

# Regeneration or Scarring: An Immunologic Perspective

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Complete regeneration of complex tissues and organs is usually precluded by fibrotic reactions that lead to scarring. Fish, salamanders, and larval anurans are among the few vertebrates capable of regenerating lost appendages, and this process seems to recapitulate ontogenic development of the structure in most respects. Recent work has revealed a capacity for excellent regeneration in certain mammalian tissues: embryonic or fetal skin and the ear of the MRL mouse. Analyses of these two systems suggest that processes of regenerative growth and patterning for the formation of new structures such as hair follicles may involve modulation of the inflammatory response to the injury in a way that reduces fibrosis and formation of scar tissue. We review evidence that this modulation includes changes in cytokine signaling and may involve properties of the extracellular matrix mediated by factors that include hyaluronic acid and “anti-adhesive substrates” such as tenascin-C. New studies and classic work on the capacity for limb regeneration in amphibians are then reviewed, focusing on the loss of this ability in prometamorphic anuran hindlimbs and the view that changing properties of the immune system may also underlie the declining regenerative potential in this system. Finally, we review recent work in comparative and developmental immunology, which raises the possibility that phylogenetic changes in regenerative capacity may be the result of evolutionary changes in cellular activities of the immune system. *Developmental Dynamics* 226:268–279, 2003. © 2003 Wiley-Liss, Inc.

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## INTRODUCTION

Even when it occurs with optimal efficiency, wound repair in most vertebrate organs is dominated by a fibroproliferative response that produces a fibrotic scar. The injured organ is patched rather than restored to its original state (Clark, 1996). Only in a very restricted number of vertebrate species and tissues is the initial phase of repair followed by perfect restoration or regeneration of an organ both structurally and functionally. Comparing the process of tissue repair with the ability of certain am-

phibians to regenerate complete limbs, Goss (1987, 1992a,b) emphasized that, although the initial responses to injury in both situations appear similar, epimorphic limb regeneration is unusual in allowing the epithelial-mesenchymal interactions and other reciprocal cell signaling needed to reproduce the missing organ.

In the past 20 years, tremendous progress has been achieved in understanding the cellular and molecular events of wound repair, but the tendency among vertebrates for

scarring rather than regeneration remains unexplained. We will review models for regeneration in which, like the amputated salamander limb, injury is followed by complete regeneration and suggest specific aspects of the process on which future attention should be focused to better understand the regenerative capacity. The activation and response of fibroblasts are of key importance in determining the nature of tissue repair: signaling events involving these cells can mean the difference between scarring or rees-

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establishing the appropriate growth and patterning events needed to actually regenerate all the missing structures of a fully functional organ.

Normal wound repair is highly dynamic, consisting of several overlapping phases (Schaffer and Nanne, 1996; Singer and Clark, 1999). Tissue injury disrupts capillaries, which immediately triggers activation of platelets to begin the clotting cascade and the events of inflammation. Neutrophils enter the injured tissue with the major function of removing bacteria, but these cells and other leukocytes release a variety of proteases, growth factors, and other cytokines with profound effects on the repair process. When epidermis is also involved, keratinocytes dissolve their junctions with one another and with the basement membrane and begin to move across or through the provisional matrix of the fibrin clot to gradually re-close the epithelial surface. Monocytes arriving at the site greatly increase the macrophage population to remove debris, dead cells, and fibrin and to facilitate changes in the wound matrix. Factors released from macrophages, keratinocytes, and other sources during inflammation activate fibroblasts to add hyaluronate and glycoproteins such as fibronectin to the extracellular matrix (ECM), after which these cells gradually shift to producing proteoglycans and collagens (Singer and Clark, 1999). The developing new granulation tissue undergoes angiogenesis, and gradually some fibroblasts become myofibroblasts, which bring about compaction of the matrix and contraction of the wound. Over the next several days, a continuing highly regulated process of collagen turnover and apoptosis, in which macrophages and fibroblasts appear to be the major players, remodels the matrix and reduces the level of vascularity, converting the granulation tissue into scar tissue.

The cellular interactions regulating the successive phases leading to the scar are poorly characterized. Hyperplastic scars with excessive collagen were recently reported in transgenic mice with mesenchymal cells overexpressing a stabilized form of

$\beta$ -catenin protein, a key component of the canonical Wnt signaling pathway (Cheon et al., 2002). Proliferating fibroblasts of granulation tissue express *Wnt* (Labus et al., 1998), and these data suggest that tight regulation of cell signaling is important in determining the extent of fibrosis in normal wound healing.

### SCARLESS WOUND HEALING AND REGENERATION IN MAMMALS

To appreciate the differences between regeneration and the usual fibrotic outcome of tissue repair, it is instructive to briefly review examples in which mammalian tissues undergo repair without scarring. Such phenomena range from the healing of corneal incisions (Fini, 1999) to the annual replacement of large complex appendages: the antlers of deer (Goss, 1992a). A dramatic example, well-studied recently, is cutaneous wound healing in the embryo and early fetus (for reviews of fetal wound healing, see McCallion and Ferguson, 1996; Garg and Longaker, 2000). Minimal inflammation and complete restoration of normal skin, including the normal pattern of reticular collagen, capillaries, hair follicles, and glands, have been demonstrated after incisional wounds in embryos and fetuses of several species. Comparisons among the many studies are complicated by different protocols, wound types and severities, species and prenatal stages of the test subjects, etc. It is clear, however, that scarless healing occurs through a gestational age equivalent to the early third trimester of humans, after which there is a transition to the normal type of scarring repair seen with adult skin. The ability of fetal skin to regenerate normal structure is at least partly site-specific, because similar wounds in the fetal myocardium, diaphragm, trachea, and the gastrointestinal tract do heal with scars (McCallion and Ferguson, 1996; Stelnicki et al., 1999; Longaker, 2001).

