Chapter 3
Limb Regeneration: Ultrastructural and Cytological Aspects

As previously indicated (Sect. 1.8), the injury of a limb in lizards induces large tissue damage that elicits a strong inflammatory reaction. Within 2–3 days after amputation, the reactive process is similar in the stump of the tail and the limb, and numerous granulocytes are present as the main phagocytes and persist in the following week (Alibardi 2009a), when also macrophages of blood origin become numerous. The latter phagocytes complete the tissue debridement but can cause scarring when they are hyperstimulated, for instance, by cauterization or repetitive cutting of the blastema (Alibardi 2009b). Macrophages are more commonly seen than granulocytes after 3 weeks after amputation.

The persistence of leukocytes in the injured limb together with the extensive exudation of fibrin that traps the microorganisms is a potent primitive innate immune defense of reptilian wounds (Huchezermayer and Cooper 2000). The permanence of active granulocytes for 1 week in the wounded tail and for over 3 weeks in wounded limbs indicates that these cells continue to be stimulated probably by a persistence of microbes and unknown chemical factors derived from tissue destruction (especially in the limb stump). In the injured tissues of the limb at 30–40 days after amputation, granulocytes within wounded tissues show an increased irregular surface and blebbing and contain activated (spongy-like) azurophil granules. These granules may store potent antimicrobial molecules that block the spreading of infection of injured lizard tissues of the stump.

3.1
Wound Healing and Blastema Formation

During the first 2–7 days after amputation of the limb, there is a strong infiltration of leukocytes among damaged tissues (muscles, connective tissue, nerves, and bone) within 0.3–0.5 mm in the stump (Alibardi 2009a; Fig. 1.8a, b).

Two to 3 days after amputation, on the surface of the limb stump a scab made of electron-dense cell remnants from blood cells and platelets, like in the tail stump, is formed (Fig. 2.14a). Beneath the scab, a region of injured or necrotic epithelial and connective cells is present in which numerous phagocytes are localized (Fig. 2.14b, c). Some of the phagocytes are poorly differentiated

epidermal cells, as indicated by the few bundles of keratin and desmosomal remnants that they contain. Other degenerating cells are granulocytes and most of their ribosomes and other organelles are lost; in addition, an intense vacuolation is present. The nuclear membrane of these degenerating granulocytes is broken and is often associated with prominent heterochromatin clumps. Other phagocytes beneath the scab, which are also localized among wound keratinocytes, are recognizable as heterophil granulocytes (the reptilian counterpart of neutrophil granulocytes in mammals). These cells contain small granules (specific, 0.1–0.2 μm) or larger granules (nonspecific or azurophilic; Fig. 2.14b, c). Another type of phagocyte is rich in small granules and is recognized as a monocyte. These cells possess a pale cytoplasm, an electron-dense nucleus with heterochromatin clumps, and cytoplasmic stout blebs. The latter feature indicates amoeboid movement and phagocytosis.

At 6–7 days after trauma, migrating epithelial cells cover a large part of the stump surface underneath the scab. Flat and elongated keratinocytes are still mixed with phagocytes, mainly heterophil granulocytes with large, azurophilic granules of 0.4–1.5 μm or, less commonly, with granulocytes containing small granules (0.05–0.2 μm). Migrating keratinocytes contain small keratin bundles and numerous, irregular pale vesicles of the endoplasmic reticulum, and secondary lysosomes. No cell junctions between keratinocytes and granulocytes are seen, and the latter cells possess cytoplasmic stout blebs infiltrated among keratinocytes. Some bacteria are seen within granulocytes, and most of their azurophilic granules at 6–18 days after amputation show a spongy texture or even broad pale areas among the dense material.

At 12–16 days after amputation, granulocytes are still numerous among the granulation tissues where numerous cells are still degenerating as in a condition of chronic inflammation. Aside from free granulocytes, clusters of phagocytes with degenerating cells are also present (Fig. 2.15a, b). This mass of cells resembles the granulomas described in mammalian tissues under chronic inflammation, and similar cell aggregates are also present at 18–22 days after amputation. The presence of granules and lobed or multiple nuclei in the degenerating cells indicates that they are granulocytes. Degenerating granulocytes contain dense and heterochromatic nuclei, and granules of different size and heterogeneous aspect or containing dense or spongy material. Pale lipid material is present in some vesicles within granulocytes, whereas most organelles are degenerated and swollen. The plasma membrane in some areas is discontinuous and the cell content is apparently released from the degenerating cells.

