BASAL LAMINA: THE SCAFFOLD FOR ORDERLY CELL REPLACEMENT

Observations on Regeneration of Injured Skeletal Muscle Fibers and Capillaries

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ABSTRACT

To explore in detail the relationships between basal lamina (BL) and regenerating cells, we have studied the reconstruction of skeletal muscle fibers and their associated capillaries in portions of rat and rabbit skeletal muscles after injury with either freezing, ischemia, or *in situ* autografting. Each type of injury produces complete necrosis of cells. The BL, however, remains intact in the area of injury and maintains a "map" of the outline of the spatial relationships between muscle fibers and capillaries. Repopulation of the defect with new cells occurs primarily along the old BL. The spatial relationship between cells, as it existed before injury, is thus reestablished. This process appears to be aided by the ability of each category of regenerating cells to grow along the cell-supporting surface of its own BL. The regenerating cells of muscle fibers and capillaries frequently form a new layer of BL. It is of the usual thickness and is deposited primarily along the outer surfaces of plasma membranes in locations in which the new cells are separated from the old BL. Where an old layer of BL is present overlying a newly formed layer, the old layer may be retained or it may be removed. Removal of redundant BL is probably mediated by interstitial cells which embrace the outside surfaces of BL of regenerated skeletal muscle fibers and capillaries.

INTRODUCTION

Maintenance of the organized structure of tissues and of organs requires continuous orderly replacement of the functioning elements. It is well known that the liver replaces itself approximately once a year, the epidermis of man roughly once a month, and the epithelium of the small intestine almost daily. While the process and rate of cell turnover has been studied in some detail in both experimental animals and in man, the problem of orderly replacement and of its exact mechanisms is only just now being examined.

In the course of experiments designed to investigate the mechanism of basal lamina (BL)¹ thickening seen in peripheral capillaries of man, particularly of patients with diabetes, it was observed that the thickening was not homogeneous. Instead, the excess material surrounding the capillaries was revealed to be composed of discrete, concentric layers, each layer resembling a single, normal layer

¹ Basal lamina is used synonymously with basement membrane and basement lamina.

of BL. On the basis of this and other findings, it was proposed that repeated episodes of cell death and cell replacement had occurred, with each layer representing the residual evidence of one cell generation (18). As a part of this notion, the concept was advanced that the old, denuded BL serves as a microskeleton, or scaffolding, for regenerating cells. Volkmann (14), for example, observed that if the "sarcolemma" was not damaged, almost complete restoration of the structure and function of skeletal muscles occurred, whereas scar formed if continuity of the sarcolemma was destroyed. Clark (2) found that the regeneration of skeletal muscle fibers will occur in the direction of old fibers, even if the piece of muscle is excised and then reimplanted at right angles to its original orientation.

In order to pursue further the details of how BL is used as the microskeleton for reconstitution and orderly repair, a series of experiments was designed to examine the regeneration and repair of controlled, necrotic lesions produced in skeletal muscles of rabbits and of rats. The observations described here indicate that capillaries are reconstituted by proliferation and/or migration of cells along the BL supports of the previous structure. The regeneration of skeletal muscle fibers appears also to follow along the old BL of the fibers. The new capillaries and muscle cells deposit a new layer of BL in certain circumstances. The old BL layer may be removed, or, as occurs frequently in capillaries, it may persist.

MATERIALS AND METHODS

Necrosis followed by regeneration of muscle cells and capillaries was induced with ischemia or cold. Ischemic necrosis was produced in rabbits either by grafting an excised piece of gracilis muscle *in situ* or by interrupting the blood supply to the anterior tibial muscle. Cold injury was induced by freezing circumscribed areas of gracilis muscles in rabbits and rats.