The minimal inflammatory response and scarless quality of healing in fetal skin initially suggested the importance of the sterile aqueous environment within the amnion for

this process, but at least two kinds of experiments indicate otherwise. Marsupials such as the opossum are born at a developmental stage equivalent to that of a late first trimester human fetus and are raised in the mother's pouch. Despite the absence of a sterile aqueous environment, incisional wounds in newborn opossums heal with minimal inflammation and no scars (Armstrong and Ferguson, 1995). Conversely, it was shown, that sheep skin from an adult or late fetus grafted onto a young fetus and subsequently wounded by incision forms scar tissue, despite its exposure to amniotic fluid and perfusion by fetal blood (Longaker et al., 1994). Such experiments indicate that scar-free healing is not a function of the prenatal environment and instead point to the degree of skin differentiation or the maturity of other organ systems as more important determinants of efficient repair or regeneration (McCallion and Ferguson, 1996).

Certain components of the dermal ECM and their rates of synthesis and degradation during repair differ significantly in embryos and adults. Fetal skin is enriched with high molecular weight hyaluronic acid (HA) in a highly hydrated state and this glycosaminoglycan has a major effect on events in the fetal wound microenvironment. HA directly promotes fibroblast migration, protects cells against free radical damage, influences both the amount and nature of collagen production, and forms a matrix that binds protein inhibitors of serine proteinases such as plasmin, cathepsin G, and activators of matrix metalloproteinases (Chen and Abatangelo, 1999). Synthesis of HA persists longer in fetal than in adult wounds, and this can moderate the inflammatory response in several ways to reduce scarring (Chen and Abatangelo, 1999). High molecular weight HA, in contrast to its degradation fragments, inhibits both angiogenesis and immigration of leukocytes (Savanti et al., 2000; Balazs and Larsen, 2000). Thus, the HA-rich microenvironment of fetal wounds may account at least in part for their reduced vascularity and much lower level of macrophage infiltration and

inflammation compared with the granulation tissue of adult wounds.

Collagen fibrillogenesis in fetal skin wounds occurs rapidly and with little subsequent remodeling produces a reticulum of collagen types I, III, IV, and VI indistinguishable from that of the surrounding dermis (Chin et al., 2000). Fibroblasts of adult wounds, in contrast, deposit primarily type I collagen fibers, which aggregate into fibrillar bundles and provide the new tissue with increasing tensile strength but which continue to develop gradually and form a scar as HA and other ECM components break down (Lindblad, 1998; Chin et al., 2000).

Differences in the concentrations and locations of various proteoglycans and glycoproteins such as fibronectin between fetal and adult wounds seem to be less significant than the disparity in levels of HA and types of collagens. One important exception is tenascin-C, which accumulates more rapidly and at higher concentrations in fetal than in adult wounds (Whitby and Ferguson, 1991). Interestingly, tenascin-C is also found in other injured tissues where the outcome is regeneration rather than scarring (see below). This factor exists as a large asterisk-shaped complex of six similar subunits and is one of a structurally diverse group of "de-adhesive" ECM components, which produce an intermediate type of cell adhesion. Such matrix components allow cell shape changes and motility but prevent both the apoptosis associated with weak substrate adhesion and the differentiation associated with formation of focal adhesions and stress fibers (Murphy-Ullrich, 2001.) The presence of tenascin-C in the environment can actually antagonize the proadhesive effects of collagen, fibronectin, laminin, etc., a property that may help maintain the undifferentiated state. In addition to its de-adhesive properties, tenascin-C also has immunomodulatory effects, inhibiting the activation of T-cells and their secretion of IL-2 (Hemesath et al., 1994; Hibino et al., 1998). The rapid accumulation of this matrix component in fetal wounds may help to suppress the proinflammatory effects of cytokines released in

the immediate aftermath of such injury (McCallion and Ferguson, 1996).

In addition to the persistence of HA and abundance of tenascin-C, the lack of inflammation in fetal wounds may also involve reduced platelet degranulation and a failure to form distinct fibrin clots. Diminished release of platelet factors, together with features of the ECM mentioned above, may in turn explain the lower amount of infiltration by neutrophils and macrophages. These cells together with platelets are major sources of transforming growth factor- $\beta$  (TGF- $\beta$ ) in wounds, which is of particular interest because of this factor's well-known association with fibrosis in many clinical and experimental contexts (Border and Ruoslahti, 1992; Shah et al., 2000).

