
2.1 Certain Tissues in an Organ Regenerate Spontaneously

Experiments in induced tissue or organ regeneration clearly require an appropriate selection, both of exogenous reactants and of the assays that define the products. Less obvious, though equally important, is the need to make a rational choice of the experimental volume for the intended study of regeneration. Criteria for such choices will be discussed in the present chapter as well as in the next. In this chapter we will discuss a fundamental characteristic of specific tissues that need to be deleted in order to generate the experimental volume in a study of induced regeneration. This characteristic is the intrinsic inability of these tissues to regenerate spontaneously.

In the preceding chapter we found out that certain types of tissues sustain reversible injury whereas other tissues are injured irreversibly. In this chapter we will distinguish between the two kinds. Clearly, in studies of induced regeneration, the investigator seeks to focus on tissues that do not regenerate spontaneously (nonregenerative tissues). While spontaneously regenerating tissues (regenerative tissues) can be restored without help from the investigator, nonregenerative tissues must be induced to regenerate and form the critical objective of such experimental study. Such tissues must be carefully deleted from the defect; if not, their residual presence will lead to the erroneous conclusion that regeneration has been induced. On the other hand, tissues that regenerate spontaneously in the defect are expected to be present at the end of the healing process irrespective of whether the study has led to repair or regeneration. The distinction between regenerative and nonregenerative tissues appears to be relatively sharp. It is discussed in detail further using four well-known experimental paradigms, two each from the literature of wound healing in skin and peripheral nerves. The literature of induced regeneration for these two organs is currently richer than for other organs.

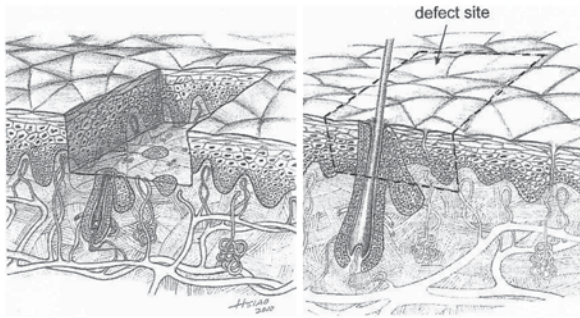


Fig. 2.1 The epidermis is a regenerative tissue. Following controlled injury (stripping or blistering), which leaves the dermis intact (*left*), the epidermis recovers its structure completely at the *site of the defect* (*right*). Hair follicles are ensheathed in epidermal tissue and participate in the regenerative process. (Adapted from Asmussen and Sollner 1993)

2.1.1 The Epidermocentric Viewpoint in Studies of Skin Wound Healing

The morphology of skin is illustrated in Fig. 2.1. A more detailed description of the structure and function of the tissues comprising skin appears in Chap. 4.

Briefly, the skin is an organ with an approximately two-dimensional geometry, consisting of an epidermis attached to the *basement membrane*; the latter is attached to the dermis. Its most critical function is protection of the organism from injury and infection originating outside the body. The epidermis is a specialized tissue consisting of several layers of epithelial cells (keratinocytes) that protects the organism from dehydration, bacteria, ultraviolet radiation, as well as insults from chemical substances. Protection is afforded principally by the outermost cell layer of the epidermis, the stratum corneum, consisting of dead, keratinized cells. The epidermis is supported by the dermis, a tough layer often about ten times the thickness of the epidermis, consisting primarily of collagen and elastin fibers as well as blood vessels. The dermis protects mechanically the thin, epidermal layer against shear and other mechanical insults. Highly vascularized, the dermis supplies the epidermis with metabolites and oxygen, both transferred through the basement membrane.

We consider the response of skin to different types of injuries. In a large majority of studies the outcome has been measured in terms of processes that involve the epidermis. The emphasis on restoration of the epidermis is frequently motivated by the clinical urgency for wound closure: An open wound invites bacteria and, if large enough, leads to dehydration of the organism; both are life threatening contingencies in the clinical setting. This approach has been carried over in several experimental studies of induced skin regeneration; it suggests that a favorable outcome of the healing process is rapid completion of processes of epithelial cell proliferation, migration, and finally, differentiation. This widespread viewpoint of the healing response emphasizes, almost exclusively, the fate of the keratinocytes while neglecting the dermis. We will refer to this as the epidermocentric viewpoint.