The animals used were male Sprague-Dawley rats (Sprague-Dawley, Inc., St. Louis, Mo.), weighing 200–300 g, and albino rabbits of both sexes, weighing 3–4 kg; they were obtained from Totem Farms, Kirkland, Washington. Rats were anesthetized by inhalation with Metofane (methoxyflurane) and rabbits with intravenous administration of Diabital (sodium pentobarbital), 30 mg/kg of body weight. To prevent infection after operations, all animals received a single intramuscular dose of procaine penicillin G, 50,000 units/kg of body weight.

For light microscopy, the tissues were excised and immediately submerged in neutral Formalin. Longitudinal sections and cross-sections of skeletal muscles

were routinely stained with hematoxylin and eosin and, if indicated, with Masson's trichrome stain. For electron microscopy, the muscle was minced into 1 mm cubes and immediately immersed in chilled, 2% osmium tetroxide in 0.1 M *s*-collidine buffer at pH 7.48. After 1 hr of fixation the tissues were postfixed in 10% neutral Formalin for 1 hr., dehydrated in ethyl alcohol, and embedded in Epon 812 via propylene oxide. For thin sectioning, three blocks showing cross-sections of skeletal muscle fibers were chosen by examination of thick sections of four to ten randomly selected blocks. Thin sections were stained with 3.5% aqueous uranyl acetate and with Millonig's lead tartrate stain.

EXPERIMENTS

In Situ Autografting of Rabbit Gracilis Muscle

Using the method described by Clark (2), gracilis muscles were exposed in eight rabbits, and a rectangular portion, about 15 mm square, was resected from each muscle. The excised piece was returned immediately to its original site and sutured into position with silk sutures. The skin was closed with sutures.

Each graft was sampled only once. To obtain the specimen the muscle was exposed and the graft, with the adjacent rim of muscle, was excised and bisected longitudinally. One-half was used for examination with the light microscope, and the other half for electron microscopy. Two grafts were sampled on the 2nd day and 1, 2, 3, 4, 6, and 8 wk after surgery.

In one animal the excised pieces of both gracilis muscles were grafted perpendicular to the longitudinal axis of the muscle and sampled for light microscopy 4 wk later.

Ischemic Necrosis of Rabbit Anterior Tibial Muscle

Clark and Blomfield (3) have described a way of producing ischemic necrosis in the anterior tibial muscle of rabbits. We used this model, but modified it so that the arterial obstruction could be terminated after a desired time interval. A suture (Mersilene No. 2, Ethicon, Inc., Somerville, N. J.) was threaded with a No. 1, semicircular, noncutting needle through the skin 1 cm below the tibial tuberosity around the anterior tibial muscle. The needle was brought out through the skin at the point of entry, completing a suture loop which passed between tibia, interosseous membrane, and fibula on one side and the anterior tibial muscle

on the other (Fig. 1). The ligature encompassed the muscle, its artery, vein, and nerve. At the skin level the suture ends were crossed and another loop was formed. A "V"-shaped spring was inserted into the outer loop, stretching it as well as the inner loop. The anterior tibial muscle, its artery, vein, and nerve were thus compressed. The circumference of the outer loop was adjusted so that the tips of the spring were 1 inch apart; in this position the spring exerted a static force of 2.8 kg on the suture. To test for completeness of arterial closure, India ink was administered intravenously to two rabbits during the time the loops were in place, and it was found that the anterior tibial muscles were the only nonblackened muscles in the body. To terminate the ischemia after the desired period of time, the spring and the suture were removed.

In a preliminary series of experiments it was found that ischemia lasting 2 hr and 40 min produced complete necrosis. Ischemia of shorter duration produced unpredictable amounts of necrosis; if it persisted for a longer time, the necrosis was complicated by interstitial hemorrhage.

Ten muscles which were ischemic for 2 hr and 40 min were examined with the light and electron microscopes 1 day and 1, 2, 3, 4, 5, 6, 7, 12, and 13 wk after the injury. Attempted reinjury after 13 wk was not successful, apparently because the original repair generated collateral circulation from the bone, connective tissue, and tendon.