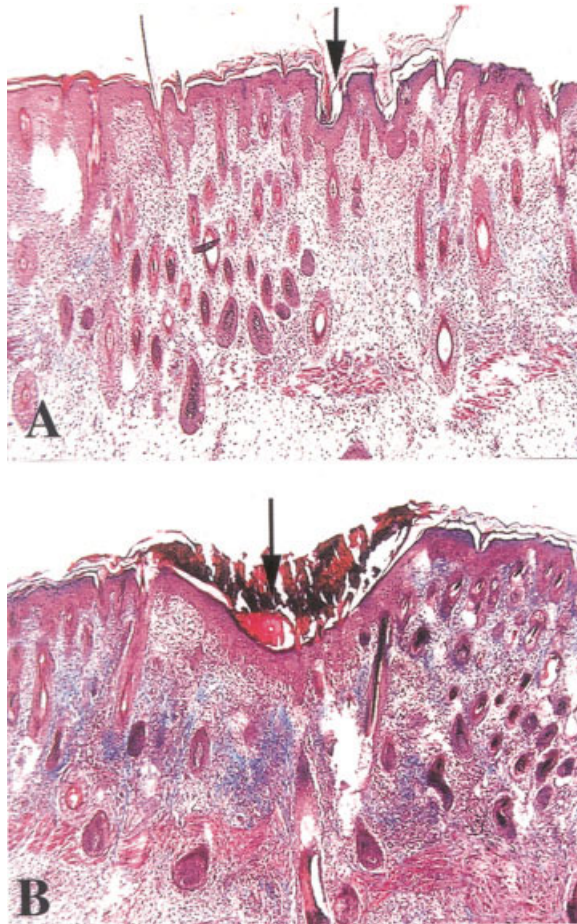
When fetal skin is injured more severely by cauterizing with a hot needle to produce localized necrosis, macrophages do immigrate in significant numbers, inflammation occurs, and a scar subsequently develops (Hopkinson-Woolley et al., 1994; McCallion and Ferguson, 1996). Importantly, such experiments demonstrate that fetal monocytes/macrophages are able to respond to chemotactic signals, accumulate in wounds, and mount an inflammatory response and that fetal dermis is in fact capable of scarring. Moreover, TGF- $\beta$  has been directly implicated in this response. Exogenous TGF- $\beta$ 1 applied to fetal wounds induces a major fibrotic reaction and scar formation, (McCallion and Ferguson, 1996) and reducing the levels of TGF- $\beta$ 1 and 2 in adult skin wounds by using either antibodies, antisense oligonucleotides, or a synthetic antagonist inhibits fibrosis and scar formation (Shah et al., 2000; Huang et al., 2002). As discussed by Stelnicki et al. (1999), the effects of TGF- $\beta$  and other cytokines released during inflammation clearly determine the extent of scarring or regeneration after injury.

Signaling cascades that act earlier than TGF- $\beta$  in the wound response may play a major role in the regulation of fetal wound healing. Production of proinflammatory cytokines such as interleukin-6 (IL-6) is minimal in fetal skin wounds com-

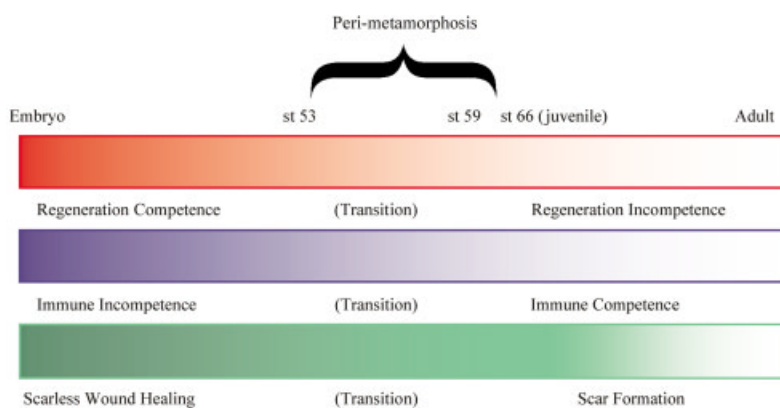
pared with those of adults (Liechty et al., 2000a). Moreover, fetal fibroblasts produce significantly less IL-6 and IL-8 than fibroblasts from adults when stimulated with platelet-derived growth factor (Liechty et al., 1998, 2000a). Because IL-8 recruits neutrophils to sites of inflammation and IL-6 both recruits and directly activates monocytes/macrophages, reduced production of these cytokines in fetal wounds explains further the relative absence of these leukocytes and the greatly reduced inflammatory response. This hypothesis was tested by using IL-10, a potent anti-inflammatory cytokine made during fetal development that deactivates macrophages and inhibits expression of both IL-6 and IL-8. Wounds in fetal skin of IL-10 knockout mice contained a significantly greater density of inflammatory leukocytes compared with controls and formed definite scars (Fig. 1; Liechty et al., 2000b). These data support the view that inflammation is actively suppressed in fetal wounds, leading to the observed paucity of macrophages and other leukocytes, less paracrine release of TGF- $\beta$  and other fibrogenic cytokines, and therefore, less scarring overall.

Stelnicki et al. (1999) have reviewed data showing that genes involved in patterning embryonic skin, including genes required for normal development of limbs, jaws, and axial skeleton, are differentially expressed during fetal but not adult skin wounds. Given the multiplicity of cytokine signals in cutaneous wound healing and the central role of factors from leukocytes (Gillitzer and Goebeler, 2001), future work on scarless healing must examine the possibility that proinflammatory factors in the adult wound microenvironment directly inhibit expression of genes required for tissue patterning. Several cross-signaling mechanisms have been described in which proinflammatory mediators exert antagonistic activities in gene transactivation (Verrecchia and Mauviel, 2002; Ghosh, 2002).

Another example of organ regeneration in mammals is the closure of full-thickness holes in the pinna of the ear that involves cartilage regeneration as well as scar-free heal-



**Fig. 1.** Sections of fetal mouse skin from the study of Liechty et al. (2000b) showing normal (A) and interleukin (IL)-10 knockout skin (B) 7 days after wounding by incisions at sites indicated by the arrows; stained with Masson's trichrome. Blue staining indicates dense collagen. Increased collagen deposition and loss of hair follicles, both indicative of scarring, are seen in the absence of the anti-inflammatory cytokine IL-10. (Used by permission of the *Journal of Pediatric Surgery*.)



