

Heterotopic neogenesis of skeletal muscle induced in the adult rat diaphragmatic peritoneum: Ultrastructural and transplantation studies

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Summary. During the course of a mild chemical peritonitis, new skeletal muscle fibers develop and persist over a twelve-month interval in the diaphragmatic peritoneum. Light and electron microscopic studies revealed that the ectopic fibers developed from myoblasts and myotubes to fully differentiated muscle cells in the same manner as normally situated skeletal muscle. The ectopic fibers were separated from the intrinsic muscle by dense connective tissue and an elastic lamina. Diaphragms taken from normal rats and transplanted to the omentum of isogenic recipients also developed skeletal muscle neogenesis in the same ectopic location as in the normal diaphragm. Satellite cells, reactive fibroblasts in the peritoneum, mesenchymal stem cells or blood-borne myoblast precursor cells could be the source of these ectopic muscle fibers. The results of the present studies, however, cannot provide conclusive evidence for the origin of the new muscle fibers. Regardless of their source, the methods employed may represent a unique model for the development and prolonged maintenance of skeletal muscle fibers in a heterotopic location *in vivo*.

Key words: Myogenesis, Rat diaphragm, Transplanted diaphragm, Ultrastructure skeletal muscle, Chemical peritonitis

Introduction

In adult mammals, smooth muscle can sometimes be found at sites usually devoid of such cells. An example of such a site is the intima of atherosclerotic arteries. New skeletal muscle cells in adults develop in pre-existing skeletal muscle. The rat's diaphragmatic

peritoneum may provide an exception to this rule. After chemical irritation of the diaphragmatic peritoneum, new muscle fibers developed. These new fibers arose, not in the muscular layer of the diaphragm, but in the submesothelial layer, separated from the pre-existing diaphragmatic muscle by collagenous connective tissue and an elastic membrane (Levine and Saltzman, 1994). The new muscle fibers had a further unique property: they were oriented perpendicular to the pre-existing intrinsic diaphragmatic muscle fibers. This geometric relation suggested that lines of force generated by the repeated contractions of the diaphragm were somehow involved in the development of the ectopic muscle fibers.

Other clues to the origin of the ectopic fibers came from the environment in which they were found. The chemical peritonitis which engendered the new skeletal muscle fibers also elicited a granulation tissue composed of abundant fibroblasts and moderate numbers of mononuclear inflammatory cells, surmounted by hyperplastic mesothelial cells (Levine and Saltzman, 1994).

The present study combined ultrastructural analysis of the ectopic muscle cells and their relation to other cells around them in their original environment in the diaphragmatic peritoneum, with a light microscopic study of ectopic muscle cells in a completely altered environment following transplantation of the entire diaphragm. The original purpose of the transplantation experiments was an attempt to produce neomyogenesis in an immobile diaphragm and thereby elucidate the relation between the perpendicular orientation of new muscle fibers and lines of force generated by repeated, rhythmic contractions of the normally situated diaphragm. In addition, however, transplantation produced a diaphragm with severe muscle damage and intense regenerative activity. The contrast with the minimum muscle damage in the normally situated diaphragm provided important evidence on the cellular origin of the new muscle fibers.

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Materials and methods

Lewis rats were maintained on Purina rodent chow 5001 and tap water in plastic cages with hardwood litter. After an overnight fast, a chemical peritonitis was produced by intraperitoneal injection of sodium dodecylsulfate (SDS), 2.0 mg/ml, 5 ml/100 g body weight. At intervals thereafter (1, 4, 7, 14, 42 days; 2, 6 and 12 months), the rats were anesthetized by intramuscular injection of ketamine hydrochloride and acepromazine maleate. The entire diaphragm and its skeletal attachments were quickly dissected out and smaller segments were cut to approximate longitudinal and transverse sections of muscle fibers. These sections were processed for light and electron microscopy. Stains used for light microscopy included Masson trichrome, phosphotungstic acid-hematoxylin and hematoxylin and eosin. Segments of tissue for electron microscopy were fixed in 5% glutaraldehyde, 4% paraformaldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide in buffer, dehydrated in graded ethanol and propylene

oxide and embedded in epoxy resin (Embed 812, Electron Microscopy Science, Ft. Washington, PA, USA). Thick sections (1-3 μm) were viewed with light microscopy and those containing abdominal peritoneal surface were selected for electron microscopy.