In these cell clumps, most pale and few darker cells are present, both representing degenerating cells (Fig. 2.15b). Sparse junctional remnants join these cells together, forming an epithelioid structure. Sparse lymphocytes are also seen among damaged tissues or within blood vessels at 6–16 days after amputation. Sparse among these granuloma-like tissues, empty spaces, derived from tissue degeneration, are present in the limb stump, another sign of the intense inflammatory reaction produced in this organ after amputation.

At 18–22 days after amputation, the cell composition of the granulation tissues of the limb stump changes and numerous fibroblasts and collagen fibrils become prevalent, as well as numerous macrophages (Fig. 2.15c). The differentiated fibroblasts are surrounded by irregular bundles of parallel collagen fibrils, which form large collagenous fibers. The latter give rise to the dense, irregular fibrotic dermis of the scarred limb. Fibrobasts contain numerous ribosomes and sparse parallel cisternae of rough endoplasmic reticulum. Basophil granulocytes are also seen among the irregular dense connective tissue at 18–22 days. A complete and continuous basement membrane separates the differentiated epidermis from the underlying, fibrous connective tissue.

In conclusion, the limb stump initially contains interstitial tissue made of prevalent hematogenous elements involved in microbe phagocytosis and cell debridement. The limb granulation tissue is replaced in 6–10 days by mesenchymal cells in the tail stump, but remains prevalently hematogenous in the limb, up to 14–18 days after amputation, when fibrocytic fibroblasts cause the rapid evolution of the granulation tissue into scar connective tissue. In comparison with the tail stump, the reepithelialization, healing, and inflammatory period in the limb are extended for 10–20 days longer with respect to that in the tail. The intense inflammation can lasts over 30 days when the bone remains protruding from the stump (Barber 1944; Kudokotsev 1960). The presence of areas containing pus in limbs at 12–22 days after amputation indicates a chronic inflammatory status in the limb stump and may also stimulate some immune reaction. Neither granulomas nor cyst or pus is formed in the tail stump. Granulocytes and macrophages remain in the stump for over 20 days after amputation, and later numerous macrophages remain among the tissue of the limb, whereas these phagocytes are less frequently seen and appear to be not activated in the regenerating tail (Alibardi and Sala 1988a, b). The tail appears to regenerate from mesenchymal cells largely derived from autotomy planes of the connective septa and intervertebral fracture planes (Quattrini 1954; Bellairs and Bryant 1985), perhaps containing stem cells. Although autotomy planes are absent in the limb, the presence of stem cells is not known. Another phenomenon that can increase the number of blastema cells in both the tail and the limb stump is the epidermal–mesenchymal transformation (Hay 1996). It is unknown whether an epidermal–dermal transformation may occur during lizard tail and limb regeneration.

3.2 Scar Formation in the Limb as Compared with the Inducement of Tail Scarring

In mammals, granulocytes do not produce chemokines for fibroblast recruitment and proliferation like macrophages, but are mainly involved in microbe destruction. The extensive damage of tissues in the limb stimulates a persistent secretion of fibroblast growth factor, platelet-derived growth factor (PDGF), and transforming

growth factor beta-1 (TGF-β_1) from macrophages which results in the recruitment and proliferation of fibroblasts capable of synthesizing a high amount of collagen and therefore inducing scarring (Kovacs and DiPietro 1994; Ferguson and O'Kane 2004). During chronic inflammation in mammals, lymphocytes and wound keratinocytes produce interleukin-1 and interferon gamma, two molecules that stimulate macrophage proliferation and indirectly scarring. In the case of lizards, no biochemical information is available on similar molecules.

The intense inflammatory reaction and the presence of a granulomatous reaction within the limb stump do not allow the establishment of a mesenchymal population, and the rapid formation of a continuous dense lamella in the basement membrane blocks any dermal–epidermal interaction to sustain the elongation of the initial limb outgrowth. These combined effects determine the origin of a variably short scar. Even in the few cases of initial blastema formation, the permanence of leukocytes and of macrophages among injured tissue causes the initial mesenchymal cell population to rapidly differentiate into fibrocytes, whereas the wound epithelium forms a basement membrane.