**Fig. 3.** Time course of *Xenopus* development showing the correlation between the changes in regenerative capacity, immune system, and scarless wound healing. The transition periods occur around the time of metamorphosis. Note that the experiments on scarless wound healing used *Rana catesbeiana* (Yannas et al., 1996).

ing of two skin surfaces. Of the many species examined, this process normally occurs only in the two families

of the order *Lagomorpha*, represented by rabbits and pikas, and fails in all rodents, including those



**Fig. 2.** Larval *Xenopus* hindlimbs 21 days after amputation at the mid-tibia and -fibula level, indicated by the dotted lines. Regeneration-competent stage 53 limbs have produced nearly complete limbs, whereas regeneration-incompetent stage 59 limbs have produced only the cartilaginous spike-like outgrowths discussed in the text.

with very rabbit-like ears such as certain species of cavy (Goss, 1987). Regeneration in the ear is of particular interest because it shares certain aspects of the epimorphic, i.e., blastema-based, regeneration seen with vertebrate appendages. As described by Joseph and Dyson (1966) epidermal cells migrate across the cut edge of the wound and form an epithelium much thicker than the adjacent epidermis and lacking an underlying dermal layer. Unlike most excisional wounds, holes in the rabbit ear heal with very little skin contraction, perhaps due to this skin's tight binding to the fibrous perichondrium. By 2 weeks after the injury, the thickened epithelium covers a large "blastema" of fibroblastic cells similar histologically to granulation tissue and extending out from the edge of cartilage. Cells of the cut perichondrium become more loosely arranged, begin to proliferate, migrate out into the developing blastema, and chondrogenesis is under way with formation of new elastic fibers by 4 weeks after injury (Joseph and Dyson, 1966). By this time, the new epidermis begins formation of hair follicles. During the following weeks, outgrowth of new cartilage is

rapid and differentiation of the skin continues with the appearance of hair and sebaceous glands. Circular holes, 1 cm in diameter, can close completely with normal cartilage and skin in 6 to 8 weeks (Goss, 1987). The process is significantly faster in male rabbits than in females and much faster still in rabbits concurrently healing midline incisions through the abdominal wall (Joseph and Dyson, 1970).

A breakthrough toward eventually understanding this model of tissue regeneration was the recent serendipitous discovery that mice of the MRL strain closed full-thickness 2-mm-diameter ear punches in a manner very similar to that of rabbits (Clark et al., 1998). Mice of other strains re-epithelialized the wound edge and formed scar tissue around the hole with little closure. Injured ear tissues of MRL mice were swollen and showed markedly more cell proliferation and migration, angiogenesis, and ECM production. Holes generally close in approximately 1 month, with complete reformation of the cartilage plate occurring later (Clark et al., 1998; Heber-Katz, 1999). Heber-Katz (1999) reported significant differences in expression of various proteases and their inhibitors in ear tissues of MRL and control mice. Moreover, by using immunohistochemistry, Heber-Katz showed that tenascin was present in the regenerating ear tissue, but not controls, an observation of particular interest in light of tenascin's potential importance in fetal skin wound healing. The responses to excision in ears of MRL mice are consistent with the view that differences in the inflammatory and growth responses determine the outcome. Heber-Katz and colleagues have more recently reported a process that appears to resemble heart regeneration as described in newts. They showed that ventricles of MRL mice when injured transmurally with a cryoprobe heal the wound with granulation tissue, which is replaced by proliferating cardiomyocytes that restore normal myocardium tissue, unlike hearts of control mice, which undergo extensive fibrosis after the same injury (Leferovich et al., 2001).

Mice of the MRL strain display im-

munologic abnormalities and have been intensively studied as models for certain autoimmune diseases. Genetic analyses of their capacity for ear tissue regeneration have clearly shown it to be a complex multigenic trait (McBrearty et al., 1998; Masinde et al., 2001) involving genes with additive effects and at least one dominant repressor gene (Li et al., 2001a). By using microarrays to examine genes differentially expressed in ears as early as 24 hr after punch injury in MRL and nonhealing B6 mice, Li et al. (2001b) identified many genes that collectively help delineate differences that lead to regeneration or scarring. Of the known genes (non-ESTs) whose expression is increased more than two-fold after injury in MRL but not in B6 mice, nearly half (10 of 21) are genes involved directly in wound repair, i.e., genes whose products represent basic components for tissue regrowth and only three are related to inflammation. In contrast, 10 of 18 of the known genes similarly up-regulated in B6 but not in MRL mice are involved in inflammation, and only three are related to tissue growth. Of interest, in light of the potential importance of thrombin in dedifferentiation events of newt limb regeneration (Tanaka et al., 1999), expression of the thrombin inhibitor heparin cofactor II showed the greatest increase (11.3-fold) in ears of B6 mice compared with those of MRL mice. Of the five known genes in which Li et al. (2001b) found expression *decreased* more than two-fold in MRL mice, two coded for procollagen and one for selenoprotein P, a plasma protein with properties of an extracellular antioxidant whose expression is inhibited by TGF- $\beta$ 1 (Mostert, 2000). Collectively, the data of Li et al. (2001b) indicate that tissue regeneration in the ear is accompanied by changes in gene expression expected to dampen the normal inflammatory response and to enhance tissue growth.

This work with two new models of mammalian tissue regeneration indicates that inhibition of specific proinflammatory cytokines or decreased expression of genes that promote inflammation may contribute to improved wound healing. However, tis-

sue regeneration is clearly impaired when the immune system and inflammation are severely restricted, e.g., by corticosteroids and in wounds with increased levels of proinflammatory cytokines such as in sepsis or chronic ulcers (Clark, 1996; Singer and Clark, 1999). A more likely interpretation of the current work is that tissue regeneration depends on a precise balance between pro- and anti-inflammatory factors that in turn determines whether the inflammation is resolved with fibrosis and scarring or with the expression of tissue and organ-specific patterning genes that can direct formation of histologically normal and functional tissues.

