

# Fibroblasts that Proliferate Near Denervated Synaptic Sites in Skeletal Muscle Synthesize the Adhesive Molecules Tenascin(J1), N-CAM, Fibronectin, and a Heparan Sulfate Proteoglycan

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**Abstract.** Four adhesive molecules, tenascin(J1), N-CAM, fibronectin, and a heparan sulfate proteoglycan, accumulate in interstitial spaces near synaptic sites after denervation of rat skeletal muscle (Sanes, J. R., M. Schachner, and J. Covault. 1986. *J. Cell Biol.* 102:420–431). We have now asked which cells synthesize these molecules, and how this synthesis is regulated. Electron microscopy revealed that mononucleated cells selectively accumulate in perisynaptic interstitial spaces beginning 2 d after denervation. These cells were identified as fibroblasts by ultrastructural and immunohistochemical criteria; [<sup>3</sup>H]thymidine autoradiography revealed that their accumulation results from local proliferation. Electron microscopic immunohistochemistry demonstrated that N-CAM is associated with the surface of the fibroblasts, while tenascin(J1) is associated with collagen fibers that abut fibroblasts. Using immunofluorescence and immunoprecipitation methods, we found that fibroblasts isolated from perisynaptic regions of denervated muscle synthesize N-CAM, tenascin(J1), fibronectin, and a heparan sulfate proteoglycan in vitro. Thus, fibroblasts that selectively proliferate in interstitial spaces near synaptic sites are likely to be the cellular source of the interstitial deposits of adhesive molecules in denervated muscle.

To elucidate factors that might regulate the accumulation of these molecules in vivo, we analyzed the expression of tenascin(J1) and fibronectin by cultured

fibroblasts. Fibroblasts from synapse-free regions of denervated muscle, as well as skin, lung, and 3T3 fibroblasts accumulate high levels of tenascin(J1) and fibronectin in culture, showing that perisynaptic fibroblasts are not unique in this regard. However, when they are first placed in culture, fibroblasts from denervated muscle bear more tenascin(J1) than fibroblasts from innervated muscle, indicating that expression of this molecule by fibroblasts is regulated by the muscle's state of innervation; this difference is no longer apparent after a few days in culture. In 3T3 cells, accumulation of tenascin(J1) is high in proliferating cultures, depressed in confluent cultures, and reactivated in cells stimulated to proliferate by replating at low density or by wounding a confluent monolayer. Thus, synthesis of tenascin(J1) is regulated in parallel with mitotic activity. In contrast, levels of fibronectin, which increase less dramatically after denervation in vivo, are similar in fibroblasts from innervated and denervated muscle and in proliferating and quiescent 3T3 cells. Together, these results suggest that the selective perisynaptic accumulation of tenascin(J1) after denervation may be part of a general program of cell activation that is induced by a mitogenic signal acting locally on ordinary fibroblasts. As axons grow toward original synaptic sites which they preferentially reinnervate, they are likely to encounter perisynaptic fibroblasts, and may be influenced by fibroblast-derived adhesive molecules.

IT is well-known that denervation of vertebrate skeletal muscle leads to numerous changes in the contractile, electrical, metabolic, and molecular properties of the muscle fiber membrane and sarcoplasm (see Engel and Banker, 1986 for reviews). There is also evidence that denervation alters interstitial spaces between muscle fibers: mononucleated cells proliferate in denervated muscle, and fibrosis accompanies denervation atrophy (e.g., Murray and Robbins,

1982a,b; Salonen et al., 1985; Connor and McMahan, 1987). We have recently observed a denervation-induced change in the molecular composition of intramuscular connective tissue that precedes atrophy and is localized to synaptic areas: the neural cell adhesion molecule (N-CAM)<sup>1</sup>, the tenascin-

1. *Abbreviations used in this paper:* M-HSPG, matrix-associated heparan sulfate proteoglycan; N-CAM, neural cell adhesion molecule.

(J1) glycoprotein<sup>2</sup>, fibronectin, and a matrix-associated heparan sulfate proteoglycan (M-HSPG) all accumulate in interstitial spaces near denervated synaptic sites (Covault and Sanes, 1985; Sanes et al., 1986).

We focused our attention on these spaces because they constitute the terrain that regenerating axons traverse in returning to original synaptic sites, which they preferentially reinnervate (reviewed in Sanes and Covault, 1985). In this regard, it is intriguing that neurons are known to be capable of interacting with all four molecules that accumulate perisynaptically: N-CAM is one of a group of membrane-bound cell adhesion molecules that mediates adhesion between neurons (Edelman, 1986) and between neurons and myotubes (Rutishauser et al., 1983; Bixby et al., 1987). Antibodies to tenascin(J1), a glycoprotein first identified in muscle (Chiquet and Fambrough, 1984), inhibit neuron-astrocyte adhesion (Kruse et al., 1985). Fibronectin and M-HSPG, both components of the extracellular matrix, have each been shown to promote neurite outgrowth in vitro (fibronectin: reviewed in Sanes, 1989; M-HSPG: Hantaz-Amroze et al., 1987). The coordinate and localized response of these four adhesive macromolecules to denervation raises the interesting possibility that perisynaptic connective tissue may provide an attractive substrate for regenerating axons (discussed in Covault et al., 1987). We have therefore investigated the cellular basis of this response.

## Materials and Methods

### Antibodies

Affinity-purified polyclonal antibodies to N-CAM (Covault and Sanes, 1986), monoclonal antibodies to M-HSPG (Eldridge et al., 1986), J1 antiserum, and affinity-purified J1 antibodies (Kruse et al., 1985) were prepared and characterized in our laboratories. Monoclonal antibodies to J1-160/180 and J1-200/220 (=tenascin) were produced by A. Fraissner, J. Kruse, and B. Pesheva (University of Heidelberg), using standard techniques. A monoclonal antibody to human plasma fibronectin (which recognizes an epitope on rat fibronectin) and affinity-purified serum antibodies to fibronectin were supplied by J. McDonald (Washington University, St. Louis, MO). Monoclonal antibodies to embryonic rat myosin (Gambke and Rubenstein, 1984), the Schwann cell surface antigen 217c (Fields and Dammerman, 1985), Thy 1.1, and desmin were obtained from Neil Rubenstein (University of Pennsylvania, Philadelphia, PA), Barbara Ranscht (La Jolla Cancer Research Foundation, CA), Serotec (Indianapolis, IN), and Biogene (Dublin, CA), respectively. Fluorescein- and rhodamine-conjugated second antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Atlantic Antibodies (Scarborough, ME) and Boehringer Mannheim Biochemicals (Indianapolis, IN), horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Cappel Laboratories, Inc. (Cochranville, PA), and alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Boehringer Mannheim Biochemicals.