Diaphragms were transplanted as described (Levine and Saltzman, 1995). An inbred strain of rats was used so that grafts would not be rejected (Lewis rats, Harlan-Sprague-Dawley, Inc., Indianapolis, IN). Normal donor rats, 7-10 weeks old, were killed with carbon dioxide. The diaphragms were excised and the central tendon and crural portions were discarded. The costal diaphragm was cut in halves or quarters, mounted on millipore filters cut to the same shape, and soaked in cold saline with penicillin 100 U/ml and streptomycin 100 $\mu\text{g}/\text{ml}$. The recipient rats of the same age and sex were anesthetized with ketamine hydrochloride 35 mg/kg and acepromazine maleate 3.5 mg/kg intramuscularly. The abdomen was opened and one leaflet of the greater omentum was cut loose from the stomach, thereby exposing the lesser omental sac. This maneuver provided

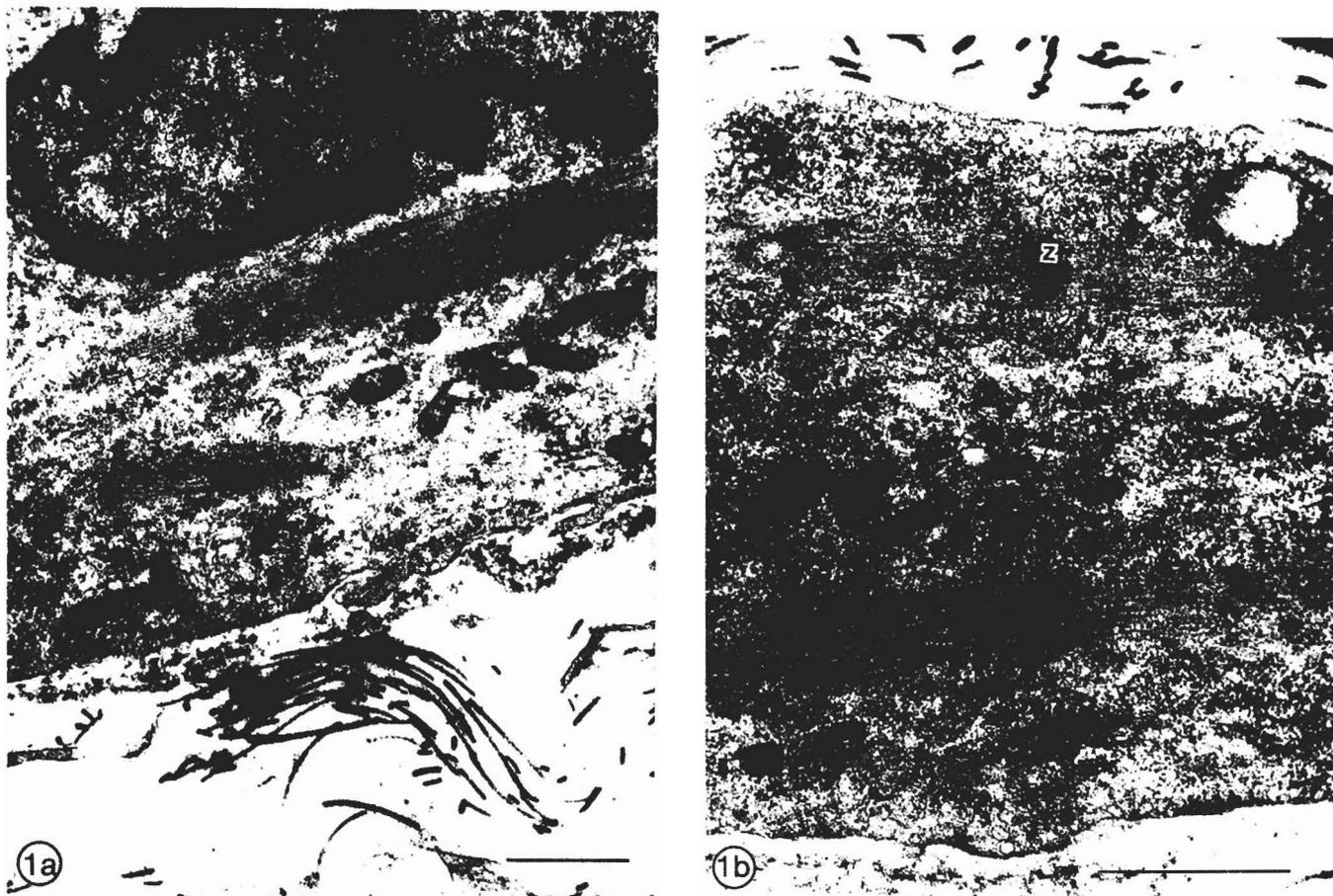


Fig. 1. A. A portion of a presumptive ectopic myoblast, located in the submesothelial connective tissue. Two myofilament bundles can be seen. In the larger bundle above the nucleus Z-bands are present. **Bar:** 1 μm . **B.** In this cell myofilaments are present, Z-lines appear as diffuse transverse densities (Z). Micropinocytotic vesicles are present close to and below the sarcolemma. **Bar:** 1 μm . Examples seen in Fig. 1A,B were present four days following administration of SDS.

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a larger area of omentum on which the graft and its filter disc support were placed. The omentum was folded over the graft from below and from the sides, and the resultant "package" was rolled into the peritoneal cavity. The wound was closed by sutures in the muscle and metal clips in the skin. Benzathine penicillin was injected intramuscularly. Two weeks later, the omentum was removed, fixed in Bouin's fluid, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Institutional and governmental regulations on animal welfare were observed.