## REGENERATION IN ANURANS

Amphibians are masters of tissue and organ regeneration. As reviewed by Thouveny and Tassava (1997), regeneration occurs in limbs, tails, jaws, and certain eye tissues in urodeles (salamanders and newts) and larval anurans (frogs and toads). Experiments designed explicitly to examine scarring in urodeles have not been reported, but numerous studies emphasize the rapidity of epithelial migration and the low level of fibrosis after injury, suggesting that scar formation is usually not significant in these animals (Schmidt, 1968; Stocum, 1995). In light of the potential for tenascin-C to influence events of regeneration and inflammation, it is noteworthy that normal epidermis of newts, unlike mammalian skin, is rich in this factor (Donaldson et al., 1991) and that tenascin is also abundant in dedifferentiating tissues of amputated newt limbs or tails (Onda et al., 1990, 1991; Arsanto et al., 1990). In the only study of scarless healing in anurans, Yannas et al. (1996) examined closure of full-thickness excisional skin wounds on the backs of larval and adult bullfrogs (*Rana catesbeiana*). The results indicate that the percentage of the original wound area closed by regenerated skin was highest in young larvae but declined rapidly during prometamorphosis as dermal contraction became more prominent. Only after metamorphosis do such



wounds show scarring (Yannas, 2001).

That developing anurans lose the capacity to regenerate amputated limbs at metamorphosis has been documented in many species (Polezhayev, 1946; Forsyth, 1946; Goode, 1967; Stocum, 1995), but has been most thoroughly studied in *Xenopus laevis* (Beetschen, 1952; Komala, 1957; Dent, 1962; Wolfe et al., 2000). Hindlimb buds appear at Nieuwkoop-Faber (1967) stage 47 and regenerate perfectly in a manner resembling embryonic regulation if amputated at any time through stage 52, which is approximately when chondrogenesis of the femur begins. The regeneration process is similar to that of urodele limbs, with rapid epithelial closure of the wound followed by thickening of this wound epithelium, distal accumulation of proliferating mesenchymal cells, outgrowth of the blastema, and finally proximal redifferentiation of new limb tissues in continuity with those of the stump and morphogenesis of the missing parts (Mescher, 1996). Amputation of developing hindlimbs at progressively later stages results in increasingly deficient pattern formation, manifested by smaller regenerates with less muscle and progressive loss of digits in an anterior-to-posterior direction (Overton, 1963; Muneoka et al., 1986).

The capacity for normal regeneration is lost first in proximal limb regions, so at any given stage, amputations at a distal level produce more complete regenerates than proximal amputations (Dent, 1962), leading to the suggestion that regenerative potential is lost as tissues of the developing limb differentiate. A significant exception to this general rule was reported by Wolfe et al. (2000), who found that regenerative capacity is greater, especially at the later stages of metamorphosis, when limbs are amputated through the ankle or tarsal-metatarsal joint rather than through the tarsus, which begins to undergo ossification at stage 55–56. They suggested that the state of ossification at the level of amputation is important for the outcome of regeneration. Another possibility is that tenascin-C, which is

associated with regeneration in many other systems as described above and is highly concentrated in developing tendons and myotendinous junctions of joints (Onda et al., 1990; Järvinen et al., 1999), may contribute to the greater regenerative capacity after amputation through these regions.

At stage 59/60, when metamorphosis is under way, or at later stages, amputation in *Xenopus* is followed by the gradual outgrowth of a heteromorphic, nonsegmented "spike" of skin-covered cartilage devoid of muscle, as shown in Figure 2 (Dent, 1962; Goode, 1967). It has been a matter of some debate whether formation of such cartilage rods represents an example of actual, albeit pattern-deficient, epimorphic regeneration (Goss and Holt, 1992) or tissue repair with excessive chondrogenesis (Korneluk and Liversage, 1984). Most evidence favors the former interpretation: the limbs close with wound epithelia and small, abnormal pseudoblastemas (Komala, 1957) or fibroblastemas (Korneluk and Liversage, 1984) derived primarily from fibroblastic periosteal cells do form. Moreover, growth of such blastemas depends on nerves (Korneluk et al., 1982; Endo et al., 2000) and the apical wound epithelia (Goss and Holt, 1992), both of which are characteristic requirements of epimorphic regeneration but not usually needed for tissue regeneration or repair. Many anurans besides *Xenopus* also form outgrowths that lack a normal skeletal pattern and muscle if hindlimbs are amputated after metamorphosis (Stocum, 1995), whereas in some species, such as *Rana sylvatica*, there is no formation of wound epithelia, pseudoblastemas, or any sort of regenerate at all (Forsyth, 1946).

The numerous histologic studies of anuran limbs amputated after metamorphosis have clearly shown that even when the cut surface is initially closed with wound epithelium, there is little tissue dedifferentiation or proliferation of mesenchymal cells. In limbs where no epimorphic regeneration normally occurs at all, the wound heals with complete skin, the cut skeletal ele-

ments form a callus of new cartilage, muscle is repaired, and a collagen-rich connective tissue scar or pad fills the distal stump areas (Forsyth, 1946; Schotté and Harland, 1943). Carlson (1974, 1978) characterizes this response to amputation as one essentially involving only scarring and tissue repair. Even in post-metamorphic *Xenopus* and other anuran limbs where pseudoblastemas and heteromorphic spikes form, there is little dedifferentiation of stump tissues and the apical wound epithelium is quickly underlain by connective tissue (Komala, 1957; Dent, 1962; Korneluk and Liversage, 1984). It is apparent from these descriptions that the failure of limb regeneration involves precocious or excessive proliferation of fibroblasts and fibrosis rather than formation and growth of a population of mesenchymal cells derived from all the limb tissues. The fibrotic tissue has long been interpreted to inhibit or interfere with the normal course of limb regeneration in some manner (Komala, 1957; Goode, 1967; Carlson, 1974).