2. The J1 antiserum used in our original studies (Kruse et al., 1985; Sanes et al., 1986) is now known to recognize two distinct groups of antigens, J1-160/180 and J1-200/220, with  $M_r$ 's of 160,000-180,000 and 200,000-220,000, respectively. It was recently shown (Faissner et al., 1988) that J1-200/220 is immunohistochemically indistinguishable from the myotendinous antigen (Chiquet and Fambrough, 1984) or tenascin (Chiquet-Ehrismann, 1986, 1988); it is also very likely to be equivalent to proteins that have been designated hexabrachion (Erickson and Taylor, 1987), GMEM (Bourdon et al., 1985) and cytactin (Grumet et al., 1985; Hoffman et al., 1988). We show here that the J1-reactive material present in muscle and synthesized by fibroblasts is J1-200/220 or tenascin-like. To promote uniformity of nomenclature without denying the possibility of differences among tenascin-like molecules, we refer to this material as tenascin(J1) herein.

### Histology

Male Holtzman rats (130-150 g) were obtained from Sasco, Inc. (Omaha, NE). The animals were anesthetized with ether before surgery. Diaphragms were denervated by intrathoracic section of the left phrenic nerve (Miledi and Slater, 1970).

Immunofluorescence analysis of muscle was carried out as previously described (Covault and Sanes, 1986). Briefly, unfixed muscles were cross-sectioned at 4-6  $\mu$ m in a cryostat, and the sections were incubated sequentially with antibody and then with a mixture of fluorescein second antibody and rhodamine- $\alpha$ -bungarotoxin.  $\alpha$ -Bungarotoxin binds specifically to acetylcholine receptors and thus marks synaptic sites; acetylcholine receptors that appear extrasynaptically after denervation are comparatively sparse and do not complicate identification of denervated endplates.

For ultrastructural localization of N-CAM and tenascin(J1), animals were anesthetized with chloral hydrate and then perfused with physiological saline solution followed by 4% paraformaldehyde in 100 mM sodium phosphate, pH 7.4. Diaphragms were dissected and incubated in the same fixative for 12-16 h at 4°C. 100- $\mu$ m cross sections were cut on a Vibratome and incubated for 12-16 h at 4°C in affinity-purified anti-N-CAM (5-10  $\mu$ g/ml), affinity-purified J1 antibodies (5  $\mu$ g/ml), or nonimmune IgG (15  $\mu$ g/ml) in PBS (150 mM NaCl, 15 mM Na phosphate, pH 7.3) plus 10 mg/ml BSA. The muscle sections were then washed in PBS plus 20% normal goat serum for 2 h, incubated overnight at 4°C in peroxidase-conjugated F(ab)<sub>2</sub> fragments of goat anti-rabbit IgG in PBS plus 20% goat serum, washed again, and then refixed for 45 min with 1% glutaraldehyde, 100 mM sodium phosphate, pH 7.4. After another wash, the sections were incubated for 30-45 min in 1 mg/ml diaminobenzidine, 100 mM citrate, pH 6, plus 0.015% H<sub>2</sub>O<sub>2</sub>, and then rinsed, refixed in 1% OsO<sub>4</sub>, dehydrated in ethanol, and embedded in Araldite.

For conventional electron microscopy, animals were perfused with 2% paraformaldehyde and 2.4% glutaraldehyde in 100 mM sodium phosphate, pH 7.4. Diaphragms were dissected and incubated in the same fixative for 2-4 h, then refixed, dehydrated, and embedded as above. Thin sections were stained with uranyl acetate and lead citrate.

### [<sup>3</sup>H]Thymidine Autoradiography

Rats were injected twice intraperitoneally with 5  $\mu$ Ci/g body weight of methyl-[<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA; 20 Ci/mmol) at 6 and at 1 h before sacrifice. Animals were then anesthetized and perfused as above. Both denervated and innervated hemidiaphragms were dissected and 100- $\mu$ m cross sections were cut on a Vibratome from the central portion of the muscle that is rich in endplates and from the distal ends that are free of endplates. The sections were refixed in OsO<sub>4</sub>, dehydrated, and embedded in Araldite. One-micron-thick sections were mounted on gelatin-coated glass slides, dipped in emulsion (NTB-2; Eastman Kodak Co., Rochester, NY), exposed for 7 d at 4°C, developed in D-19 (Eastman Kodak Co.), and fixed. The sections were then stained through the emulsion with toluidine blue.

To quantitate cellular proliferation, the numbers of muscle fibers and of thymidine-labeled interstitial nuclei were determined in each section and the ratio of labeled nuclei to muscle fibers calculated. For each time point, we examined 5-15 sections containing a total of 2,000-4,000 fibers from two animals. Thymidine-labeled nuclei clearly associated with nerve trunks, blood vessels, and muscle fibers (i.e., satellite cells) were not included in these counts. The density of interstitial cells in endplate-rich or endplate-free regions was determined in the same sections as the ratio of the number of interstitial nuclei to the number of muscle fibers in each of 15-30 fields (1,000 magnification) examined.

### Tissue Culture

To culture fibroblasts from adult muscle, skin, or lung, the tissues were cut into ~2-mm<sup>2</sup> pieces with iridectomy scissors. These pieces were placed onto collagen-coated coverslips and maintained at 37°C in DME containing 10% horse serum, 5% calf serum, glutamine, and the antibiotics penicillin and streptomycin. Cells began to migrate from the explants onto the collagenous substratum after ~2 d in vitro; cultures were analyzed 1-3 d later, a total of 3-5 d in vitro.