Results

Morphological evidence of inflammation was evident at the peritoneal surface one day following administration of the chemical irritant. Intrinsic diaphragmatic muscle appeared intact. By day 4 the thickened area between mesothelium and intrinsic muscle contained numerous collagen bundles, fibro-

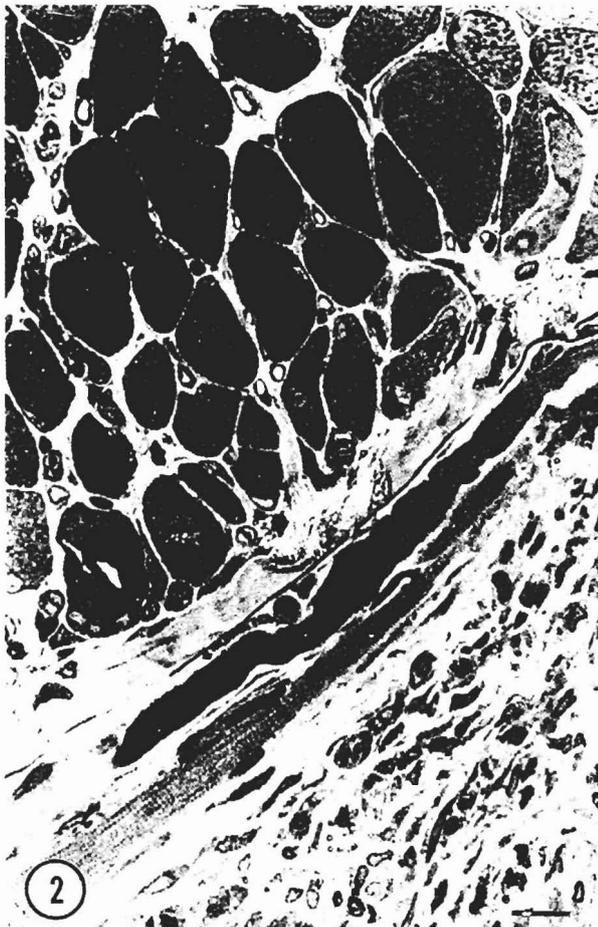


Fig. 2. Submesothelial connective tissue elements present at lower right. Portions of two ectopic striated muscle cells are present below a dense connective tissue lamina; above this transverse sections of intrinsic muscle fibers can be seen. Bar: 20 μ m. Seven days after SDS administration.

blasts, macrophages and eosinophils. In addition to these elements, larger cells with multiple central euchromatic nuclei were often seen. Ultrastructural evaluation of these cells revealed the presence of dispersed bundles of filaments. The presence of dark transverse lines (Z-lines) in longitudinally sectioned filament bundles characterized these large cells as myoblasts (Fig. 1A,B). At 7 and 14 days following administration of chemical irritant, myotubes and more fully differentiated striated muscle cells were evident. These heterotopic myoblasts, myotubes and mature muscle were always noted to be oriented perpendicular to intrinsic muscle fibers. A compact layer of dense connective tissue and an elastic lamina separated these muscle elements from the intrinsic diaphragmatic muscle fibers (Fig. 2).

The heterotopic muscle elements were surrounded by collagen bundles. Fibroblasts were often noted apposed to presumptive myoblasts. This can be seen in Figure 3, sampled from a rat 7 days following administration of SDS. The complex of fibroblast and the two presumptive myoblasts is surrounded by collagen bundles. The fibroblast is adjacent to both cells. Whether gap junctions are present remains to be resolved. Although several muscle cells could be seen in the connective tissue between mesothelium and intrinsic diaphragmatic muscle, they remained as isolated cells and did not group together in fascicles. Beginning at early intervals after the inflammatory response and continuing through later intervals, different stages of differentiation of muscle cells were observed. Portions of two different heterotopic muscle cells sampled 6 months after SDS administration, are seen in Fig. 4A,B. The area directly beneath the sarcolemma contains mitochondria, profiles of rough endoplasmic reticulum and other sarcoplasmic components. In the portion of the cell seen in Fig. 4A the nucleus encompasses the width of the cell and myofibrils are few in number. In the portion of the cell seen in Fig. 4B myofibrils are more numerous. The majority of these muscle cells retained an orientation at right angles to the intrinsic diaphragmatic muscle.

At all time intervals studied the nucleus had a euchromatic appearance. At early times after chemical insult (7-42 days) nuclei were centrally located; at later time intervals (6 months), nuclei were either central or peripheral (Fig. 5). In some instances one muscle cell contained both a central and a peripheral nucleus. All the differentiated muscle cells observed had thick Z-lines indicative of slow twitch muscle fibers. Nerve bundles were seen in the vicinity of these aberrant muscle cells, however, neuromuscular junctions were not seen in the samples studied. Heterotopic striated muscle cells were still present 12 months after the single chemical insult.