Analyses of gene expression in regenerating and nonregenerating *Xenopus* limbs support the view that regenerative failure is due to interference with normal controls on patterning genes. Most evidence from amphibian limbs indicates that patterning in epimorphic regeneration involves mechanisms that are similar to those operative during limb ontogeny (Géraudie and Ferretti, 1998), although the question of whether epimorphic regeneration recapitulates all details of ontogenesis is still unanswered. For example, Gardiner et al. (1995) found that HoxA13 and HoxA9 expression do not follow the developmental spatial colinearity rule during axolotl limb regeneration. *Xenopus* limb blastemas at regeneration-competent stages express key genes involved in patterning, including *fgf2* (Cannata et al., 2001), *fgf8* (Christen and Slack, 1997), and *fgf10* (Yokoyama et al., 2000). Also expressed are the negative regulators of differentiation and markers for dedifferentiation, *msx1*, *ld2*, and *ld3* (Shimizu-Nishikawa et al., 1999; Endo et al., 2000); *shh*, which mediates for-

mation of the anteroposterior axis (Endo et al., 2000); *Lmx-1*, which is involved in establishing the dorso-ventral limb axis (Matsuda et al., 2001); and *HoxA13*, a marker for the distal limb region (Endo et al., 2000). Regeneration-incompetent *Xenopus* limb pseudoblastemas do not express the *fgf* genes (Yokoyama et al., 2000; Cannata et al., 2001), *shh* (Endo et al., 2000) or *Lmx-1* (Matsuda et al., 2001). Such pseudoblastemas, however, do express low levels of *msx* (Endo et al., 2000), *Id2*, and *Id3* (Shimazu-Nishikawa et al., 1999), and the distal marker *HoxA13* (Endo et al., 2000). These results are consistent with the fact that the pseudoblastemas of regeneration-incompetent limbs still show limited cellular dedifferentiation and produce cartilage rods by epimorphic regeneration.

In an important study, Yokoyama et al. (2001) showed that treating stage 56 *Xenopus* limb stumps, which are almost completely regeneration-incompetent, with beads containing FGF-10 not only rapidly stimulated expression of *fgf8* in the apical wound epidermis and later mesenchymal expression of *fgf10*, *msx1*, *shh*, and *HoxA-13*, but also produced, in 7 of 11 cases, regenerates having three or four segmented digits with correct anteroposterior polarity. Although the tarsus and tibia-fibula were still missing or defective in these limbs, this work indicates that FGF-10 can up-regulate several genes involved in dedifferentiation and pattern formation and can set conditions leading to the establishment of an anterior-posterior (A-P) axis, all of which produce a dramatic improvement in the regenerative outcome. One likely interpretation of these results is that augmenting or supplying the distal FGF-10 signal in amputated stage 56 hindlimbs either substitutes for or sets up the epithelial-mesenchymal interactions, and possibly other cell-cell signaling events, required for expression of *msx-1* and *shh* and for establishment of the A-P limb pattern.

Studies with *Xenopus* limbs thus suggest that the cellular interactions that normally establish patterns of positional information required for

normal regeneration are precluded in the limb stumps of metamorphic anurans, perhaps by the fibrosis. It has long been appreciated that the wound epithelium is required to convert the repair process triggered by amputation into growth and development of an epimorphic regeneration blastema and, moreover, that the wound epithelium is functionally similar to the apical ectodermal ridge (AER) required for limb formation in the chick or mouse embryo (Stocum, 1995). The AER is induced when FGF-10 produced in the underlying mesenchyme acts on the adjacent ectodermal cells to activate expression of *fgf-8* (Capdevila and Izpisua Belmonte, 2001). FGF-10 signals the epithelial cells to express specific *Wnt* genes (*Wnt-2b* in the wing bud, *Wnt-8c* in the leg bud) and the secreted protein products of these genes act in a paracrine manner, diffusing and binding surface receptors on neighboring epithelial cells, and signaling through  $\beta$ -catenin to activate *fgf-8* expression (Kawakami et al., 2001). Secreted FGF-8 in turn diffuses back to the mesenchyme, where it completes the regulatory loop by maintaining FGF-10 synthesis there and activates a small posterior domain of *Shh* expression within the incipient limb bud (Capdevila and Izpisua Belmonte, 2001). The integrity of these reciprocal signaling pathways across the epithelial-mesenchymal interface, which for normal embryonic tissue patterning requires finely tuned regulation of the activity and availability of FGF and its receptors, involves heparin sulfate and other components of the ECM (Ornitz, 2000). This complex signaling system may be easily disrupted in the inflammatory and fibrotic microenvironments of the limb stump where the balance between tissue repair and blastema formation is determined. Even slight increases in the production or stability of collagen due to changed activity of TGF- $\beta$  may be sufficient to disrupt the FGF signaling loop and squelch events that would begin epimorphic regeneration (Ghosh, 2002).

Regeneration of amphibian limbs is enhanced by measures designed to stimulate tissue dedifferentiation

or interfere with fibrosis. Polezhaev (1972) applied additional mechanical trauma to limb stumps of prometamorphic *Rana* and obtained regenerates with five typical digits in many cases, a result similar to that with exogenous FGF-10 in *Xenopus* limbs of the same stage (Yokoyama et al., 2001). Limb stumps of post-metamorphic *Rana* produce hypomorphic regenerates after topical treatment with concentrated salt solutions (Rose, 1944) or dimethyl sulfoxide (Cecil and Tassava, 1986). These studies indicate that the loss of regenerative capacity in anuran limbs during metamorphosis is not irreversible and that local factors in the limb stump may be more important than hormonal or other systemic changes. The latter point is underscored by other work on the regenerative ability of limbs transplanted between larval and postmetamorphic *Xenopus* (Sessions and Bryant, 1988).