For short term cultures, cells were dissociated enzymatically. Normal and 7-10-d denervated diaphragms were minced and incubated in medium containing collagenase (2 mg/ml), hyaluronidase (1 mg/ml), soybean trypsin inhibitor (0.3 mg/ml), and 1 mM CaCl<sub>2</sub> for 4-5 h at 37°C (Jessen et al., 1987). After incubation, the cells were triturated, filtered through a nylon

mesh, spun in a clinical centrifuge, and resuspended in medium containing 10% horse serum, 5% calf serum, and antibiotics. About 25,000–50,000 cells were plated on polystyrene coverslips that had been placed in the wells of a 24-well plate and coated with polylysine. Because fibroblasts are a major interstitial cell type in muscle (Schmalbruch and Helhammer, 1977), and because the enzymes that were used liberate myogenic (satellite) cells poorly (Bischoff, 1974), we expected the majority of dissociated cells to be fibroblasts. Consistent with this expectation, only 1–4% of the cells were labeled by antibodies to desmin (an intermediate filament protein expressed by smooth and skeletal muscle cells but not by fibroblasts; Debussche et al., 1983) or to myosin in cultures stained 4 h after plating, while >95% of the cells were labeled by antibodies to fibronectin (which is expressed by fibroblasts).

NIH 3T3 fibroblasts obtained from American Type Culture Collection (Bethesda, MD) were maintained in medium containing 10% calf serum plus penicillin and streptomycin.

For analysis by immunofluorescence, cultures were fixed in 95% ethanol–5% acetic acid at  $-20^{\circ}\text{C}$ , and then incubated with primary and secondary antibodies, as described in Covault and Sanes (1986). Some cultures were treated with 1  $\mu\text{M}$  monensin (Sigma Chemical Co., St. Louis, MO) for 5 h before staining with antibodies, to prevent secretion of newly synthesized glycoproteins (Tartakoff, 1983).

### Immunoprecipitation

Cultures prepared as described above were labeled with 100  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine (New England Nuclear; 400 Ci/mmol) for 16–20 h at  $37^{\circ}\text{C}$ . Medium was then collected and diluted in an equal volume of PBS containing the protease inhibitors EDTA (10 mM), *N*-ethylmaleimide (10 mM), and PMSF (2.5 mM). Cell layers were rinsed in PBS and extracted with 1% NP-40 in 100 mM sodium phosphate, pH 7.4, with protease inhibitors. 50- $\mu\text{l}$  aliquots of medium or cell layer extract were incubated with 5  $\mu\text{l}$  of affinity-purified antibodies to tenascin(J1), N-CAM, or fibronectin, or with nonimmune IgG for 2 h. 6  $\mu\text{l}$  of a 50% (vol/vol) suspension of protein A-conjugated Sepharose (Sigma Chemical Co.) was then added to each tube and incubation continued for 90 min. The immunoprecipitates were washed as described by Fambrough and Bayne (1983), eluted with gel sample buffer, and analyzed by PAGE (Laemmli, 1970) followed by fluorography (Laskey and Mills, 1975). When explant cultures were labeled, the explants themselves were removed before adding [ $^{35}\text{S}$ ]methionine, leaving the cellular outgrowth undisturbed. When cultures of different densities were to be compared, aliquots containing equal numbers of TCA-precipitable counts per minute from each culture were incubated with antibodies.

### Immunoblotting

Cell layers of subconfluent and confluent 3T3 cultures were scraped off the culture dishes and solubilized in gel sample buffer. The amount of DNA in the cell layers of sibling cultures was determined by the diphenylamine method (Giles and Myers, 1965). The volumes of the samples to be analyzed were adjusted to equalize the concentration of DNA and thus the number of cells per sample. These were then analyzed by gel electrophoresis (Laemmli, 1970), electrophoretically transferred to nitrocellulose (Towbin et al., 1979), and probed with J1 antibodies, antifibronectin, and nonimmune IgG. The filters were incubated with alkaline phosphatase-conjugated second antibody and developed using nitro blue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate as substrates.

### On the Relationship of J1 to Tenascin

The J1 antiserum used in many of the studies reported here recognizes proteins of  $M_r$  ~160, 180, 200, and 220 kD in brain (Kruse et al., 1985). Recently, Faissner et al. (1988) showed that the 200- and 220-kD forms are related to tenascin and immunochemically distinguishable from the 160- and 180-kD forms. We used two methods to determine which forms are present in muscle and synthesized by fibroblasts. First, we stained denervated muscles, muscle-derived cultured fibroblasts, and 3T3 cells with two newly generated monoclonal antibodies: one (578) that recognizes the 200–220-kD (tenascin-like) forms but not the 160–180-kD forms, and another (5G4) which recognizes the 160–180-kD but not the 200–220-kD forms. Antibody 578 stained muscle sections and cultured fibroblasts in a pattern indistinguishable from that seen with J1 antiserum, whereas antibody 5G4 did not detectably stain any of these preparations (Fig. 1). Second, we determined the apparent molecular mass of J1 antigens produced in cultures of muscle fibroblasts and 3T3 cells. Both in immunoprecipitates and on immu-

noblots, J1-like immunoreactive material had an apparent molecular mass of 200–220 kD on reducing gels (see Figs. 10 and 13). Together, these results indicate that both in vivo and in vitro, fibroblasts accumulate the 200–220-kD, tenascin-related form(s) of J1, but not the 160–180-kD forms. We therefore present results obtained with monoclonal and polyclonal antibodies together in Results, and refer to the muscle and fibroblast-derived material recognized by J1 antibodies as tenascin(J1).

