Unexpected Regeneration in Middle-Aged Mice

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Abstract

Complete regeneration of damaged extremities, including both the epithelium and the underlying tissues, is thought to occur mainly in embryos, fetuses, and juvenile mammals, but only very rarely in adult mammals. Surprisingly, we found that common strains of mice are able to regenerate all of the tissues necessary to completely fill experimentally punched ear holes, but only if punched at middle age. Although young postweaning mice regrew the epithelium without typical pre-scar granulation tissue, they showed only minimal regeneration of connective tissues. In contrast, mice punched at 5–11 months of age showed true amphibian-like blastema formation and regrowth of cartilage, fat, and dermis, with blood vessels, sebaceous glands, hair follicles, and, in black mice, melanocytes. These data suggest that at least partial appendage regeneration may be more common in adult mammals than previously thought and call into question the common view that regenerative ability is lost with age. The data suggest that the age at which various inbred mouse strains become capable of epimorphic regeneration may be correlated with adult body weight.

Introduction

THE ABILITY TO REGENERATE DAMAGED ORGANS has long been thought to be the prerogative of salamanders, worms, and fetuses.¹ Most adult mammals are unable to regenerate tissues and usually heal wounds through production of a vascular space-filling tissue known as granulation tissue, which precedes production of the final dense collagen scar.^{2,3} There are few exceptions, but one of the best characterized is external ear punch hole filling in certain mammals. For instance, the rabbit ear can completely close small experimentally induced ear holes, regenerating the full array of involved tissues in a scarless process called epimorphic regeneration.^{4,5} Another exception has been the “autoimmune” Murphy Roths Large (MRL) mouse, which can also close ear holes by epimorphic regeneration without leaving a scar.⁶ In contrast, “normal” mice and most other mammals have not been shown to have this capacity. In fact, holes are routinely punched in the ears of young postweaning mice as a long-term means of identification.

In the process of beginning a study on aging and immunity,⁷ we ear-punched a number of older C57BL/6 (B6) mice and found, to our astonishment, that the ear holes nearly disappeared. To follow this up qualitatively and quantitatively, we designed extrasharp hole punchers that would reliably produce circular 2- or 2.2-mm holes, and we evaluated the macroscopic and microscopic appearance of the regenerating

tissue in BALB/c and B6 mice, comparing the regenerative capacity of 1-month-old postweaning mice with that of older mice. We found that older mice were indeed capable of completely closing these holes, and that the overall histological appearance of the phases of regeneration was very similar to that seen in amphibian limb regeneration, and in the MRL mouse. We suggest that the MRL is not genetically unique in its capacity to regenerate punched ear holes. The main genetic differences between MRL and other mouse strains control “when” rather than “whether” regeneration occurs. We further suggest that regenerative ability correlates with age and adult body size. Inbred mouse strains that grow rapidly to become large adults can regenerate quite early, whereas smaller strains gain regenerative capacity later in life.

Materials and Methods

Animals

BALB/c and C57BL/6 (B6) mice were from the National Cancer Institute (Frederick, MD), and MRL mice were from the Jackson Laboratories (Bar Harbor, Maine). Only female mice were used in all experiments because of prior data suggesting the superiority of female MRL ear hole regeneration,⁸ and because of the results of our preliminary finding that female B6 mice regenerate better than males. All experiments were conducted according to National Institutes of Health

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Histological examination

Ears were excised following cervical dislocation of the mice, fixed overnight in 4% paraformaldehyde, and processed for paraffin embedding. Transverse sections (5 μ m) of the ears were cut, as depicted in Fig. 4, A and B, and stained with Hematoxylin & Eosin (H & E), as in Fig. 4C. All histopathology was done with ears cut with a 2.2-mm cutter, unless otherwise noted.