The response of skin to two extreme types of injury, namely, a mild injury (blistering) and a very severe injury (full thickness skin excision), has been described extensively. A blister can be inflicted by a abrasion or by a very mild burn. Shear forces or thermal injury cause failure at the interface between epidermis and dermis (dermal–epidermal junction), followed by exudation of lymph fluid into the injured space (Asmussen and Sollner 1993). A similar model wound can be generated by repeated stripping of the epidermis with a tape (Nanney and King 1996). The blister typically separates the dermis and the epidermis at the level of the basement membrane; if the experiment is conducted carefully enough, the basement membrane remains mostly intact. The underlying dermis reddens, swells, and often forms a small amount of exudate but remains, otherwise, relatively intact. Since, the injury does not extend deep into the dermis, blood vessels are not injured and there is no bleeding. Soon, the necrotic epidermis forming the blister is sloughed off, leaving behind an epidermis-free surface. Keratinocytes migrate from the injured edges and reattach themselves onto the inner layer (lamina densa) of the relatively intact basement membrane (Krawczyk and Wilgram 1973; Beerens et al. 1975). Keratinocyte migration also originates in the appendages of skin (hair follicles, sweat glands, sebaceous glands) that are located in the dermis. Migration of keratinocytes finally leads to formation of a continuous cell layer (confluence) over the basement membrane and the migration stops; the cells undergo mitosis and eventually form a maturation gradient by differentiation to a multilayered and keratinizing epidermis (Fig. 2.1). No sign of the blister can be detected on the regenerated epidermis, indicating that the ruptured dermo-epidermal junction has been restored (Briggaman et al. 1971; Marks et al. 1975; Konig and Bruckner-Tuderman 1991; Stenn and Malhotra 1992).

A much more severe injury is excision of the epidermis and of the entire layer of dermis to generate a full-thickness excisional skin wound (referred to often below as a dermis-free defect). In a useful example, involving a clinical trial of human volunteers, a dermis-free defect in the forearm was studied and a richly vascularized connective tissue (granulation tissue) soon formed inside the defect. Two different processes of defect closure, contraction of the dermal edges and epithelialization, were monitored. After the 17th day, contraction of the dermal edges had led to closure of somewhat less than half of the original defect area (Fig. 2.2). The balance of defect closure, somewhat over 50 % of the original area, was contributed by epithelialization (Ramirez et al. 1969). A simple analysis of the data in this study shows that the keratinocytes from the edges migrated and proliferated over distances at least as long as 15 mm and covered an area totaling more than 5 cm².

The two familiar examples of skin wound healing described above span the range from complete recovery of physiological skin structure after careful blistering, all the way to formation of scar without recovery of physiological structure after excision of the dermis through its full thickness. The outcomes of these two healing models are as different as they can be and will be used below as paradigms of the two basic outcomes of a healing process (regeneration vs. repair). Nevertheless, in both cases, keratinocytes migrated and proliferated extensively from their original location at the edge of the injured tissue, eventually forming a regenerated epidermis over a large area. The evidence clearly shows that extensive epithelializa-

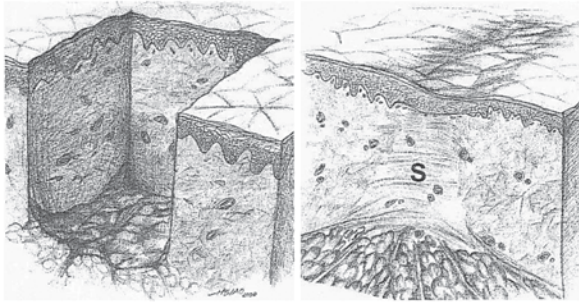


Fig. 2.2 The dermis is a nonregenerative tissue. Following excision of the epidermis and of the dermis to its full thickness (*left*), the wound edges contract and close with simultaneous formation of scar tissue (*S*) in place of a physiological dermis (*right*). Adipose tissue (fat) is shown underneath the dermis or scar. The epidermis that forms over scar is thinner and lacks undulations (rete ridges)

tion that leads to formation of a new epidermis is an intrinsic property of migrating keratinocytes rather than being a property of the type of injury.