## Results

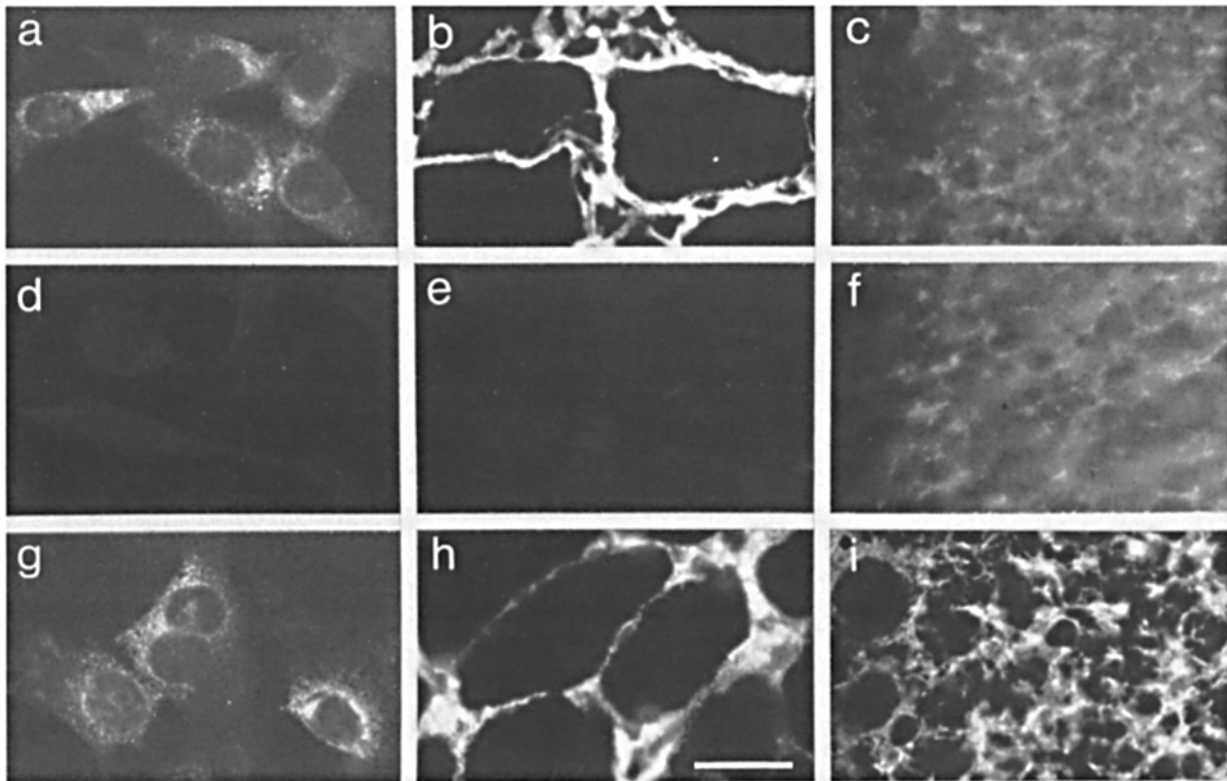
We previously showed that tenascin(J1), N-CAM, fibronectin, and M-HSPG accumulate in interstitial spaces near endplates after denervation (Covault and Sanes, 1985; Sanes et al., 1986). Elsewhere in muscle, these molecules are not coordinately regulated: whereas tenascin(J1) is restricted to denervated perisynaptic interstitial spaces (Fig. 2, *a–c*), N-CAM also appears throughout the muscle fiber surface and cytoplasm after denervation (Fig. 2, *d–f*), and fibronectin and M-HSPG are abundant in the basal lamina of both innervated and denervated muscle fibers (not shown). However, the coordinate accumulation of all four molecules in perisynaptic interstitial spaces constitutes an intriguing response of muscle to denervation. We have therefore sought the cellular source of these molecules. Our results are organized in three parts. First, we show that denervation results in the selective proliferation and accumulation of interstitial cells near synaptic sites and demonstrate that these cells are fibroblasts. Second, we present evidence that these fibroblasts synthesize the interstitial deposits of tenascin(J1), N-CAM, fibronectin, and M-HSPG that accumulate near denervated synaptic sites. Finally, focusing on tenascin(J1) and fibronectin, we compare fibroblasts cultured from several sources and under various conditions, with the aim of learning how accumulation might be regulated in vivo.

### Fibroblasts Proliferate in Interstitial Spaces near Denervated Synaptic Sites

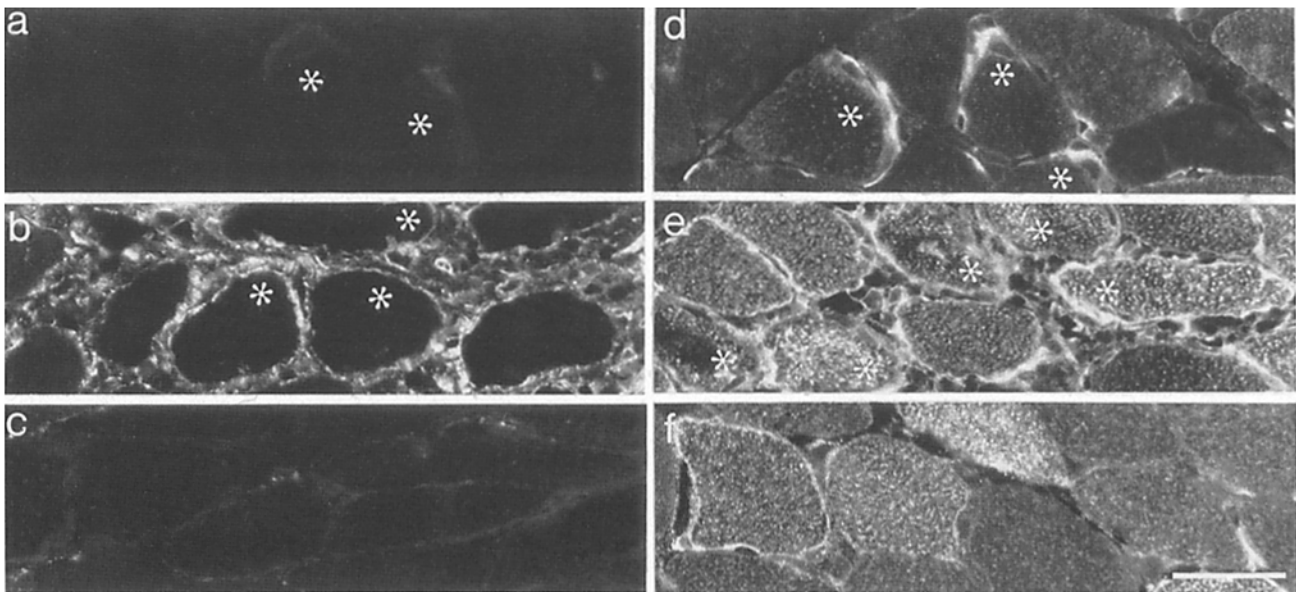
We began our study by searching for cellular changes that accompany, and might therefore underlie, the perisynaptic accumulation of adhesive molecules.