Hole cutter

Most investigators have relied on wounding devices that were not designed to cleanly cut through tissue (i.e., thumb punches), but Ferguson showed that using a sharp biopsy puncher instead of a dull thumb punch enhances features of regeneration.⁹ The disadvantage of the Ferguson system is that it requires anesthesia. To create a cleaner, more standardized injury than that which normally results from standard ear punches, and to avoid the need for anesthesia, we designed a sharp hypo-tubing-based puncher that could be used quickly without anesthesia. We made two hole-cutting instruments that use stainless steel hypotubing (as from a hypodermic needle without the taper) to cut through ear tissue. The outer diameter of the smaller cutter is 1.95 mm and reliably cuts a 2-mm hole, whereas the outer diameter of the larger-bore hypotubing is 2.15 mm and reliably cuts a 2.2-mm hole. Although operated by the thumb, as in solid-dowel thumb punches, this apparatus is spring-loaded and designed to operate quickly with just a touch of thumb pressure. A schematic of the device can be seen at (<http://ott.od.nih.gov/db/abstxt.asp?refno=1352>). It consists of a long handle attached to a housing containing an actuator that moves the cutting edge of the puncher through the housing. The inner diameter of the hypotubing was “sharpened” (chamfered) prior to use, and the hypotubing replaced after every 50 uses. We chamfered the inside diameter so that the outside diameter becomes the sharp cutting edge.

As we report for the first time below, a payoff of the new techniques was that they produced clearer patterns in both qualitative histopathologic features, quantitative changes in hole geometry, and percent closure with age at punch than had appeared before.

Hole measurements

Measurement of changes in hole size over time *in vivo* is usually performed by two people—one to hold the mouse and one to measure hole diameter with a 7 $\times$ grid-etched reticle. Commonly, the two parties then switch roles and the holder becomes the measurer and vice versa. Each measurement takes a variable number of seconds, depending upon the desired precision. One geneticist estimates that the time from removing the mouse from its cage to returning it is approximately 20 sec.^{10,11} The need for multiple measurements has never been explained explicitly, but seems designed to control for various sources of variability, such as mouse movement, tissue movement (i.e., “play”) due to the hole being in the very curved center of the mouse ear, and observer imprecision. Of particular concern is the fact that greater ac-

FIG. 1. Comparison of macroscopic appearance of complete closure of 2.2-mm holes in MRL and B6 mice. Ears of MRL mice (albino) were punched with a 2.2-mm cutter at 2 months of age, whereas B6 ears (black) were punched at 9 months of age. The appearance of the holes on the day of punch, day 0 (*left panels*), and day 32 (*right panels*), is shown for MRL and B6 strain mice. By day 32 postpunch, new tissue has completely filled both holes.

curacy is achieved only by increasing periods of restraint, which can lead to restraint stress and cortisol excess. Corticosteroids are the most potent known inhibitors of epimorphic regeneration.¹² To attempt to eliminate play in the thin pinna tissues surrounding the hole as a source of variability, we devised a technique that would: (1) simultaneously straighten the pinna and expand the hole enough to take reasonably accurate measurements, (2) be conducted without anesthesia by a single investigator, (3) provide an imprinted memory of the hole itself, (4) quickly produce a cast of the hole that could later be slowly and carefully evaluated, and (5) have a total handling time per mouse of about 3–5 sec. We compared various ink-based versus dry impression methods and finally found that moist child’s modeling clay (DAS pasta per modellare, Fila via sempione 2/C, 20016 Pero Milano Italy, <http://www.fila.it>) worked best. The mouse was held in left lateral recumbency with the left hand, and a small ball of clay was picked up with the right hand and flattened against the right thumb. Then, the clay was quickly pressed against the inside of the right external ear with the right thumb, leaving a distinct mound-shaped cast of the hole that dried quickly for measurement and storage.

To determine if clay measurements correlate with direct reticle readings, we punched holes in the right ears of 15 mice and measured the longest and shortest diameters of the holes at day 21 with both the reticle and the clay. The average diameter measurements produced by both methods were highly correlated (Supplemental Fig. 1a), although the clay tended to result in slightly higher measurements at very small hole sizes (less than 0.5 mm), and then slightly lower measurements with intermediate and larger hole sizes (Supplemental Fig. 1b).