During metamorphosis, anurans undergo major modifications in their hematopoietic, endocrine, and immune systems, all of which have global effects while also changing the constituents of local tissue environments (Yoshizato, 1998). Of these metamorphosing organ systems, the cells of the immune system are of particular interest because these cells exert their functions specifically at sites of injury, infection, or tissue rejection and often constitute an intrinsic long-term part of specific tissues such as skin and connective tissue (Jameson et al., 2002). Moreover as indicated earlier, the immune system is of primary importance in determining the response of a tissue to injury, the degree of inflammation, the extent of scarring, and the nature of the reparative response (Barbul, 1996; Slavin, 1996; Gillitzer and Goebeler, 2001). The potential importance of cells mediating the immune response to the events of amphibian limb regeneration was first recognized by Sicard (1985). We have begun to examine the relevance of metamorphic changes in the amphibian immune system for the regenerative capacity of such animals, testing the hypothesis that immunomodulatory interactions similar to those implicated in regenera-

tion of fetal skin and MRL mouse tissues are important for the regenerative capacity of urodeles and larval anurans.

## AMPHIBIAN IMMUNOLOGY

All amphibians possess the components of innate immunity, e.g., antimicrobial peptides, neutrophils, macrophages, the complement system, and factors protective against oxidative damage. Amphibians also possess immune signaling pathways such as those involving NF $\kappa$ B and molecular chaperones, as well as elements defining the adaptive or combinatorial immune system, e.g., antigen-recognizing lymphocytes, immunoglobulins (Ig) and Ig family T-cell receptors, products of the major histocompatibility complex (MHC), and recombinase activator genes 1 and 2 (Marchalonis et al., 2002). However, urodeles appear to be "immunodeficient" compared with anurans (Tournier et al., 1998), because although T and B lymphocytes and a diverse repertoire of Ig and T-cell antigen receptor genes are present, humoral immunity in urodeles is mediated only by IgM and is apparently amnesic. Moreover, assays of their cellular immunity indicate that it is very weak. Working with the axolotl, *Ambystoma mexicanum*, Tournier et al. (1998) present evidence for very restricted diversity within class II MHC in these animals and suggest this as an explanation for their subdued immune response. Nondiverse antigenic presentation could only poorly stimulate T-helper cells, resulting in extremely low cytokine synthesis and reduced cellular cooperation (Tournier et al., 1998). Low levels of cytokine production may account for the poor inflammatory responses in wounded tissue of urodeles. For a more in-depth review of amphibian immunology, see Du Pasquier and Flajnik (1999) and Robert and Cohen (1998).

By far the most widely used model in amphibian immunology is *Xenopus laevis*, which is particularly valuable because it offers a perspective on immunology not seen in most other vertebrates: the dramatic changes of metamorphosis that al-

ter every aspect of the animal. The larval immune system shows characteristics of an ancestral system functioning with fewer mechanisms for cell-mediated immunity (Robert and Cohen, 1998). The adult system appears remarkably similar to the mammalian system, with the ability to generate a potent cytotoxic response (Robert and Cohen, 1998). Moreover, the dramatic change in the immune system during metamorphosis seems to occur without memory transfer of previous antigenic stimuli (Turpen, 1998).

A fundamental difference between larval and adult *Xenopus* immune systems involves the antigen-presenting MHC class I and II proteins. MHC class I and II are critical signaling elements, which present cytoplasmic antigens to CD8+ and extracellular antigens to CD4+ T cells, respectively, thus initiating appropriate immune responses (Kaufman et al., 1990; Salter-Cid et al., 1998; Du Pasquier and Flajnik, 1999). Cells of the larval *Xenopus* immune system express only MHC class II proteins, whereas expression of both classes occurs in the adult (Du Pasquier et al., 1989). Furthermore, class II MHC expression is limited to less than 70% of the larval lymphocytes derived from spleen, whereas in adults, 100% of these lymphocytes express class II MHC (Du Pasquier and Flajnik, 1987). Class I expression begins at stage 55, as detected by flow cytometry, but levels remain low until metamorphic climax (stage 65/66; Rollins-Smith et al., 1997). The onset of MHC class I expression is concurrent with the premetamorphic period, during which levels of thyroid hormones increase, regulating metamorphic changes. Rollins-Smith et al. (1997) found that the onset of MHC class I expression, although altered by thyroid hormone manipulation, was not directly induced by thyroid hormones. Rather, they concluded that MHC class I expression was regulated by other undetermined aspects of metamorphosis.

The differential expression of MHC antigens between the larval and adult immune systems of *Xenopus* may have its origins in the fact that these different systems arise from dif-

ferent populations of precursor cells (Turpen, 1998). The developing thymus is infiltrated by three distinct waves of lymphocytes, corresponding to the larval, premetamorphic, and juvenile/adult immune systems. Cells from the ventral blood islands and a minor contribution of cells from dorsal lateral plate (DLP) mesoderm surrounding the pronephros migrate into the thymic rudiments between 4 and 13 days after fertilization (~stages 44–49). This wave differentiates and reaches peak production of lymphocytes within the thymus by approximately 20 days after fertilization (~stage 51/52; regeneration competent). However, by 42 days after fertilization (~stage 57/58; regeneration incompetent), the cells from this first wave are no longer detectable.