**Cellular Changes in Interstitial Areas.** In normal adult muscle, interstitial spaces near endplates contain collagen fibers, capillaries, and a few cells that bear elongated processes (Fig. 3 *a*). After axotomy, nerve terminals degenerate and are phagocytosed by endplate-associated Schwann cells (Miledi and Slater, 1970; Manolov, 1974). At about the same time, the number of interstitial cells and cellular processes in perisynaptic areas begins to increase. This increase was evident by 2 d after denervation, the earliest time examined (Fig. 3 *b*), became more striking during the subsequent week (Fig. 4 *a*; see also Fig. 7 *a*), and was maintained for up to 21 d, the latest time studied. By 10 d after denervation, the density of interstitial cells near endplates had increased more than fivefold (from  $0.25 \pm 0.02$  to  $1.36 \pm 0.13$  interstitial cells per muscle fiber cross section, mean  $\pm$  SEM, measured as described in Materials and Methods).

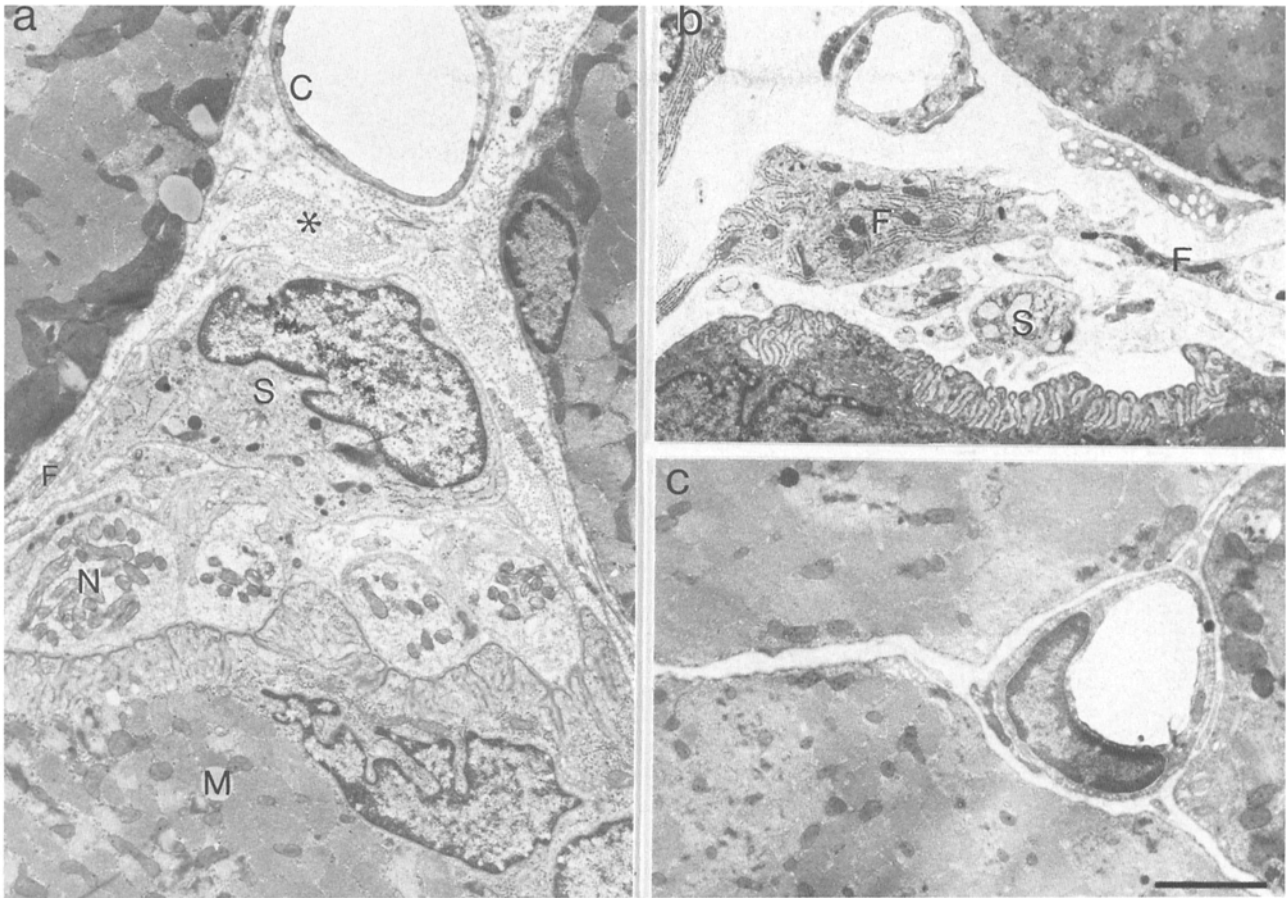
To ask whether cells accumulate selectively in perisynaptic areas, we examined sections cut from endplate-free regions of the diaphragm. (Synapses are confined to the central quarter of diaphragm muscle fibers, facilitating dissection of endplate-rich and endplate-free areas; see, for example, Merlie and Sanes, 1985.) Interstitial spaces in nonsynaptic



**Figure 1.** Staining of cultured fibroblasts (*a*, *d*, and *g*), cryostat sections from perisynaptic areas of adult denervated skeletal muscle (*b*, *e*, and *h*), and cryostat sections of cerebellum (*c*, *f*, and *i*) by monoclonal antibodies specific for the 200–220-kD forms of tenascin(J1) (578; *a–c*) and the 160–180-kD forms of tenascin(J1) (5G4; *d–f*), and by polyclonal J1 antibodies which recognizes all forms (*g–i*). Anti-J1/200–220 stains both fibroblasts and interstitial areas as does the antiserum; anti-tenascin(J1)/160–180 stains neither, although it does stain the granule cell layer in the cerebellum from a 15-d-old mouse. Bar, 25  $\mu$ m.