Twenty-five transplanted diaphragms were uniformly viable and well populated by muscle cells (Fig. 6). These intrinsic muscle cells were narrower than normal, and their nuclei were larger and often central rather than subsarcolemmal. These features indicate that these were regenerating fibers. There was no evidence of

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graft rejection. A narrow zone of connective tissue separated the intrinsic muscle from the omentum which surrounded the graft. In this zone, in sections where the adjacent intrinsic muscle was cut in cross-sections, there was a single heterotopic skeletal muscle fiber cut longitudinally (Fig. 7). Cross striations were clearly visible. Correspondingly, in sections where the intrinsic muscle was cut longitudinally, there was a row of new heterotopic muscle fibers cut in cross-section (Fig 8). Most important, the new fibers were separated from the intrinsic muscle by a narrow zone of connective tissue and by a highly refractile elastic membrane, proving that they were in an ectopic location. The ectopic fibers were focal, never universal in distribution, just as in the experiments on the normally located diaphragms, and

there was no obvious explanation for the focal distribution. They were found in 18 of the 25 successful grafts.

Discussion

These ultrastructural observations have confirmed the previous report of neogenesis of skeletal muscle fibers in the reactive tissues caused by chemical irritation of the diaphragmatic peritoneum. Also confirmed was the unique observation that the new, ectopic fibers, although separated from the intrinsic diaphragmatic muscle, were oriented at a right angle to them. In addition, the electron microscopic studies revealed that the new ectopic fibers developed and



Fig. 3. A fibroblast (fb) in close association with two presumptive myoblasts is seen surrounded by collagen bundles in longitudinal and transverse sections. Seven days following SDS administration. Bar: 1 μ m.

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matured in the same manner as ordinary skeletal muscle. However, unequivocal evidence for the origin of the new fibers was not obtained.

Several candidates may be considered as the stem cell-source for the new muscle cells demonstrated in this study. The population of resident satellite cells are the source of stem cells that sustain self-renewal and growth of differentiated muscle (Mauro, 1961). In the adult animal satellite cells are normally quiescent. During injury or disease the cells are activated to divide and

repeat the developmental myogenic pathway, thereby adding new muscle cells. In this study, injury of the intrinsic muscle by SDS injection was mild and focal. Furthermore the new muscle cells were located in an area distant from the existing muscle, surrounded by connective tissue. In addition an elastic lamina, possibly fenestrated, was present beneath the intrinsic muscle. Although possible, it is not likely that satellite cells from the intrinsic diaphragmatic muscles migrated through the connective tissue and elastic membrane that separated