FIGURE 1 Diagram of partial cross-section of rabbit's lower leg. A suture loop is placed around the anterior tibial muscle; the suture crosses at the skin level and forms another loop which is stretched by a spring, compressing the muscle and its artery, vein, and nerve.

Freezing of Rat and Rabbit Gracilis Muscles

To produce necrosis with cold injury, the gracilis muscles of rats and rabbits were exposed and freed from the adjacent structures. A superficial suture was placed on the surface of the muscle to identify the location of the injury. The round, flat end of an aluminum disk, measuring 0.6 cm in diameter, was cooled by flowing CO_2 gas. The apparatus was similar in design to that described by Hass and Taylor (5). It was applied for approximately 1 min to the muscle or until the full thickness of the muscle was frozen. The skin was closed with sutures. This injury was applied to two gracilis muscles of rabbits and six gracilis muscles of rats. Samples were taken from rabbits on the 2nd and 14th days and from rats on the 1st, 2nd, 4th, 7th, 14th, and 35th days after injury.

In animals in which the injury was repeated, the gracilis muscle was exposed, and the site, identified by the previously placed suture, was frozen again. In two rabbits, four muscles were frozen again at successive 2-wk intervals. They were examined 2 wk after the second, 2 wk after the third, and similarly after the fourth freezing. One muscle was removed 5 wk after the fifth freezing. In rats, five gracilis muscles were frozen three times in succession: the interval between the first two times was 1 wk, and between the second and third freezing was 2 wk. Samples were taken 1, 2, 3, 4, and 5 wk after the third freezing.

OBSERVATIONS

The nature of the injuries produced and the patterns of regeneration resulting in the three experimental models have many features in common. Therefore, the description of the injury, of the events in the interstitium, and of the reconstitution of muscle fibers and capillaries applies to all experiments. Where significant differences occur, as for example in the fate of old BL, they are described separately.

Light microscopy of longitudinal sections of specimens obtained at different intervals after injury reveals that the regeneration proceeds in a zone which moves in time and space along the dead muscle fibers, starting at the junction of viable and dead tissue. The young, regenerated muscle fibers are basophilic, bandlike structures, half the diameter of mature muscle fibers, with a row of centrally placed nuclei. They are oriented in a parallel arrangement in the exact direction of the old, dead muscle fibers. These young muscle cells extend into

the zone of active repair on one end and imperceptibly blend with mature muscle fibers on the other. Because of this gradual transition to mature muscle fibers, the original junction of dead and viable muscle is obscured; only in the grafted muscle can its exact location be approximately ascertained by the location of suture remnants. Within 3 wk, most cell debris has been removed and the area of injury is replaced by mature or maturing muscle fibers.

The sequence of stages in repair of injury to the skeletal muscle fibers and their sustaining blood vessels is clearly discernible with the light microscope. With the electron microscope the exact dating of events is complicated by a sampling problem because the various stages of repair move with time within a narrow zone along the injured muscle fibers. In what follows, the sequence of changes represents a composite view as obtained from many samples of ongoing regeneration from different portions of the area of injury taken at various times.

The Injury

Both the infarcted and frozen areas of muscle appear as a yellow slough 1 and 2 days after injury. Microscopically, most cells are dead: the plasma membrane and nuclei have disappeared and the cytoplasmic constituents have undergone fragmentation and clumping. Only BL, erythrocytes, and occasional muscle cells on the surface of the grafts are preserved and appear undamaged. The debris of the dead cells is not diffusely scattered in the damaged tissues but retained within the confines of the respective BL of different cells and structures (Fig. 2).

Inflammatory Reaction

Inflammatory response to ischemia or cold injury is inconspicuous. Initially, a transient and sparse infiltrate of neutrophils and eosinophils is present; this is followed by the appearance of macrophages. Polymorphonuclear leukocytes are seen mainly in the interstitium and only rarely inside of BL tubes of muscle fibers and capillaries. They do not appear to destroy BL. Later, preceding or during the regeneration of muscle fibers, large mononuclear cells, having many features characteristic of macrophages, appear in the interstitium (Fig. 2). These mononuclear cells contain rough endoplasmic reticulum, mitochondria, phagocytic vacuoles, and lysosomes. They do not seem to disturb the integrity of BL.