**Figure 2.** Distribution of tenascin(J1) and N-CAM in adult muscle. Cryostat sections cut from endplate-rich regions of innervated muscle (*a* and *d*), endplate-rich regions of 1-wk denervated muscle (*b* and *e*), or endplate-free regions of 1-wk denervated muscle (*c* and *f*) were stained with anti-tenascin(J1) (*a*, *b*, and *c*) or anti-N-CAM (*d*, *e*, and *f*) plus fluorescein–second antibody. Synaptic sites were identified by doubly staining the sections with rhodamine- $\alpha$ -bungarotoxin; their positions are marked by asterisks. Anti-N-CAM stains both pre- and postsynaptic elements in normal muscle but does not stain extrasynaptic areas; in denervated muscle, anti-N-CAM stains interstitial spaces near synaptic sites as well as the surface and cytoplasm of muscle fibers. Tenascin(J1) is absent from normal and denervated muscle fibers, but abundant in interstitial spaces near denervated synaptic sites. Bar, 25  $\mu$ m.

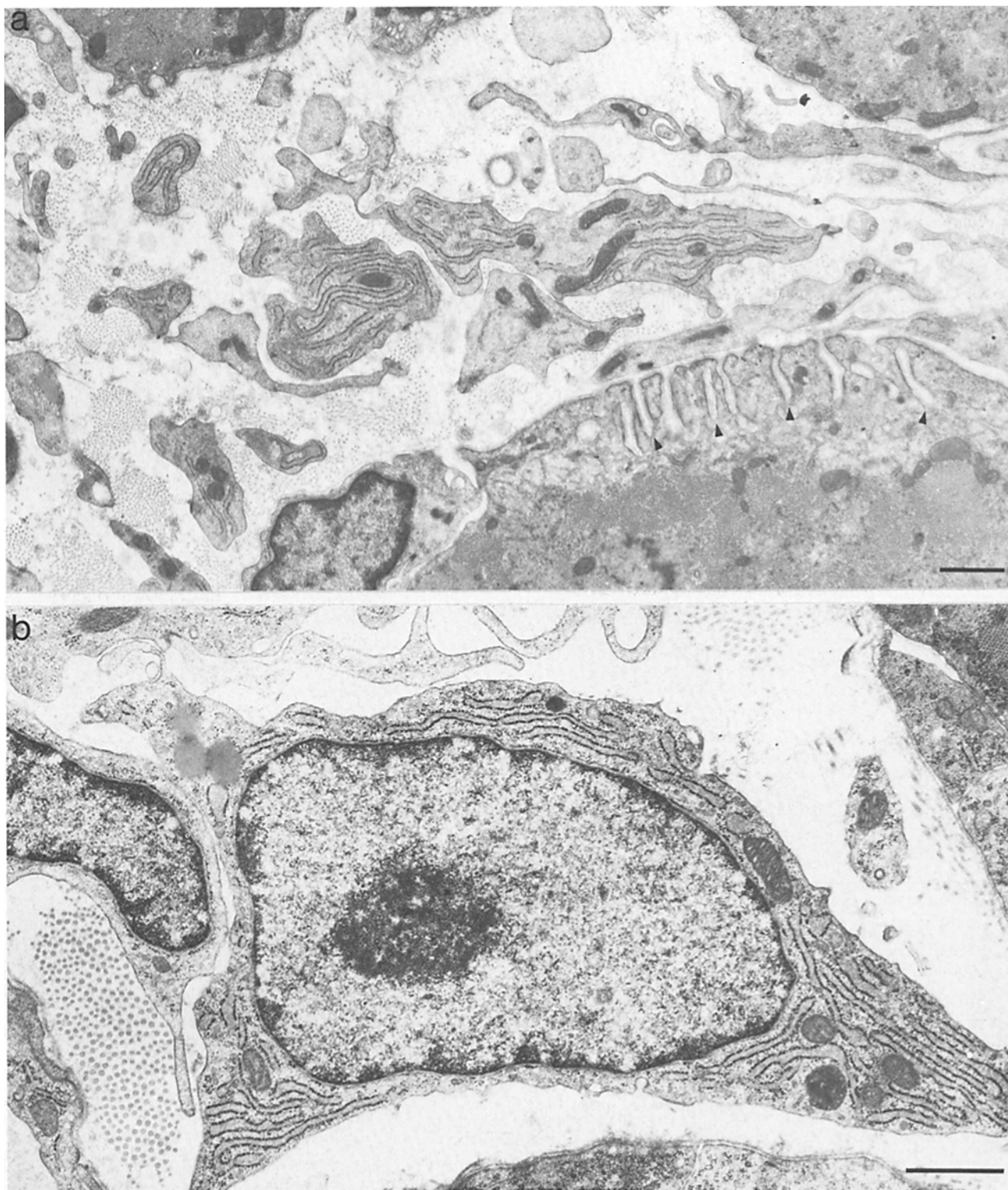


**Figure 3.** Electron micrographs of synaptic sites in normal (*a*) and 2-d denervated (*b*) muscle illustrate the cellular changes that accompany denervation. In normal muscle, motor nerve terminals (*N*) abut the infolded postsynaptic membrane of the muscle fiber (*M*), and are capped by a Schwann cell (*S*). Interstitial spaces contain capillaries (*C*), collagen fibrils (\*), and a few fibroblast processes (*F*). After denervation, nerve terminals have been phagocytized by Schwann cells, while other cell processes have become more numerous. (*c*) In nonsynaptic areas, interstitial areas remain narrow and cell-poor after denervation (2-d denervated muscle; section cut  $\sim 3$  mm from synapse-rich area). Bar, 2  $\mu\text{m}$ .

areas bore the same complement of collagen fibers, capillaries, and process-bearing cells seen near synapses. However, spaces were typically narrower extrasynaptically than perisynaptically, and contained somewhat fewer cells ( $0.15 \pm 0.02$  per muscle fiber cross section extrasynaptically vs.  $0.25 \pm 0.02$  perisynaptically). After denervation, interstitial spaces distant from synapses remained narrow (Fig. 3 *c*), and the number of interstitial cells increased only modestly (to  $0.37 \pm 0.03$  per muscle fiber cross section at 10 d after denervation). Because synaptic sites on adjacent muscle fibers are not in precise register, it has been difficult to determine the distance over which interstitial cell density falls from high perisynaptic to low extrasynaptic levels; however, examination of electron micrographs and of anti-Thy-1-stained cryostat sections (see below) as well as reconstructions of synaptic zones from serial sections all suggest that the decline occurs over a distance of a few hundred microns from the denervated synaptic site. In any event, it is clear that interstitial cells accumulate selectively near endplates in denervated muscle.