We conclude that, during healing processes in the skin, regeneration of the epidermis occurs spontaneously and therefore cannot be used as a reliable indicator of a successfully induced regenerative outcome. These considerations limit the value of the epidermocentric viewpoint in an analysis of induced regeneration of skin and focus instead attention to the stroma.

2.1.2 The Axonocentric Viewpoint in the Study of Nerve Wound Healing

There is a profound topographic difference between the organization of tissues in the skin and the peripheral nerve trunk. Whereas tissues in skin are layered in a largely planar configuration, in a nerve trunk the tissues are wrapped around each other concentrically in a cylindrical arrangement. A peripheral nerve trunk measures about 1 mm in diameter for a typical rat sciatic nerve and comprises one or more bundles (fascicles), each consisting of many elementary conducting units (nerve fibers). Its main function is transmission of electrical signals from the spinal cord to the periphery. Many mature nerve fibers comprise an axon surrounded by a sheath of the protein myelin (myelinated axon), the later provided by the wrappings of many Schwann cells around the axon perimeter, and a tubular basement membrane that lines the external surface of Schwann cells. Other nerve fibers lack myelin (nonmyelinated axons). An axon is a long, fiber-like extension (cytoplasmic process) of a nerve cell (neuron). Individual nerve fibers are surrounded and supported by “nonneuronal” tissues arranged cylindrically around the fibers. Proceeding from a nerve fiber radially toward the periphery of the nerve trunk, we encounter the following nonneuronal tissues: the endoneurium, comprising a loose stroma (endoneurial stroma) and specialized blood vessels that establish a blood-nerve barrier;

a tight, multilayered, and highly specialized tissue that provides a diffusion barrier, the perineurium; and, when the nerve trunk comprises more than one fascicle, a strong sheath that surrounds all fascicles, the epineurium. The permeability barriers provided by the endoneurium and the perineurium protect the space immediately outside the nerve fibers from changes in chemical composition, thereby preserving the electrical conductivity of the fibers. A detailed description of the structure and function of a nerve trunk is presented in Chap. 5.

Investigators have treated injured peripheral nerves with a variety of agents that are hypothetical reactants for inducing regeneration. In these studies, outcome measurements are collected by studying cross sections of regenerated nerves and typically consist of counts of myelinated and unmyelinated axons, measurements of the average thickness of the myelin sheath that surrounds an axon, as well as data on the distribution of axon diameters. This is a decidedly axonocentric view. It is based on the well-known fact that interruption of axon continuity causes loss of the ability to conduct electrical signals that nerves uniquely possess. In studies of peripheral nerve regeneration very little attention has been traditionally paid in the literature to nonneuronal tissues. Does the large number and important functional role of axons, not to mention the experimental ease of counting them, merit having axons be counted as the frequently single, often exclusive, measure of outcome to be considered in a study of induced nerve regeneration?