The second wave of thymic infiltration overlaps the first, but these cells seem to lie dormant in the thymus until day 24 (stage 53) when they begin to proliferate, reaching peak production at 40 days after fertilization (stage 56). Cells from the DLP mesoderm provide a higher percentage of the cells in this second wave of thymic infiltration and, unlike the cells of the first wave, a portion of these cells will last and become part of the adult immune system. Finally, around the age of sexual maturation, the third and final wave of thymic infiltration occurs and gives rise to the majority of lymphocytes with adult immune function. The different origins and phases of lymphocyte production give rise to the distinctly different larval and adult immune systems as well as the transitional period separating the two (for reviews, see Du Pasquier et al., 1989; Turpen, 1998; Robert and Cohen, 1998; Rollins-Smith, 1998; Du Pasquier and Flajnik, 1999).

As the larval and adult immune systems are derived from different populations of precursor cells, it is perhaps not surprising that they show structural and functional differences. Investigators have used various techniques to determine the activity of ectothermic immune systems and two of the most widely accepted methods are skin grafting and the mixed lymphocyte reaction (MLR). Skin grafting determines the



ability of the immune system to reject nonself tissue, which in the amphibian model is characterized by total loss of pigment in the transplanted tissue (Du Pasquier and Bernard, 1980; DiMarzo and Cohen, 1982a,b; Barlow and Cohen, 1983; Obara et al., 1983; Izutsu and Yoshizato, 1993). The MLR tests the ability of the immune system to respond to foreign antigens by means of clonal expansion of responding T cells.

Izutsu and Yoshizato (1993) found that adult *Xenopus* rejected autografts of larval skin that had been cryopreserved while the frogs developed. Furthermore, Kaye et al. (1983) showed that the larval and adult immune systems were sufficiently different that proliferative responses between larval and adult splenocytes were detectable with MLR. Along with these data, several other studies have consistently shown that the larval immune system is not only different from the adult immune system but is also less competent. Skin grafts from minor-histocompatibility mismatches are not rejected by larval immune systems but are by the adult immune system. Of interest, during the premetamorphic immune transition, even major-histocompatibility mismatches are tolerated (reviewed in Rollins-Smith, 1998). This premetamorphic tolerance may be the result of a down-regulation of receptors for fundamental cytokines such as IL-2 (Ruben et al., 1992). The transition between the two immune systems must then be accomplished in such a way that lymphocytes of the larval system do not react with cells of the emerging frog tissues and that lymphocytes of the new system do not attack larval tissues. This seems to be accomplished by suppression of immunity during metamorphosis so that neither larval nor adult antigens are recognized as nonself (i.e., both are "hidden") during this transitional period (Ruben et al., 1989).

Although complete cytokine profiles during metamorphosis do not exist, it is likely that as new immune elements are introduced during this time period, differences in cytokine expression will occur, such as those

implicated during the transition from fetal wound healing to scar formation (Liechty et al., 2000b). With the emergence of components of the adult immune system capable of recognizing larval antigens (Izutsu and Yoshizato, 1993) "hidden" during normal development (Ruben et al., 1989), the question remains, Are those larval antigens, which are likely to be expressed on dedifferentiating cells, also "hidden" or are they more accessible to emerging elements of the adult immune system? If these dedifferentiation antigens become progressively more accessible to the developing adult immune system, then it is likely that elements of that system will respond to those antigens as nonself, thus providing a possible mechanism for the decline in regenerative capacity during metamorphosis.

We hypothesize that changes in immune competence are important for the gradual loss of regenerative potential that accompanies metamorphosis. The relative "immunodeficiency" of urodeles and larval anurans presents an interesting parallel with their capacity for epimorphic regeneration (Fig. 3). The components of adaptive immunity that emerge during anuran metamorphosis are more similar to those of mammals and are likely to account for the mammal-like response to limb amputation that occurs in adult frogs, namely fibrosis and formation of a distal pad of scar tissue rather than cell patterning and growth in a regeneration blastema. The changing profile of circulating lymphocytes and cytokines produces a higher degree of immune reactivity in the postmetamorphic frog, which is manifested in the amputated limb stump as a stronger inflammatory response than that of younger limb stumps. This greater degree of inflammation results in fibrosis that may interfere with the signaling required to establish a pattern like that which follows amputation of limbs at earlier stages.

## CONCLUDING PERSPECTIVE

We have reviewed work with new mammalian models of regenera-

tion, relating it to anuran limb regeneration, and briefly summarized the evidence from amphibian developmental immunology that shows "immunodeficiency" in urodeles and a shift toward greater immune reactivity during anuran metamorphosis. The possible role of immune cells in interfering with the events of regeneration has received little attention from developmental biologists interested in epimorphic regeneration and its loss in mammals. Yet, the cells and cytokines of the immune system are central to the processes of tissue repair and wound healing. In mammals and adult frogs, the interplay of these factors in the wound environment affects mesenchymal cells/fibroblasts in a way that produces fibroplasia, fibrosis, and scarring. The more primitive immune system of urodele amphibians may be the key to the excellent regenerative properties of these animals. In fetal mammalian skin and limbs of young frog larvae, the wound microenvironment allows the signaling and patterning of mesenchymal cells so that perfect restoration of the damaged organ can be achieved. Unique but uncharacterized features of the immune systems of MRL mice and rabbits somehow seem to reduce inflammation after injury and allow a greater degree of regenerative growth in those mammals.

We suggest that additional insights into how inflammation and fibrosis are regulated by factors produced by lymphocytes and other cells of the immune system will open a new window toward understanding the loss of epimorphic regeneration during both ontogenic and phylogenetic development. Such insights are currently emerging rapidly from laboratories studying a wide spectrum of immune and inflammatory disorders. Given the evidence that inflammation and scarring are tightly regulated by cytokine signaling and that these processes inhibit tissue or organ regeneration, it should be clear that studies of regulation among cells of the immune system are directly relevant to the work of developmental biologists investigating epimorphic regeneration.

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