**Identification of Perisynaptic Cells.** We used ultrastructural and immunohistochemical methods to identify the cells that accumulate perisynaptically in denervated muscle.

Among the cell types that occupy interstitial spaces between muscle fibers are Schwann cells, perineurial cells, capillary endothelial cells, and fibroblasts (Murray and Robbins, 1982*b*). In addition, muscle satellite cells, normally confined beneath the muscle fiber basal lamina, may migrate into connective tissue spaces after denervation (Schultz, 1978). However, several lines of evidence indicated that the majority of cells that accumulate perisynaptically after denervation are fibroblasts. First, electron microscopy showed that these cells were free of detectable basal lamina, rich in rough endoplasmic reticulum, and bore numerous elongated processes (Fig. 4), all typical features of fibroblasts (Jackson, 1964; Porter and Pappas, 1959). In contrast, perineurial, capillary endothelial, and Schwann cells are coated by a basal lamina; perineurial and capillary endothelial cells also contain numerous pinocytotic vesicles in their cytoplasm (Asbury and Johnson, 1978). Furthermore, Schwann cells are stained by the monoclonal antibody 217c but not by anti-Thy-1, while fibroblasts are Thy-1 positive and 217c negative (Fields and Dammerman, 1985; Brockes et al., 1977); we found that tenascin(J1)-rich interstitial spaces near denervated endplates were Thy-1 rich but 217c poor (Fig. 5). Finally, while we had no antibody that distinguished satellite cells from fibroblasts,

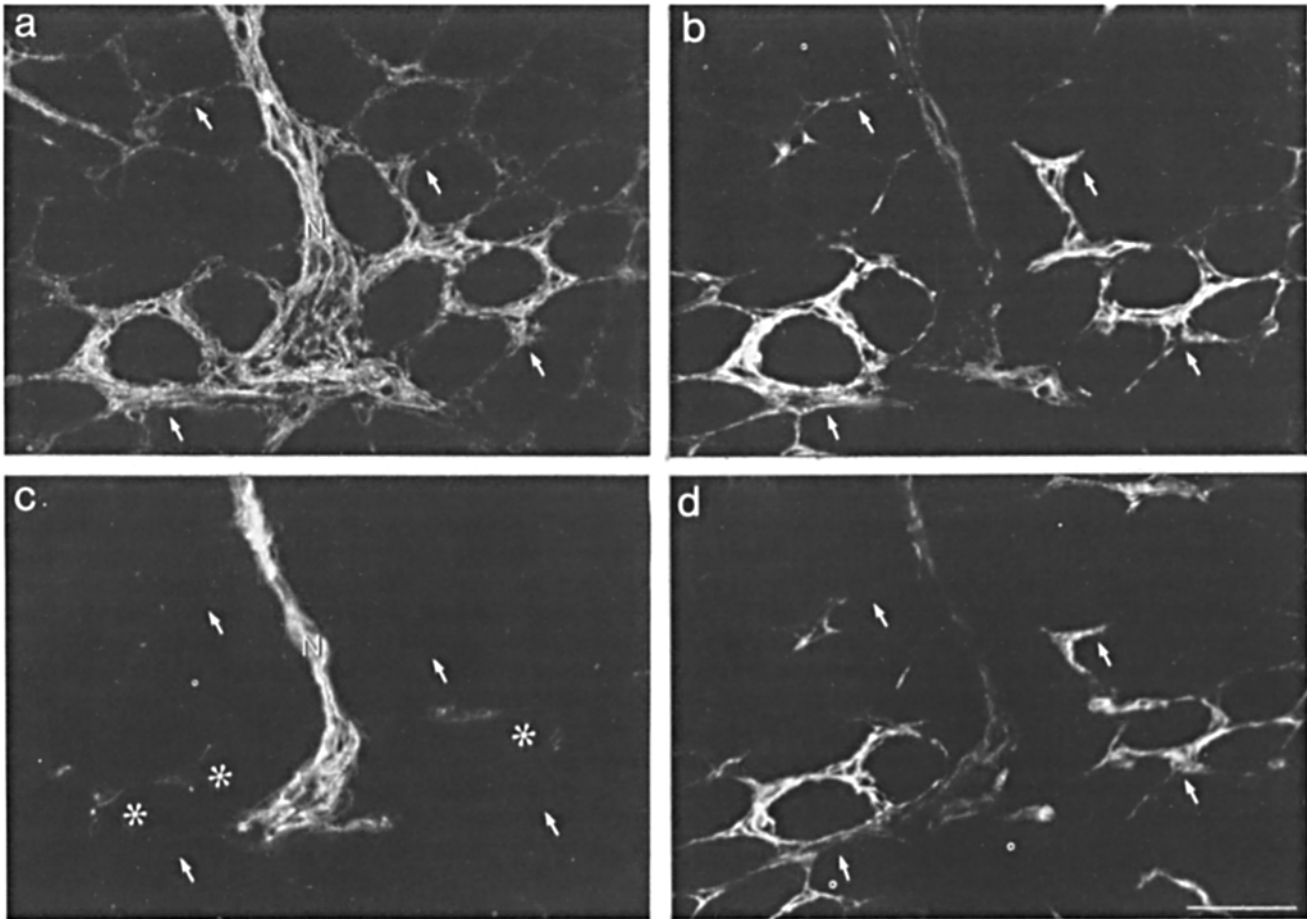


**Figure 4.** Electron micrographs of cell processes (*a*) and somata (*b*) near synaptic sites in 10-d denervated muscle show that these cells are fibroblastic in morphology: they bear long, slender processes, contain prominent rough endoplasmic reticulum and are uncoated by basal lamina. Some of the junctional folds in the postsynaptic membrane are indicated by arrowheads. Bars, 1  $\mu\text{m}$ .

ultrastructural studies indicated that satellite cells differentiated to form small-diameter myotubes, as described by Schultz (1978); at no stage did the differentiating satellite cells resemble fibroblasts (not shown). Thus, fibroblasts

comprise most of the population of cells that accumulate near endplates after denervation.