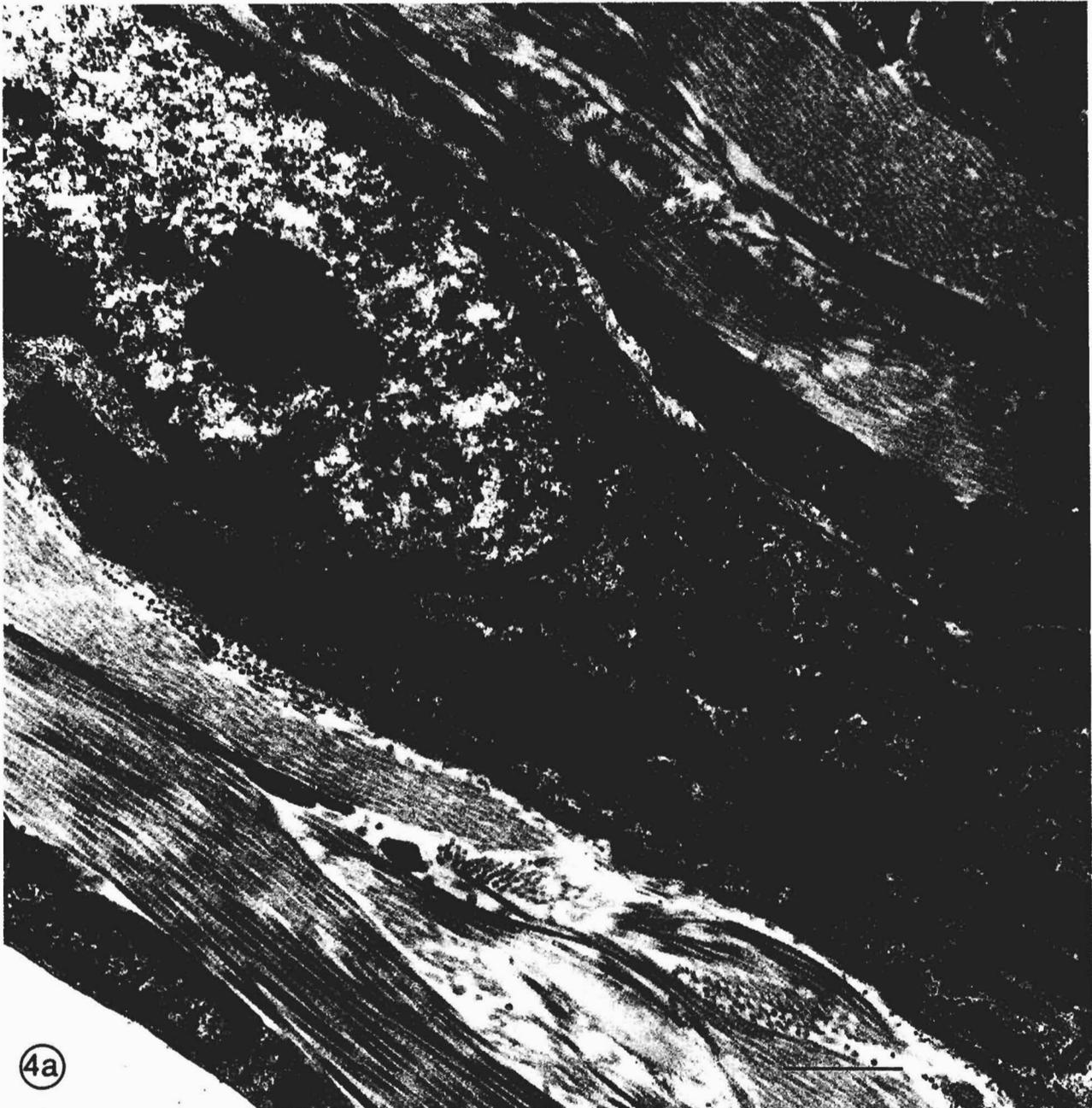


Fig. 4. A, B. Representative examples of ectopic striated muscle cell seen 6 months after administration of SDS. Bar: 1 μ m.

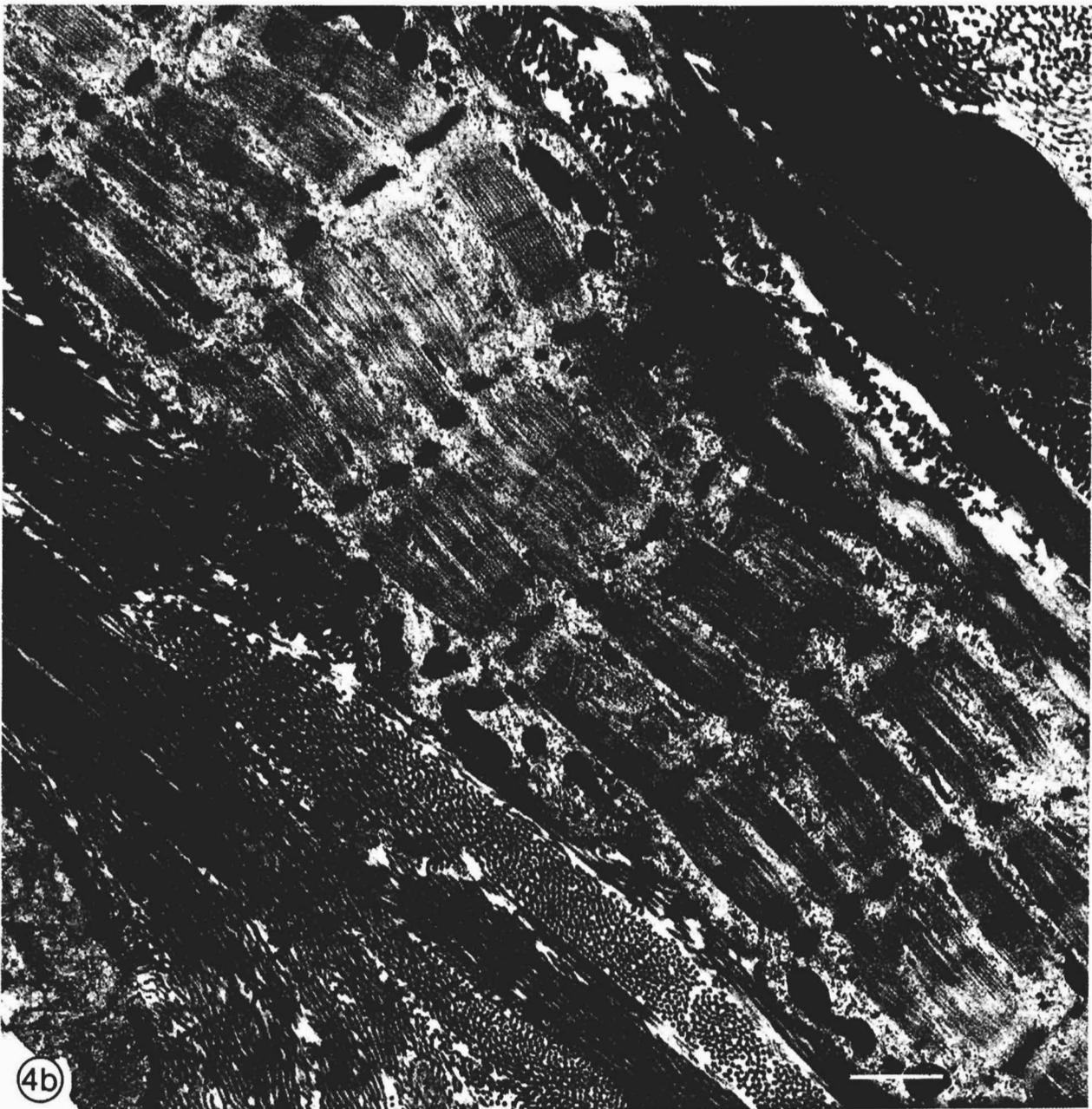
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intrinsic muscle from the new muscle fibers. If satellite cells were capable of making such a migration, one might expect neomyogenesis, not only below the peritoneal mesothelium, but also in the connective tissue layer adjacent to the intrinsic muscle. No muscle cells were found in this location.