The confirmation that a strong inflammatory reaction, aside from the sparse recruitment of stem or undifferentiated cells, is the main cause for the unsuccessful limb regeneration derives from the study of the process of scarring in the tail after cauterization, or from other experimental manipulations (Marcucci 1914–1915; Bellairs and Bryant 1985). After cauterization of the tail stump (Fig. 1.9g), the damaged surface forms a dark necrotic tissue that produces a thick scab at 14–16 days after cauterization (Alibardi 2009b. Beneath the scab a soft dark mound of less than 1 mm in length is formed, which initially resembles a blastema. In the following week, the soft mound can grow to 1–3 mm, but it rapidly turns into a pale and scaled scar by about 1 month after cauterization (similar to those shown in Fig. 1.11g, h). The scaling pattern and the pigmentation of the outgrowths are irregular as pigment cells are distributed at random in the epidermis and also in the dermis.

Aside from tissue necrosis, cauterization also produces damage affecting the permeability of blood vessels with the consequent loss of a large amount of fibrin. The latter is still present among tissues at 14–20 days after cauterization. The ultrastructural analysis of the soft outgrowths at 14 days after cauterization shows numerous fibroblasts with well-developed ergastoplasm, surrounded by a fibrinous exudate containing collagen fibrils. The latter form a characteristic "alveolate intercellular matrix" among densely packed fibroblasts (Fig. 1.13e). The cytoplasm of these fibroblasts contains sparse bundles of microfilaments, an indication that these cells may represent myofibroblasts, the key cell leading to scarring in mammalian tissues (Wynn 2008). Numerous macrophages are present among fibroblasts and both cell types appear trapped within a dense collagenous network of bundles. Another common cell type encountered among fibroblasts is the electron-dense granulocyte with large, 0.2–0.5-μm granules with a characteristic spongy texture and an irregular cell surface with frequents blebs. Also, the numerous melanophores present in the outgrowth appear trapped within the fibrin–collagenous extracellular

matrix. The regenerated epidermis contains a continuous dense lamella that is contacted by anchoring fibrils from fibroblasts. Hemidesmosomes are commonly present, like in the normal epidermis. The rapid formation of a differentiated basal lamina with a continuous dense lamella in the cauterized tail stump as well as in the amputated limb contrasts with the presence of a discontinuous lamella dense in the blastema of the regenerating tail (Alibardi 1994a, b; 1995; Alibardi and Toni 2005).

From 16 to 20 days after cauterization, the connective tissue of the outgrowth forms scar tissue, made of elongated fibrocytes within a dense matrix, like in the stump of the limb. At 20–30 days after cauterization, fibrocytes adopt a perpendicular orientation with respect to the basement membrane of the epidermis, like in the limb. The presence of numerous macrophages, heterophils, or other types of granulocytes for over 20 days after injury in both cauterized tissues of the tail and the stump of the limb probably stimulates the recruitment of scarring fibroblasts, a phenomenon well known in mammalian chronic inflammation. Cauterization stimulates the excessive migration of macrophages that, through the liberation of fibrogenic cytokines (TGF-β_1, PDGF, etc.), attract the numerous fibrocytes responsible for the production of scar connective tissue. It is likely that cauterization in lizards gives rise to a population of myofibroblasts, like in mammalian fibrosis (Wynn 2008; Alibardi 2009b). The latter cells determine the rapid contraction of the wound and also the deposition of a large amount of collagen. In the tail scars, not only the cell composition is changed in comparison with normal blastema, but also the degradation of the collagen may take place at a lower rate than its production, with the consequent net increase of collagen fibrils.

The above observations indicate that the lizard model of regeneration can be utilized to study scarring mechanisms in amniotes in general as it resembles the process present in mammals. The condition of the mesenchyme and that of the extracellular matrix in the normal regenerative tail blastema are comparable to those present in mammalian embryonic tissues or in tissues of fetuses (Adzick and Longaker 1992; Martin 1997). It is known that mammalian fetal wounds produce little or no inflammation and that they repair well without scarring (Ferguson and O'Kane 2004). Among other growth factors, also the TGF-β_1, produced by macrophages, stimulates fibrocyte recruitment. In contrast, in embryonic/fetal wounds the amount of an isoform of transforming growth factor, transforming growth factor beta-3, is increased and this isoform favors regeneration without scarring. This factor is also present in lizard tissues after wounding and regeneration (Alibardi, unpublished observations).