Reconstruction of Injured Muscle Fibres

In uninjured muscle the multinucleated myocytes run through the entire length of the muscle; in injured muscle those portions of fibers which were exposed to injury are dead and demarcated from the proximal and distal viable segments. The first indication of ensuing repair is an increase in the size and number of nuclei along the junction of dead and viable fiber segments. Within the first 3 days a cellular zone forms at this junction which then advances slowly through the zone of the dead muscle fibers in the direction of their long axis. In front of this zone is dead muscle, and in its wake are muscle cells in various stages of regeneration. The leading edge of the cellular repair zone is occupied by large mononuclear cells inside the BL tubes of muscle fibers (Fig. 3). Frequently these cells are closely aligned with the inside surface of BL. There appear to be two cell types: one has prominent phagocytic vacuoles which contain electron-opaque material of varying densities, lysosomes, prominent rough endoplasmic reticulum, free ribosomes, and occasional lipid droplets; the other cell type has principally rough and smooth endoplasmic reticulum, free ribosomes and filaments which resemble myofibrils, as well as parallel arrays of myofibrils (Fig. 4). These cells resemble embryonic skeletal muscle cells growing in vitro (13). A third cell type, which is also seen occasionally, has characteristic features of both, the macrophage-like cells and developing muscle cells.

These cells appear to have their origin within the BL tube. Similar cells are not present in the interstitium nor are they seen traversing the BL. Whether they originate from dedifferentiated muscle nuclei or from preexisting reserve cells (10) is not apparent from these experiments.

As the cell debris is removed, the individual cells become larger and fewer in number until only one cell occupies the cross-section of a muscle fiber. These fibers are now smaller than adult ones, have centrally placed nuclei, and contain an abundance of myofibrils. They are surrounded by a loosely fitting, pleated BL representing the residuum of the old, original muscle fiber (Fig. 5).

At about this stage a new layer of BL appears along the portions of the plasma membrane which is not in apposition with the old BL layer. In areas where plasma membrane is in close proximity to the old BL layer, a new BL does not appear. The result is focal duplication of BL (Figs. 5 and 6).

There is no indication within the cytoplasm nor on the surface of the new cell how the new BL is formed.

The extent of replacement of dead muscle fibers with new ones differs among the experimental models. In the infarcted tibialis, anterior and fro-

zen gracilis muscles of both animals, the reconstitution is almost complete, rendering the injured area indistinguishable from the uninjured portions. In the grafted piece, however, only a part of the dead muscle fibers is replaced. Grossly the regenerated piece is thinner than the adjacent unin-

FIGURE 2 1-wk old autograft of rabbit gracilis muscle. Section is from the central portion of the graft where regeneration is still absent. The cell debris of muscle fibers and capillaries is contained by BL (muscle BL, long arrows; capillary BL, short arrows). In the upper right quadrant an "empty" BL tube is present. A macrophage (*M*) is seen in the interstitium. $\times 3000$.

FIGURE 3 Rat gracilis muscle 4 days after injury with cold. Large cells, containing electron-opaque material of different densities, occupy the BL tubes (long arrows) of muscle fibers. A remnant of capillary, consisting only of BL (short arrow), cell debris, and erythrocyte is in the interstitium. All structures have a single layer of BL. $\times 6000$.

FIGURE 4 Rat gracilis muscle 8 days after injury with cold. Portions of three cells (labeled A, B, C) are aligned along the inside surface of single layered BL (long arrow). Cells A and C contain myofibrils characteristic of skeletal muscle fibers while the cell B has the features of a macrophage. $\times 9000$.

jured muscle, and microscopically it contains fewer muscle fibers and excessive amounts of collagen. Among the collagen bundles and fibroblasts, acellular BL tubes are encountered during the first three wk of regeneration. Later they are fewer or completely absent.