Consider the response of axons and Schwann cells in a peripheral nerve following two types of injury: a mild injury (crushing of nerve trunk) and a severe injury (complete cutting of nerve or transection; also referred to as resection or division). In detailed studies of rat peroneal and sural nerves that had been crushed using smooth-tipped forceps, observations were made at the crush site and adjacent to it. It was reported (Haftck and Thomas 1968) that the tubular basement membrane (*BM tube*) that surrounded a crushed nerve fiber persisted at the crush site; the tube diameter became shrunken but the tube wall did not rupture. Axon cytoplasm (axoplasm), myelin, and Schwann cell cytoplasm inside the BM tubes were all displaced out of the crushed site. Even though separated by a clear gap at the crush site, the displaced tissues were retained inside the intact tubes. In the regions adjacent to the crush site, the BM tubes accommodated this displaced material by becoming distended but not rupturing. Following release of the crushing force, the shrunk BM tubes rapidly filled once more at the crush site with the tissues that had been displaced, and structural recovery across the defect followed (Haftck and Thomas 1968). Not only the axoplasm, but the myelin sheath as well recovered its structure following a carefully administered crush. By 2 weeks, the myelin sheath had degenerated to the point where very little myelin could be detected; however, by 4–10 weeks, regeneration of the myelin sheath was complete (Goodrum et al. 1995, 2000). It has been shown that normal function was eventually restored following mild crushing (Madison et al. 1992). We conclude that, following this mild injury that severed the axons and induced degeneration of the myelin sheath, but left the BM tubes intact, axons recovered the continuity of their structure and the nerve fiber functioned physiologically once more (Fig. 2.3).

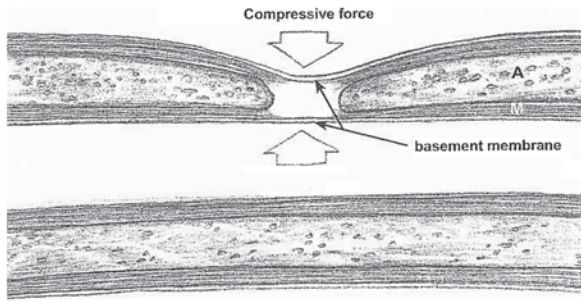


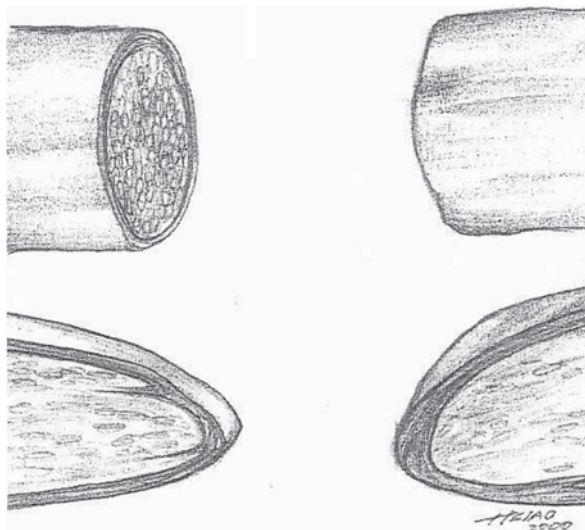
Fig. 2.3 Axons and their myelin sheath inside the nerve fiber shown above are regenerative tissues. After a mild crushing injury, the axoplasm (*A*) separates and the myelin sheath (*M*) degenerates on either side of the crushed nerve fiber. The *basement membrane* has remained intact throughout (*top*). Regeneration of the nerve fiber occurs after a few weeks (*bottom*)

We now review the response of axons and Schwann cells in a peripheral nerve to a complete cut through the entire diameter (transection); clearly, this is a much more severe injury than mild crushing (Fig. 2.4). Since, we are concerned with the potential for spontaneous (unaided) regeneration, we focus on the response of a transected nerve in which the stumps were not ensheathed in the commonly used tubular prosthesis (tubulation) (the response of the tubulated configuration is a clear case of induced, rather than spontaneous, regeneration and is discussed in detail in Chap. 5). Of the two nerve stumps resulting from transection, only the proximal one was still connected to the cell body (neuron). When the gap separating the stumps was sufficiently long, the proximal stump bulged out, forming a semispherical mass (neuroma); a neuroma-like structure also formed at the distal stump (Chamberlain et al. 2000a).

A neuroma is the product of a repair process in a peripheral nerve. It comprises highly disorganized and poorly vascularized connective tissue. Embedded in it are Schwann cells and a large number of tangled axons, some of which are myelinated; most axons are reported to end blindly inside the neuroma, or to be nonmyelinated and to be oriented in a highly irregular manner (Cajal 1928; Denny-Brown 1946; Young 1948; Aguayo et al. 1973; Wall and Gutnick 1974; Jenq and Coggeshall 1985b; Olsson 1990; Sunderland 1990; Zochodne and Nguyen 1997; Chamberlain et al. 2000a). Certain authors reserve the use of the term “neuroma” for the outgrowth of the proximal stump alone; the two repaired stumps differ somewhat in the presence of elongating axons and of a proliferating perineurium in the proximal, but not the distal, stump (Thomas 1988).