The graft experiments added important evidence against a role for satellite cells. The grafts were studied two weeks after transplantation, when necrotic debris had been removed and intense regeneration was underway. The regenerative process should have stimulated the satellite cells, yet the neogenesis was no

more widespread than in the normally situated, non-transplanted diaphragm. Furthermore, the new muscle fibers were still ectopically located and had the usual orientation perpendicular to the intrinsic fibers.

Modern methods for activation of skeletal muscle specific genes have been used to demonstrate the development of skeletal muscle, *in vitro*, from a wide variety of non-muscle cells. These have included amniocytes and chorionic villus cells (Sancho et al., 1993), pericytes (Grounds, 1990), smooth muscle (Van Neck et al., 1993), osteoblasts (Grigoriadis et al., 1988), thymic cells (Grounds et al., 1992; Spuler et al., 1994)



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and CNS cells (Wier and Lennon, 1981; DeVitry et al., 1994; Tajbakhsh et al., 1994). Weintraub et al. (1989) have shown in studies with MyoD that expression of these gene products is able to activate myogenesis in cell lines derived from mesodermal, ectodermal and endodermal tissue. Gown (1990) has stated that "different nonmuscle cells can acquire, under certain circumstances, muscle-like features - representing a common final pathway for cells of different types that respond to a particular set of signals in response to particular local needs." Cossu (1997), Breton et al. (1995) and Salvatori et al. (1995) have reported that myogenesis can be induced in a mononucleated fibroblast by signals derived from neighboring myogenic cells.

In our study fibroblasts were often seen in intimate relation to the new muscle fibers. The entire submesothelial area contained fibroblasts but neogenesis was found only in its deepest part, adjacent to the elastic membrane. The thickness of the submesothelial zone varied greatly, but neogenesis occurred where the zone was either thick or thin and where fibroblasts were either numerous or scanty. In the grafts there was even less fibroplasia, perhaps because no SDS had been used, but neogenesis occurred anyway. From our morphological studies there is no direct or indirect evidence for the conversion of fibroblasts to skeletal muscle cells.

The peritoneal mesothelium could have been the source of the new muscle cells because the location of the new muscle fibers, just above (superficial to) the