In the grafts which were placed perpendicular to the original course of muscle fibers, the regeneration is also incomplete. The fibers which have regenerated are oriented in the direction of the dead fibers, that is, perpendicular to the course of the muscle fibers of the undamaged muscle.

FIGURE 5 Rabbit gracilis muscle, 1 wk after excision and *in situ* reimplantation. The cross-section of muscle fiber is occupied by one cell with a centrally placed nucleus. The outside of the cell is surrounded by a redundant BL (long arrows). A second layer of BL is apparent in several sites (short arrows and *insert*) where the outer, pleated BL layer is some distance away from the plasma membrane. The inner BL follows the contours of plasma membrane. $\times 6500$.

FIGURE 6 Rat gracilis muscle 1 wk after injury with cold. The old BL (long arrow) is pleated; a second BL (short arrow) is present along the plasma membrane in sites where the old BL is some distance away. $\times 13,000$.

FIGURE 7 Rabbit anterior tibial muscle, 1 wk after infarction. Two elongated interstitial cells (IC) with the characteristics of fibroblasts are arranged along the muscle fibers. One fibroblast is aligned particularly close to the outer surface of the muscle fiber which is lined in part by two layers of BL (arrows). $\times 6400$.

Reconstruction of Capillaries

Initially, the endothelial cells and pericytes of capillaries lie dead within the areas injured by ischemia or cold. Their plasma membranes and nuclei have disintegrated (Fig. 2). The cell debris is confined by the BL of endothelial cells and pericytes. Intact red blood cells or their ghosts are seen occasionally among endothelial cell debris.

Regeneration of capillaries is not synchronous with the reconstruction of muscle fibers. The new capillaries begin to develop after the regeneration of muscle fibers is well underway. The new cells which appear in the BL tube of the old capillary contain abundant rough endoplasmic reticulum and free ribosomes. Frequently, cell debris is seen between the BL and the new cell. At the time when

the lumen becomes apparent, first as a simple slit and later as an opening containing flocculent material and erythrocytes, a new layer of BL begins to appear closely applied to the outer plasma membrane of the endothelial cell (Fig. 8). The new layer of BL generally appears around the entire perimeter of capillary, except in sites of the capillary circumference in which the old BL is in close apposition with the plasmalemma of the new endothelial cell. Pericytes generally appear adjacent to endothelial cells, and they too become enveloped by a single layer of BL. Between the pericyte and endothelial cell, only one layer of BL is present. Such newly formed capillaries are complete, consisting of an endothelial tube, pericyte, and BL, and are located within the BL tube of the old capillary. In this way the new capillaries develop

FIGURE 8 Regenerating capillary from rabbit anterior tibial muscle 1 wk after infarction. Along the outer plasma membrane of the endothelial cell a new layer of BL appears to be developing (short arrows). The entire structure, including some cell debris, is surrounded by the BL of the original capillary (long arrows). Most of the old BL is composed of one layer, except in a short section where two layers are present: the space (S) delineated by these two layers is probably the site of a pericyte in the original capillary. The lumen of the new capillary contains an erythrocyte. $\times 12,500$.

two layers of BL, a new inner one and an outer, old one. There are, however, exceptions: In some parts of capillary circumference only a single BL layer may be present, as if a new one has not been formed or the old layer has been removed. On the other hand, three layers may be present in certain parts of the capillary perimeter as shown in Fig. 8: The inner one belongs to the new endothelial cell, a middle layer probably represents the BL which separated a pericyte from an endothelial cell in the original capillary, and, finally, an outermost layer which probably covered the outside of the pericyte in the old capillary.

In experiments with repeated injury, multiple layers of BL are frequently found. They occur most commonly in capillaries and are rare in muscle fibers. The layering is generally not concentric around the perimeter of the capillaries but occupies only segments of the circumference. The number of layers corresponds only roughly to the number of times the injury was repeated.