Axons and associated Schwann cells have been observed to have elongated or migrated into and through the tissues of a growing neuroma over distances at least as long as a few millimeters. Considering that the diameter of most myelinated axons in the rat sciatic nerve, a popular model for studies of regeneration, is about 1–5 μm , a distance of a few millimeters corresponds to an axon elongation of almost ten times its diameter. Reports of an axonal elongation through a neuroma, over a distance of about 1–2 mm, have appeared on several occasions (Cajal 1928;

Fig. 2.4 Most of the supporting tissues (stroma) associated with nerve fibers are not regenerative. Although axons with their myelin sheath are regenerative following complete transection of the nerve trunk, the remainder of the tissues in the nerve trunk are not (e.g., endoneurial stroma). Following transection (*top*), each of the stumps eventually becomes a neuroma (neural scar), a clump of fibrous tissue that has closed largely by contraction (*bottom*)



Denny-Brown 1946; Wall and Gutnick 1974). In other studies, axons managed to cross an untubulated 4-mm gap in the rat sciatic nerve (Archibald et al. 1991) or an untubulated 2-mm gap in the mouse sciatic nerve (Buti et al. 1996) and established substantial recovery of nerve function. In a more striking report, axons were observed to pass through the proximal neuroma, along the sling stitch that surgically united the untubulated stumps, and finally entered the distal stump, a distance of about 10 mm (Noback et al. 1958). Following a detailed study of this phenomenon, it was concluded that “nerve fibers can certainly grow in neuromas” (Denny-Brown 1946).

The two healing outcomes described above are examples of spontaneous response of the nerve fiber to extreme cases of nerve injury: restoration of a physiological nerve fiber (regeneration) following mild crushing (Fig. 2.3) and formation of nonphysiological neuroma (repair) following transection (Fig. 2.4). Axons appear to have the intrinsic ability to elongate over substantial distances, though not necessarily in a straight line, independently of whether other tissues, such as the endoneurium or perineurium, are undergoing regeneration or repair. Schwann cells are also capable of proliferation and myelination of axons independently of the extent of injury to the nerve trunk. Axon elongation and myelination appear to be intrinsic properties of axons and Schwann cells; they are not properties of the severity of the injury. However, nerve regeneration is incomplete unless there is regeneration of all anatomical structures that are responsible for physiological nerve function.

We conclude that neither the incidence of axon elongation nor that of axon myelination appears to be, by themselves, either sensitive or conclusive evidence of recovery of overall physiological function in an injured peripheral nerve. The available evidence limits, therefore, the value of the axonocentric viewpoint in an

analysis of induced regeneration of peripheral nerves and leads instead to consideration of the endoneurial stroma as an indicator of at least equal importance.

2.1.3 Spontaneously Regenerative Tissues

Let us summarize the results of the four well-documented experiments described above with models of skin and peripheral nerve injury. The combined evidence showed that an increase in the severity of injury did not suppress the incidence of spontaneous proliferation and migration of keratinocytes in skin; nor was the spontaneous elongation of axons and the myelinating activity of Schwann cells suppressed in nerves. We conclude that keratinocytes and axons, as well as Schwann cells, are intrinsically capable of restoration of the original specialized functional tissues (epidermis and myelinated axons, respectively) and that they exhibit this property after two very distinct injuries. Even when the injured organ as a whole does not recover its structure, these individual tissue components show a remarkable ability to migrate and proliferate (skin), or elongate and become myelinated (nerve).

