumference (Tank, 1981; Rollman-Dinsmore and Bryant, 1982). Furthermore, the patterning information of the skin has been shown to determine the pattern of the final regenerate in some experiments. For example, when grafts are made so that the skin is symmetrical but the internal tissues are normal, the regenerated limbs are in many cases symmetrical and in accord with the symmetrical arrangement of the skin (Tank, 1979; Slack, 1980, 1983; Maden and Mustafa, 1982). The importance of the skin for normal regeneration has been highlighted by recent experiments (Tank, 1983, 1984) in which flank skin, grafted to replace limb skin, completely inhibits regeneration.

Given the importance of the dermis in regeneration, it is important to consider the cell types which may be contributing to the blastema. The dermis of the axolotl is composed of two morphologically distinct cell types, fibroblasts and pigment cells, arranged diffusely in a network of extracellular fibers (Holder and Glade, 1984). Pigment cells can be eliminated as being important for the regeneration process since the limbs of the axolotl mutant d/d, which lack these cells, regenerate perfectly well (see Wallace and Wallace, 1973). Fibroblasts are by far the most abundant cell type in the dermis and are almost certainly responsible for the dermal contribution to the blastema, as well as for the patterning role of the dermis. It is important to note that the dermis itself comprises approximately 50% of all the observable loose connective tissue fibroblasts present in a histological section of the axolotl limb (Tank and Holder, 1979 and our unpublished results). Hence it is conceivable that the entire regenerate, with the exception of muscle (see Namenwirth, 1974; Dunis and Namenwirth, 1977; Lheureux, 1983) may be derived from fibroblasts. This interpretation is consistent with earlier observations based solely on histological examination of the regeneration process (see Schmidt, 1968).

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