In all specimens and most notably in the grafted

pieces of skeletal muscle, the BL appears to be repopulated always with the correct cell types. As far as it is possible to make out, capillaries are not seen developing within the large redundant BL of skeletal muscle fibers, nor is there evidence of muscle cells growing inside capillary BL tubes. Equally remarkable is the fact that the regenerating cells invariably grow on the cell side of the BL and are never seen aligning on the outside BL surface, that is, the surface facing the connective tissue.

The Fate of the Old BL Layer

Regenerated muscle fibers and capillaries frequently possess two layers of BL covering portions of their surfaces (Figs. 5–10). In skeletal muscle fibers the BL is commonly folded and redundant. Such duplication is seen after each of the three types of injuries. During the first 3 wk, approximately 60% of the surface area of new muscle fibers is covered by a double BL. Later, between 3 and 5 wk, it is seen less commonly and is almost ab-

FIGURE 9 Capillary cross-section from rabbit anterior tibial muscle 13 wk after infarction. The capillary is surrounded in part by more than one BL layer. The outer layer (long arrows) is discontinuous while the inner layer (short arrows) is closely applied to the outer plasma membrane of the endothelial cells. Multiple layers of BL are present adjacent to the pericyte (P) while the pericyte is separated from the endothelial cell by a single investment of BL. $\times 13,300$.

FIGURE 10 Rabbit anterior tibial muscle 8 days after ischemic necrosis. The small vessel is partly lined by two layers of BL (arrows and *insert*). Interstitial cells (*IC*) having the appearance of fibroblasts are embracing a portion of the vessel's circumference. $\times 8600$.

sent after 5 wk. At this stage the muscle fibers have generally a single, tightly fitting investment of BL.

In the interval between the appearance and disappearance of two BL layers, the regenerated muscle fibers develop a seemingly characteristic relationship with some fibroblast-like interstitial cells (Fig. 7). These cells are large and elongated and possess multiple cell processes. They are not covered by BL. Substantial amounts of rough endoplasmic reticulum and distended cisternae filled with granular, electron-opaque material are frequently present, whereas phagocytic vacuoles and lysosomes are uncommon or absent. These fibroblast-like cells appear to embrace the muscle fibers, and their plasma membrane follows the contours of the outer surface of the BL of skeletal muscle fibers. Most commonly these embracing cells are

seen on the portions of muscle fibers where the BL is duplicated, although not all sites with two BL are covered by these cells. Only rarely do the embracing cells occur over portions of muscle fibers which are lined by a single BL layer. 5 wk after injury, when most muscle fibers have a single BL layer, these embracing cells are almost entirely absent.

In contrast to the BL of skeletal muscle fibers, the fate of excessive BL layers in regenerated capillaries depends on the type of injury: After ischemia by either excision or infarction, the outer layer of BL may be present for many weeks (Fig. 9). After freezing, however, the layering of BL is present only in the early stages of regeneration and is absent in tissues obtained 3 wk after injury.

The capillaries, after they have completely re-

generated and have formed a new layer of BL, also become partly surrounded by fibroblast-like interstitial cells which follow the contours of the outer BL surface with their plasmalemma (Fig. 10). Capillaries are rarely encircled completely, most commonly, several cell processes are applied along different positions of capillary circumference. These embracing cells are similar in appearance to those embracing the skeletal muscle fibers: they possess prominent rough endoplasmic reticulum and distended cisternae containing granular, electron-opaque material. Phagocytic vacuoles and lysosomes are rare. They are most commonly present during the first 3 wk of regeneration. Later, they become rarer and are absent in specimens taken 5 wk after injury.

DISCUSSION

It has been recognized for roughly 100 yr that skeletal muscle cells regenerate along the tracks left by dead portions of muscle fibers. It has been also recognized that nerves regenerate, in an orderly rather than random pattern, along the tracks of old nerves. Waldeyer (15) in 1865 ascribed the role of guiding the regenerating muscle cells to sarcolemmal tubes, an observation which has been confirmed repeatedly since then (1-3, 12-14, 18). However, it is only since the advent of the electron microscope that we can examine in reasonable detail at the ultrastructural level some of the features of this process.