FIG. 2. Age-at-punch effect. Mice punched at middle age are quantitatively better regenerators than young postweaning mice. 2-mm holes were punched in the ears of BALB/c (a,c) or B6 (b,d) mice at various ages, and measured weekly. (a and b) Kinetics of closure. (c and d) The percent hole closure at 2 weeks postpunch (*y* axis) for mice punched at different ages (*x* axis). By Dunn's multiple comparison test, postweaning mice (punched at either 1 or 2 months) did not differ significantly. Therefore, we combined these into one group for statistical comparison with mice punched at later ages. For BALB/c, postweaning mice differed from mice punched at 7 months by $p < 0.001$, and from mice punched at 8 months by $p < 0.01$. For B6 mice, postweaning mice differed from mice punched at 5 months by $p < 0.01$, and from mice punched at 8 months by $p < 0.001$; $n = 8-10$ mice/group.

Quantitative analysis of hole closure

Percent hole closure was calculated by subtracting the area of the experimentally measured hole from the area of the original punched hole (which equals the area of new regenerating tissue at the hole margin), dividing by the original hole area and multiplying by 100 to get “% hole closure.”

Percent hole closure = $(O - E) \times 100 / O$, where O (area of original circular hole) = $\pi r^2 = 3.14 \text{ mm}^2$ for 2-mm holes, and E is the area of the experimentally measured hole. As revealed by our direct impression method, the closing holes are very rarely circular, suggesting that past estimates of percent hole closure, which are based on the area of a circle, are inherently inaccurate.¹² The holes are usually elliptical, hav-

FIG. 3. Similar histological appearance of regenerated ear hole tissues in middle-aged B6 and young post-weaning MRL mice at 4 months postpunch. Ears of B6 mice that had been punched at 8 months of age, and MRL mice punched at 2 months of age were examined at 120 days postpunch. Sections taken through the center of the position of the original hole show numerous regenerating cartilage islands intermixed with abundant new whitish adipocytes, and with some new sebaceous glands and hair follicles. Original magnification, 50 $\times$.

FIG. 4. Regenerative histological features become more pronounced with increasing age at punch. (a–c) Schematic showing how the histological sections were obtained and viewed. Cross sections through the ear (b) that extend above and below the hole create “tongues” of tissue when laid on glass slides, as seen in c, a section taken 6 days postpunch in a young B6 mouse. Original magnification, 100 $\times$. Primitive bluish-staining connective tissue (encircled in yellow) has begun to fill the space between the cut cartilage and the new epithelium, and constitutes early blastema tissue. (d–k) Comparison of B6 mice punched at different ages with MRL punched at 2 months of age. Sections were taken 6 days post punch (early blastema stage). (d–g) H & E staining, bright field. (h–k) Visualization under broad-spectrum fluorescein isothiocyanate (FITC) filter. Black areas represent areas containing no protein. White circle demarcates site of suspected chondro–epithelial interaction.

ing one long and one short axis. Because the area of an ellipse equals the product of half the major axis times half the minor axis times π , then $E = \pi[(M/2) \times (m/2)]$.

Statistical analysis of hole closure data

Variance among the medians of hole size at 2 weeks post-punch for mice punched at various ages was analyzed globally by Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks. Then, by Dunn’s multiple comparisons test, because hole closure of mice punched at 1 or 2 months of age was not found to be significantly different, we com-

bined these two groups into a single group for comparisons with mice punched between 5 and 8 months of age (see Fig. 2).

Results

As ear hole closure has been best studied in young MRL mice, we wanted to know if events occurring in our older B6 mice resembled what had previously been described in young “autoimmune” MRL mice.⁶ We first examined the overall macroscopic appearance of closed holes in young MRL and older B6 mice at 1 month postpunch to look for any obvious

FIG. 5. Correlation of adult body weight and hole closure. Using hole closure data from Li et al.¹⁴ and body weight data from the Jackson Laboratories Phenome database,^{16,17} we examined the correlation between hole closure and body weight, plotting the average adult body weight (female and male at 6 months) versus hole closure at 30 days postpunch. Spearman correlation analysis shows $p < 0.0096$. The solid line is the regression line, and the dashed lines show 95% prediction intervals.

differences. None were apparent. In both strains, holes filled so completely with new tissue that it was sometimes difficult to find the site of the original hole (Fig. 1).