2.2 Other Tissues Are Nonregenerative

A reactant does not have regenerative activity unless it leads to synthesis of a tissue that does not regenerate spontaneously. Preparation of an experimental defect for the study of induced regeneration should, therefore, be based on thorough excision of nonregenerative tissues prior to the experimental study of a reactant with unknown activity and critical assays of induced regeneration should be focused on the identification of these nonregenerative tissues at the completion of the study. In this section we will identify these tissues.

2.2.1 The Dermis Is Nonregenerative

The structure of the physiological dermis was described briefly above; a more detailed description appears in Chap. 4.

The adult mammalian dermis does not regenerate spontaneously. This can be observed most clearly in the response to a severe injury, such as the excision of the epidermis and of the dermis down to its full thickness (dermis-free defect). The resulting defect closes spontaneously by contraction of edges and synthesis of epithelialized scar (Fig. 2.2). The epidermis of scar is thinner than that in physiologic skin and there are few, if any, undulations (rete ridges) in its dermal–epidermal junction; in the subepidermal region of scar, skin appendages are typically absent. The connective tissue layer of scar (dermal scar) is largely avascular, rarely has nerve endings, and the collagen fibers are packed tightly with their axes oriented largely in the plane of the epidermis rather than packed almost randomly, as in physiological dermis. When only part of the thickness of the dermis has been excised (partial thickness skin wound), as

in a donor site used in harvesting a split-thickness graft, the skin appendages in the residual dermal layer form centers from which epithelial cells proliferate and eventually reepithelialize the thin scar tissue that forms over the entire donor site. The inability of dermis to regenerate has been documented abundantly in animal studies (Billingham and Reynolds 1952; Billingham and Medawar 1955; Billingham and Russell 1956; Ross and Benditt 1961; Luccioli et al. 1964; Peacock and Van Winkle 1976; Goss 1992) and in studies with humans (Ross and Odland 1968; Peacock 1971, 1984; Madden 1972; Boykin and Molnar 1992; Butler and Orgill 2005). Very few exceptions to this rule have been reported: Unlike the ear of sheep and dogs, the rabbit ear has been reported to regenerate after a full thickness hole has been punched through it (Goss and Grimes 1972, 1975; Goss 1992).

The appendages of skin do not regenerate spontaneously (Martin 1997). It might have been expected that the presumptive epidermal origin of these appendages (Young et al. 2006) would have prevailed and that, like the epidermis itself, hair follicles, sebaceous glands, and sweat glands would be capable of spontaneous regeneration; but such is not the case.

2.2.2 The Endoneurial Stroma Is Nonregenerative

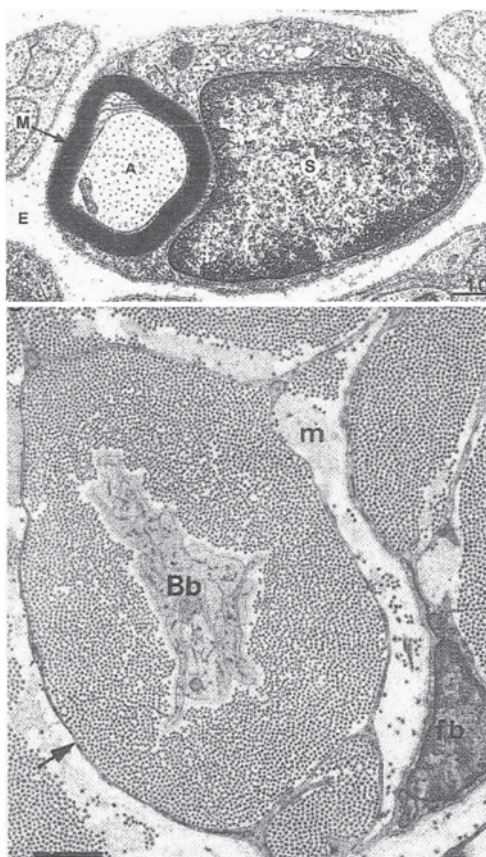
The structure of the endoneurium has been outlined briefly above; it is described in greater detail in Chap. 5.