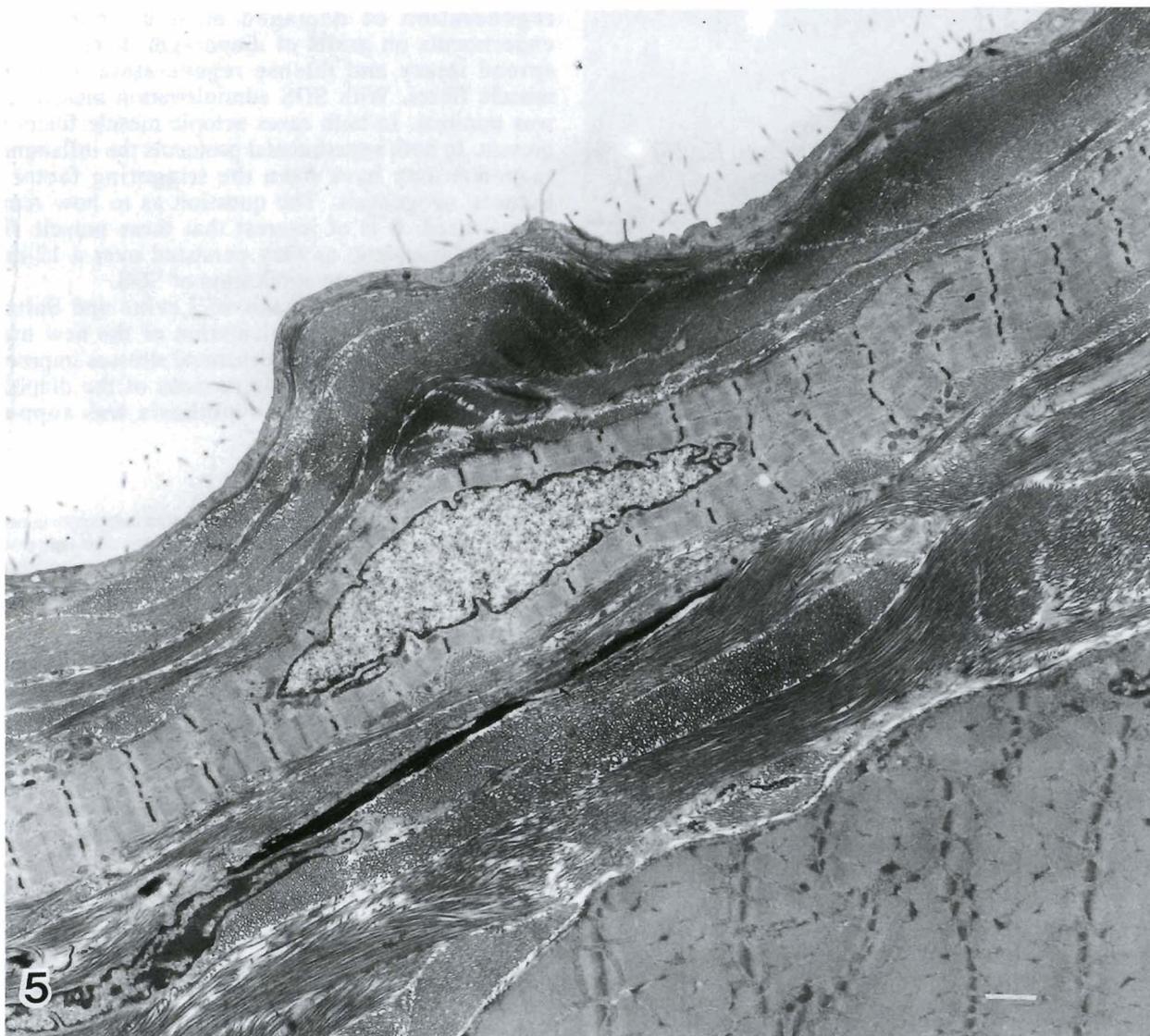


Fig. 5. Mesothelial cells seen at top of figure. An ectopic striated muscle cell with central euchromatic nucleus is seen surrounded by components of dense connective tissue. Six months after SDS. Intrinsic diaphragmatic muscle is seen at bottom. Bar: 1 μ m.

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elastic lamina is the location of the mesothelial cells before the reactive fibroplasia was started by the SDS injection. However, despite their favorable location, there is no direct evidence from our studies to implicate mesothelial cells.

Mesenchymal stem cells are thought to reside within connective tissue (Young et al., 1995). During development of the diaphragm myoblasts differentiate within the septum transversum and emigrate to form part of the future diaphragm muscle. Most of the septum

transversum then gives rise to the non-muscular central tendon of the diaphragm (Larsen, 1997). Connective tissue from the central tendon ensheaths diaphragmatic muscle fibers. Perhaps this connective tissue contains dormant myoblasts that are activated on "stimulation" (SDS or graft) and repeat their embryological fate.

Satellite cells, fibroblasts, mesothelial cells and mesenchymal stem cells are potential local sources of myogenesis. Alternatively myoblast precursor cells could enter from the blood stream (Bateson et al., 1967). Wakitani et al. (1995) have reported that *bone marrow* mesenchymal stem cells appear to have the capacity to be induced to differentiate *in vitro* into myogenic and adipocytic phenotypes. Ferrari et al. (1998) have demonstrated *in vivo* that bone marrow-derived cells can migrate into areas of induced muscle degeneration, undergo myogenic differentiation and participate in the regeneration of damaged muscle fibers. In our experiments on grafts of diaphragm there was widespread injury and intense regeneration of intrinsic muscle fibers. With SDS administration muscle injury was minimal. In both cases ectopic muscle fibers were present. In both experimental protocols the inflammatory response may have been the triggering factor that induced neogenesis. The question as to how remains unanswered. It is of interest that these muscle fibers were not transient, as they persisted over a 12-month interval following one application of SDS.

The original hypothesis of Levine and Saltzman (1994) that the unique orientation of the new muscle fibers was related to the mechanical stresses imposed by the constantly repeated contractions of the diaphragm must be amended. This hypothesis was supported