To evaluate more fully the effect of age on the regenerative capacity of conventional (non-“autoimmune”) strains of mice, we compared 2-mm ear-hole closure rates in B6 and BALB/c mice that were punched at different ages. At all ages, the rate of closure seems to have two phases, an early rapid phase that lasts for about 2 weeks, followed by a much slower phase, during which the process is gradually completed (Fig. 2A,B). Although these two phases are characteristic of all ages, the details differ with age. The initial (“rapid”) phase was quite slow in mice punched at 1 month and increased with age. In addition, young mice never completed the process of macroscopic hole closure (BALB/c mice averaged 40%, and B6 averaged 65% replacement tissue), whereas mice punched at 8–9 months averaged 90–97%; many of them closed completely.

Figure 2, c and d, shows the percent hole closure at 14 days postpunch for mice that were punched at different ages. We chose day 14 because most growth had occurred by this point. For both BALB/c and B6 mice, ear closure improved with age, peaking at 8–10 months and then declining somewhat at 12 months. We conducted ANOVA on the 1–8 months at punch data for both strains and found statistically significant differences between the 1–2 months and middle-aged months at punch (see legend to Fig. 2 for details).

To determine if the biological processes underlying the ear closure in middle-aged B6 and BALB/c mice are similar to the well-known regeneration of young MRL mice, or if the older non-autoimmune mice were simply filling the holes with fibrotic scar tissue, we undertook histological analysis. Figure 3 compares the appearance of ear holes at 120 days postpunch from an MRL and a B6 mouse punched at 2 and 8 months of age, respectively. The holes have filled in similarly, with regrowth of adipocytes, chondrocytes, sebaceous glands (Fig. 4), and hair follicles. The main difference in the appearance of regenerated ear tissue in MRL and B6 mice is that melanocytes have also regenerated in the dermis of the B6 mouse. That these elongated brown cells, which appear in all B6 histopathology images at all ages at punch, were not seen in MRL and are indeed melanocytes was confirmed by inspection at 400 $\times$ magnification (Supplemental Figs. 2 and 3). Thus, the hole closure in middle-aged B6 mice is indeed regenerative.

To examine what histological differences might underlie the age-related improvement in regeneration, we examined the regenerating tissues of increasingly old B6 mice at day 6 after punch, using both H & E bright-field illumination (where protein appears pink) and fluorescence imaging (where the eosin/protein complex glows yellow). The most obvious age-related difference in B6 was in the thickness of the early apical epidermis (Fig. 4f), which at 9 months at punch was much like the classical “apical epidermal cap” seen in MRL mice (Fig. 4g) and in amphibian limb regeneration. Young B6 mice developed thinner apical caps. In other respects, both young and old B6 mice showed strikingly amphibian-like regenerative features. Under bright-field illumination, beginning at about day 3 postpunch, a bluish-staining primitive “myxomatous” connective tissue appeared under the new epithelium, instead of the pink-staining granulation tissue that precedes scar formation. Using fluorescence imaging, the myxomatous tissue appeared as a black region with no basement membrane above it, suggesting that these regions were low in protein and high in carbohydrate (most likely hyaluronate¹³), similar to the myxomatous matrix of amphibians and embryos. At all ages, the regenerating epidermis generated downgrowths oriented toward the mature cartilage (demarcated by a white circle), a feature similar to classical embryonic epithelial-mesenchymal interactions. Thus, regeneration is not limited to one “autoimmune” mouse strain, but occurs in common non-autoimmune strains, improves with age, and exhibits the stages of classical amphibian epimorphic regeneration (Supplemental Figs. 4 and 5).

That MRL mice are not alone in their ability to regenerate had been hinted at earlier. Geneticists looking for the genes that might govern the MRL mouse’s ability to regenerate studied the four founder strains from which the MRL mouse was created, and found that, of the four, the “Large” (LG) mouse also had the ability to completely close experimental ear holes.¹⁵ Because the MRL mouse is also an extremely large mouse, we were intrigued by the possibility that size might correlate with early regeneration ability. In the only previous study to explore the influence of body size on ear hole closure, Li et al. compared the *rate* of ear-hole closure of several strains punched at 5 weeks and concluded that body weight had little influence.¹⁵ We revisited this question, comparing instead, the *extent* of closure at a single late time point (day 30) with adult mouse strain body weight at

6 months. We obtained our closure data directly from Li, as a colored graph of Fig. 1 from their paper. For body weight data, we used the average adult weight (including males and females) of each strain.^{16–18} Figure 5 shows the comparison for 18 of the 26 strains in the Li study for which body weight data at 6 months were available, revealing that there is indeed a correlation between average adult body weight and the extent of hole closure.