Following peripheral nerve transection, and provided that the untubulated stumps were separated by several millimeters at the beginning of a study, each stump heals individually by formation of a capsule of neural scar (neuroma) around the edge of the stump (Fig. 2.4; Cajal 1928; Denny-Brown 1946; Chamberlain et al. 2000a). A neuroma was formed when the nerve was transected by both, a scalpel as well as by use of a laser (Fischer et al. 1983).

Clear and irreversible changes have been observed in the connective tissue of the intrafascicular space (endoneurial stroma), both in the distal and proximal stumps, following nerve transection. In the distal stump, by 4 weeks after transection, collagen accumulation (endoneurial fibrosis) had occurred; the average diameter of the new collagen fibrils was 25–30 nm (i.e., about 50% of the value in normal endoneurial stroma). Collagen fibrils surrounded columns of Schwann cells (Büngner bands), leftovers from degeneration of nerve fibers (Wallerian degeneration; Salonen et al. 1985, 1987a). By 20–30 weeks after transection, the Schwann cell columns had become shrunken, they showed decrease in laminin content, and occasionally had become fragmented, with dispersion of fragments inside the intrafascicular space and replacement of fragmented Schwann cells by collagen fibrils (Salonen et al. 1987b; Røytta and Salonen 1988; Giannini and Dyck 1990). Finally, as long as 26 months after transection, the site of previous nerve fibers was indicated by sharply demarcated domains of approximately circular outline consisting of densely packed longitudinally oriented collagen fibrils, with diameters that were smaller than those in the uninjured endoneurium (Fig. 2.5; Bradley et al. 1998).

In the proximal stump, some of the morphological changes following repair were very similar to those observed in the distal stump; others were unique to the

Fig. 2.5 The endoneurial stroma is non-regenerative. *Top*: A nerve fiber before transection, showing the axoplasm (*A*), myelin sheath (*M*), associated Schwann cell (*S*), as well as a very thin basement membrane (not labeled) surrounding *M* and *S*. The entire nerve fiber is surrounded by the endoneurium (*E*), a loosely structured stroma (not clearly visible in this photo; a clearer image of collagen fibers in intact endoneurial stroma is shown in Fig. 6.2). Scale (*bottom right*): One half of the 1- μ m scale bar in the original photo is shown. *Bottom*: 26 months after transection the domain previously occupied by a nerve fiber in the distal stump is now filled with a sheaf of collagen fibers, shown above mostly as *dots*, that enclose groups of Schwann cell processes (*Bünger bands*, *Bb*) encircled by a basement membrane. The entire sheaf of fibers is enclosed inside a thin fibroblast process (*arrow*). The fibroblast in the *lower right* (*fb*) has been partly transformed to a perineurial cell. Microfibrils (*m*) are observed outside the sheaf. Scale: 1 μ m. (*Top* photo from Burkitt et al. 1993, Copyright 1993, Harcourt Publishers Ltd. *Bottom* photo from Bradley et al. 1998)



proximal stump. The following changes were common to both stumps: Following transection, continuous extrusion of intrafascicular contents was observed (endoneurial bulge; Archibald and Fisher 1987) and a significant mass of collagen was deposited in the stump (fibrosis; Eather et al. 1986). The collagen fibrils that were deposited immediately outside Schwann cells in the proximal stump had an average diameter of 30 nm, compared with 50 nm in normal endoneurial stroma (Morris et al. 1972b). In the repaired proximal stump, the original uni- or difascicular structure of the normal nerve trunk disappeared and was replaced by a collection of small fascicles, filled with small-diameter axons, each fascicle surrounded by its own multilaminar perineurium (compartmentation; Morris et al. 1972d). Compartmentation (also referred to as micro- or minifasciculation) was not observed in the distal stump; nor was formation of Schwann cell columns observed in the proximal stump (Morris et al. 1972d). Subdivision of a single fascicle into many was accompanied by significant loss in cross-sectional area occupied by endoneurial stroma (Morris et al. 1972d). The subdivision of the injured nerve trunk into many fascicles has been observed in early studies (Cajal 1928). Compartmentation, typically accompanied by decrease in axon diameter compared with the intact nerve, has been

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