In the experiments described in the present paper, the reconstitution of tissues after several different ways of producing death or destruction of portions of muscle fibers and their associated small blood vessels is engendered by cell regeneration along the scaffolding of BL and by clean-up of cell debris within the old BL structures. As far as one is able to make out from the experiments, the endothelial cells follow along the BL of endothelial tubes, and the regenerating muscle cells follow along the BL tubes of muscle fibers even if the BL tubes have been transected and oriented perpendicular to the direction of viable muscle fibers. This feature is of special interest because it implies that endothelial cells have some means of recognizing their own kind of BL, and that the same holds true for muscle cells. Furthermore, it is interesting that the inside of the BL tube and not the outside appears to be the pathway which can be "recognized" by the newly proliferated or regenerating cells.

Another interesting feature seen in both animal

species and after several different means of inducing injury is that the new cells which form capillaries and striated myocytes produce a new discrete layer of BL of normal thickness. In the experiments described, two layers could be clearly distinguished after the first episode of necrosis and, in some places, multiple layers after several episodes of injury. These observations are compatible with the notion that, at least in certain tissues or with certain cell types, the production of BL is a "quantized" event. The evidence which appears to support this concept stems in part from observations made on skeletal muscle capillaries of patients with diabetes mellitus (16) where the thickened BL is not homogeneous but is composed of multiple concentric layers of BL resembling, at least superficially, growth rings seen on cross-sections of tree trunks. Additional evidence for this concept stems from the experiments reported earlier (18) in which the BL of rat muscle was labeled with silver by feeding the animals silver nitrate in drinking water for 20 months. The silver nitrate was discontinued and replaced by fresh water at the time *in situ* autografts of gracilis muscles were performed. 3 wk later, most capillaries and muscle fibers which had regenerated within the transplanted pieces of muscle had two layers of BL: the outer layer contained electron-opaque granules, resulting from feeding the silver, while the inner BL layer which was in apposition to the plasma membrane was free of granules. The experiment was interpreted as showing that after cell death the silver-labeled BL provided a scaffolding for the regenerating cells and that the inner BL layer was formed after termination of silver feeding.

These observations and the experiments presented here seem to indicate that the capillaries as well as skeletal muscle cells can make a new BL layer during their differentiation from precursors to fully differentiated cells. Whether they will develop a new BL layer or not appears to have some relationship to the distance which exists at a certain stage of cell development between the plasma membrane of the newly formed cell and the old BL. If the plasma membrane is immediately opposed to the old BL, then a new BL layer is apparently not formed but the old BL layer becomes the BL of the new cell. If, however, the old BL is separated from the plasma membrane of the new cell by some distance, then the new cell develops a new layer of BL in apposition to its plasma membrane. Wherever this occurs, the cell is lined by two layers of BL as depicted in Figs. 5-10. It appears

from our observations that a separation of the order of the width of a single BL layer is enough to incite the formation of a new BL layer.

At least two reasons for the nonalignment of the new cell's surface with the old BL are apparent. In skeletal muscle, for example, the old BL is thrown into folds and the plasma membrane of the new cell touches only the innermost surfaces of the BL folds and ridges (Figs. 5 and 6). In regenerating capillaries, on the other hand, the endothelial cell tube is separated from the BL of the original capillary by cell debris as shown in Fig. 8. When the new layer of BL is formed, the cell debris usually becomes encased between the two layers.

There seem to be differences among tissues in regard to whether or not regenerating cells will produce a new layer of BL. Skeletal muscle fibers, muscle capillaries, renal tubules (4), and nerves of skin (11) are examples of tissues where two layers of BL are commonly present after a single episode of injury and regeneration. In contrast, the alveolar epithelium and septal capillaries of the lung (17) and the epidermis in the monkey (6) and mouse (9) seem to utilize the old BL as their immediate footing and do not form a new layer of BL. The epidermis in chick embryo, however, may develop two layers of BL after it has been detached and reapposed to the mesenchyme *in vitro* (8). The reason for these differences is not apparent. They are not seemingly related to the nature of injury.