Discussion

Taken as a whole, our data show that regeneration is not the unique property of one or a few odd “autoimmune” genotypes of mice, such as the MRL mouse or its predecessor, LG¹⁴, but that it seems to be a generic property of mouse ears in many strains. This property has previously been missed because investigators routinely use mice at quite young ages, usually 4–8 weeks. The ability to regenerate increases with age and then regresses after 1 year. The regeneration seen in middle-aged mice mimics the stages of epimorphic regeneration seen in amphibians, proceeding through the classical stages of an early “apical epidermal cap,” a carbohydrate-rich swollen blastema, and a thinning and differentiating “palette” stage during which internal structures such as dermis, cartilage islands, fat tissue, blood vessels, and hair follicles/sebaceous glands (and melanocytes in B6 mice) form and fill the hole.

Our finding that strains differ less in their intrinsic capacity for ear-hole regeneration than in the age at which they express that ability led us to reassess the predominant paradigm and to look for age-related features that might correlate with the ability to regenerate. Revisiting an earlier study in which ear-hole closure was measured at 5 weeks of age in 26 mouse strains,¹⁵ we saw that body size correlated strongly with the ability to close the holes (Fig. 5). Thus, large strains seem to have regenerative capacity early in life, whereas the smaller strains (like B6 and BALB/c) acquire it later. What might be the biological basis for this finding?

We can envisage two different possibilities: The first possibility is that there is a biological age (or stage of development) at which regeneration capacity becomes possible, and large strains reach it more quickly than smaller strains because large mice are aging more rapidly.¹⁹ In this scenario, the actual regenerative process in young large-strain mice is the same as that seen in older small-strain mice. The second possibility is that large-strain mice may actually go through two different tissue replacement phases as they age: a generative phase and a regenerative phase.²⁰ During their extensive early growth period, while their ears are growing rapidly, they fill ear holes with the same generative process that they use to enlarge the ear. Once growth slows down, however, “regeneration,” with the classic processes of dedifferentiation and redifferentiation, becomes the mechanism of ear-hole closure. We are now undertaking global gene expression analysis to distinguish these two main possibilities.

Although it might be argued that our data are not comparable with prior studies, because we use new methods of inducing and measuring holes, several of the earlier studies actually had data similar to ours that were either missed or underinterpreted. For example, in the first description of the regenerative phenotype of the MRL mouse in 1998, the B6 mouse was presented as a prototypical nonregenerative

“scarring” control.⁶ A key histopathologic image, however, showed the presence of newly regenerated cartilage islands in both the MRL mice and the B6 “control” at 4 months post-punch (Fig. 5 in ref. 6), but those in the B6 seem to have been missed. In another study, Ferguson’s group found that neither B6 nor BALB/c ear hole tissues actually scar, and concluded that there is some limited regeneration of multiple tissue types in both strains.⁹

It seems counterintuitive that aging should enhance regenerative ability. Yet our data clearly point in this direction. There is a classic case in humans, where children that fall off bicycles often end up with large, stretched, scars under their chins, whereas adults with similar accidents have far less obvious sequelae.²¹ Ashcroft and co-workers had shown, in fact, that older adults heal excisional wounds induced in their upper inner arm with a far higher-quality process, resulting in more nearly normal dermal architecture, than do children. In particular, they found a “regenerative pattern” of elastin and fibrillin arcades at the dermo-epidermal junction in wounds of aged subjects.²² This finding has now been corroborated by Ferguson’s large human study.²³ Thus, counterintuitive as it may be, our data clearly suggest that at least some forms of regeneration seem to get better with age. We are presently analyzing the differences between young and older mice in attempts to discern the critical differences.

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Authors Disclosure Statement

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