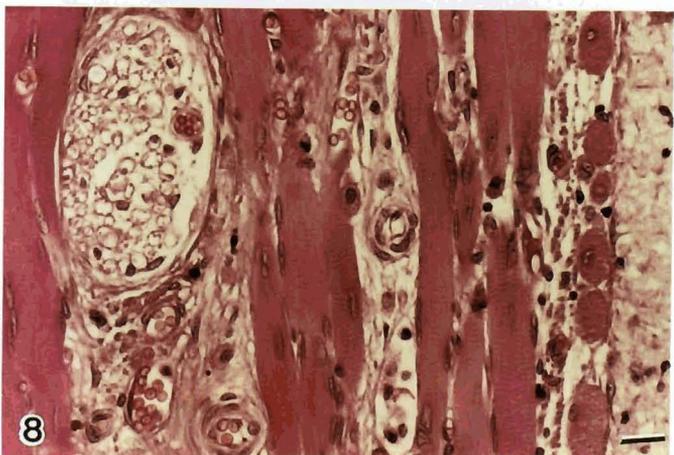
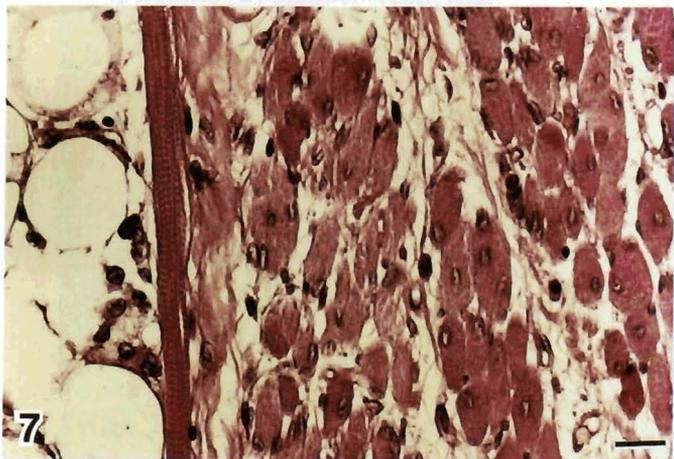
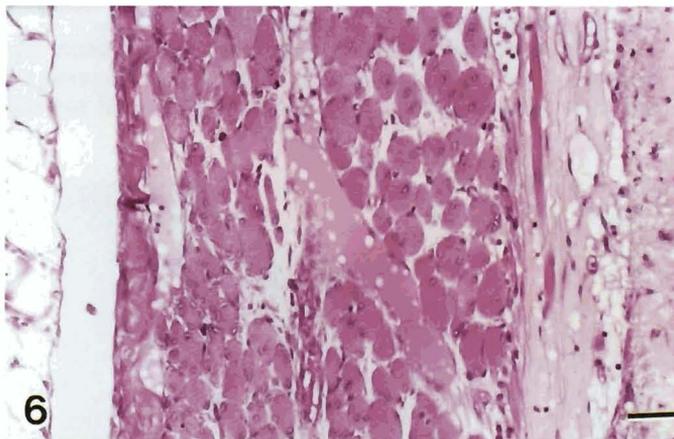


Fig. 6. Two weeks after grafting, the transplanted diaphragm is between the omental fat (left side) and the supporting filter disc (extreme right side). The intrinsic muscle fibers are cut transversely, but there is a single ectopic fiber cut longitudinally between the intrinsic muscle and the filter disc (right side). Hematoxylin-eosin. Bar: 40 μ m.

Fig. 7. The transplanted diaphragm (two weeks after grafting) occupies the center and right portions of the photograph. Note the enlarged, centrally located muscle nuclei. The intrinsic muscle cells are cut transversely or obliquely. Between the diaphragm and the omental fat (left side of picture), there is a single muscle fiber with prominent cross-striations that has been cut longitudinally. Careful examination will reveal that this ectopic muscle fiber is separated from the intrinsic diaphragm muscle by a thin membrane and some connective tissue. All these details correspond exactly to the reaction of the normally situated diaphragm to a chemical irritant. Hematoxylin-eosin. Bar: 20 μ m.

Fig. 8. Another transplanted diaphragm (center and left) which differs from Fig. 7 in that the intrinsic fibers have been cut longitudinally and many of them show cross-striations. Between fiber groups there are some macrophages and connective tissue, and, in the upper left corner, a degenerating nerve. The supporting membrane filter is at the extreme right. Between it and the diaphragm there is a single line of new muscle fibers that have been cut transversely. The development of ectopic muscle fibers oriented at a right angle to the intrinsic muscle fibers of the transplanted diaphragm corresponds to the reaction to an irritant of the normally located diaphragm. Hematoxylin-eosin. Bar: 20 μ m.

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indirectly by the production in arteries of longitudinal smooth muscle cells oriented at right angles to the normally circumferential smooth muscle (Wagenaar and Wagenvoort, 1978). In addition, mathematical analysis had confirmed that the new smooth muscle fibers were aligned in the axis of least mechanical stress (Kockx et al., 1993). However, the transplanted diaphragm, separated from its skeletal attachments, surrounded by the supporting disc on one side and omentum and connective tissue on the other side, and lacking innervation, was certainly immobile and not subject to mechanical stress. Nevertheless, ectopic neogenesis of skeletal muscle developed in the same position in the immobile graft as in the normally situated and constantly moving diaphragm. Therefore, the development and orientation of new skeletal muscle in our model cannot be attributed to contractile forces.

The conversion of non-muscle cells to myoblasts *in vitro* has been extensively studied (Miranda et al., 1990). Our model is of interest because it is one of the very few studies in adult mammals that suggests neogenesis of skeletal muscle from non-muscle cells *in vivo*.

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