Evidence for turnover of BL in the conventional sense was not observed. However, the fact that at several weeks after the episodes of freezing or ischemia the muscle fibers within the area of injury had only one BL layer indicates that there is a mechanism for removal of old BL of skeletal muscle fibers. This is not an instantaneous process, and the disappearance of old BL lags for some time after the removal of the cell debris has been completed and cell regeneration is well underway. It seems that the original BL of skeletal muscle fibers remains intact at least long enough to provide the template for harmonious reconstruction of the damaged muscle.

How or by what means the old BL is removed is not clear. The interstitial cells which embrace the muscle fibers and capillaries may be involved in this process: their location, their close apposition to the outer surface of BL, and the timing of their appearance and disappearance suggest that this may be the case. Their configuration and cytoplasmic constituents indicate that they are fibroblasts. One can then construe as part of their func-

tion the reestablishment of structural relationships of collagen with the surface of BL.

The old BL is not invariably removed. In capillaries its fate appears to depend on the type of primary injury. As depicted (Fig. 9), 13 wk after ischemic necrosis the capillaries may still be encased by two layers of BL. In contrast to this, the outer layer of BL is absent in samples removed approximately 3 wk after injury by cold, although it is present in the early stages of regeneration.

In considering the role of BL in maintenance and tissue repair, certain generalizations may be made in the light of what is already known about the BL, particularly about its development during embryogenesis, its chemical composition, and anatomic distribution. The evolving scheme, as it is presented below, suggests that there are several factors which effect the cell-BL relationships and that BL and cells must possess certain properties which make these interactions possible.

In the embryo the BL is formed in very early stages of organogenesis, presumably as the result of "macromolecular complexing" at the inductive tissue interfaces, such as occur between embryonal mesenchyme and developing epithelium (7). There the BL seems to be a physical substrate for orderly orientation of epithelial cells. Its matrix consists of collagen-like protein and contains immunochemically and histochemically detectable glycoprotein which may well represent an accumulation of "cell surface associated material" (7). The character of BL does not apparently change significantly as the animal grows. In the adult state it is found invariably at the interface of parenchymal cells and the compartments occupied by connective tissue.

If cells are lost as the result of normal turnover or injury (4, 12, 17, 18), the BL generally remains physically intact and maintains by its presence the spatial plan of the tissue by containing within one of its surfaces the connective tissue compartments and provides with the other a physical substrate for growth and appropriate orientation of regenerating parenchymal cells. The polarity of BL and its apparent specificity for cell types are probably inherent in its structure in the form of cell surface associated material which is built into it since the time of embryonal tissue interactions.

The denuded BL may be an important stimulus for initiation of cell multiplication. The parenchymal cells will seemingly grow and multiply as long as cell-free BL surfaces are available, and will stop as soon as the BL is populated. The absence of cell-

specific surface which is devoid of cells may then at least in part represent an important negative signal which sets the size limit to reconstruction.

Not all new cells apparently have to be in contact with the old BL to complete the regenerative process. In areas where they are separated from it, a new layer of BL will appear along the plasma membrane. Whether interactions between the new parenchymal cells and the connective tissue cells, similar to those occurring in embryonal organogenesis, are required for this process is unknown. The fact that considerable distances exist between the two tissue components does not exclude this possibility (7).

Finally, the BL seems also to have the important function of limiting the extent of connective tissue regeneration of confining fibroblasts to the spaces delineated by the "connective tissue" face of BL. The boundaries of this space change, however, if, during regeneration, a new layer of BL has formed. Because the new BL represents a new interface between parenchymal cells and the connective tissue compartments, the old BL layer comes to be in the domain of the connective tissue. The nature of the molecular factors and mechanisms involved in the process of "recognition" and of removal remains to be ascertained.

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