**Proliferation of Fibroblasts.** The accumulation of fibroblasts near denervated endplates could result from the migra-

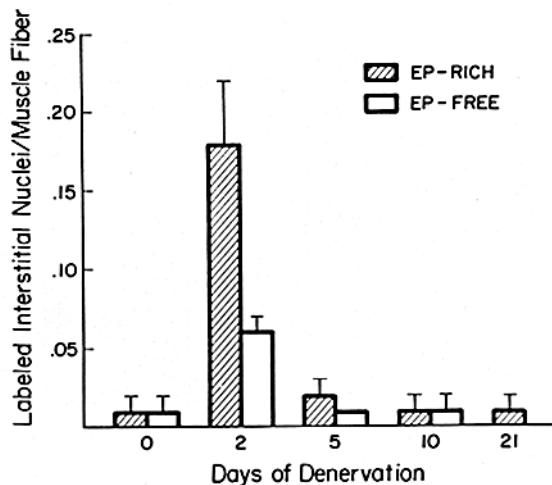
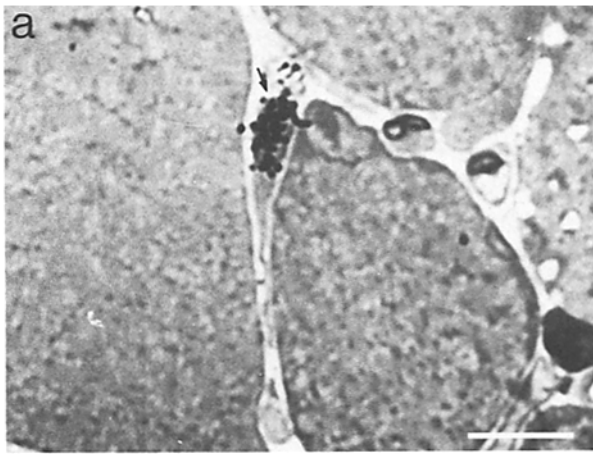


**Figure 5.** Tenascin(J1)-rich interstitial spaces in denervated muscle are labeled by a fibroblast marker (anti-Thy-1) but not by a Schwann cell marker (anti-217c). Near-serial cryostat sections of 9-d denervated muscle stained with mouse anti-Thy-1 (*a*) or anti-217c (*c*) plus rabbit anti-tenascin(J1) (*b* and *d*). Synaptic sites, identified by staining an adjacent section with rhodamine- $\alpha$ -bungarotoxin are indicated by asterisks in *c*. Corresponding points on the four panels are indicated by arrows. Anti-tenascin(J1) stains perisynaptic interstitial areas strongly and denervated intramuscular nerve branches (*N*) weakly. Both anti-Thy-1 and anti-217c stain nerve branches, but only anti-Thy-1 stains interstitial spaces. Bar, 50  $\mu$ m.

tion of cells from nonsynaptic or extramuscular compartments, and/or from the proliferation of resident precursor cells. As a test of the latter possibility, we used [ $^3$ H]thymidine autoradiography. Animals were injected with [ $^3$ H]thymidine 2, 5, 10, or 21 d after denervation, then sacrificed shortly after injection (see Materials and Methods); muscles were fixed, sectioned, and processed for autoradiography to determine how many cells had synthesized DNA and could therefore be presumed to be mitotically active. We found that denervation results in a transient increase in interstitial cell proliferation (Fig. 6). By 2 d after denervation, proliferation had increased in both endplate-rich and endplate-free areas, but the density of labeled cells was considerably higher in perisynaptic areas of denervated muscle (18-fold over normal) than in nonsynaptic areas (sixfold over normal). By 10 d after denervation, the mitotic index had dropped to control levels. Because the period of maximum proliferation occurred soon after denervation and was transient, we conclude that the accumulation of interstitial cells near denervated endplates results, at least in part, from the selective proliferation of precursor cells in this part of muscle.

### **Perisynaptic Fibroblasts Synthesize Adhesion Molecules**

**Immunoelectron Microscopy of Denervated Muscle.** We used electron microscopic immunohistochemistry to ask if the interstitial deposits of N-CAM and tenascin(J1) near denervated endplates are associated with fibroblasts that proliferate in this region. N-CAM was consistently present on the surface of fibroblasts that accumulated near denervated endplates (Fig. 7). Examination of N-CAM-positive cells at high magnification confirmed that they showed the characteristic features of fibroblasts: prominent rough endoplasmic reticulum in the cytoplasm, lack of basal lamina, and elongated processes emerging from the cell body (Fig. 8 *a*). Muscle fiber surfaces and satellite cells were also stained by anti-N-CAM (see also Covault and Sanes, 1985; Sanes et al., 1986), and Schwann cells that directly apposed the junctional folds were occasionally weakly N-CAM positive. However, N-CAM was not detected on the surface of other cell types that occupy these spaces, including capillary endothelial cells, mast cells, macrophages, and the perineurial cells



**Figure 6.** Denervation results in the selective proliferation of interstitial cells near synaptic sites. (a) A [ $^3\text{H}$ ]thymidine-labeled interstitial cell (arrow) in denervated muscle, visualized by light microscopic autoradiography. (b) A histogram showing the amount of interstitial cell proliferation in endplate-rich (▨) and endplate-free (□) regions at different times after denervation determined from autoradiograms as described in Materials and Methods. Bar, 10  $\mu\text{m}$ .

of terminal nerve branches. Thus, the bulk of the perisynaptic interstitial N-CAM previously detected by immunofluorescence (Covault and Sanes, 1985) is associated with fibroblasts.

The distribution of tenascin(J1) in denervated interstitial spaces differs from that of N-CAM. J1 antibodies stained collagen fibers and smaller collagen-associated fibrils (Fig. 8 b; see also Sanes et al., 1986) that were stained poorly if at all by anti-N-CAM (Fig. 8 a). In contrast, J1 antibodies did not stain the surfaces of any interstitial cells, including the fibroblasts, which were N-CAM positive. However, tenascin(J1)-positive fibrils in the extracellular matrix were consistently concentrated near fibroblast processes (Fig. 8 b): nearly all such fibrils that directly abutted the fibroblast surface were tenascin(J1) positive, and the fraction of fibrils that were tenascin(J1) positive decreased with distance from fibroblast surfaces. Thus, tenascin(J1) and N-CAM are both associated with perisynaptic fibroblasts in denervated muscle, but in different ways.

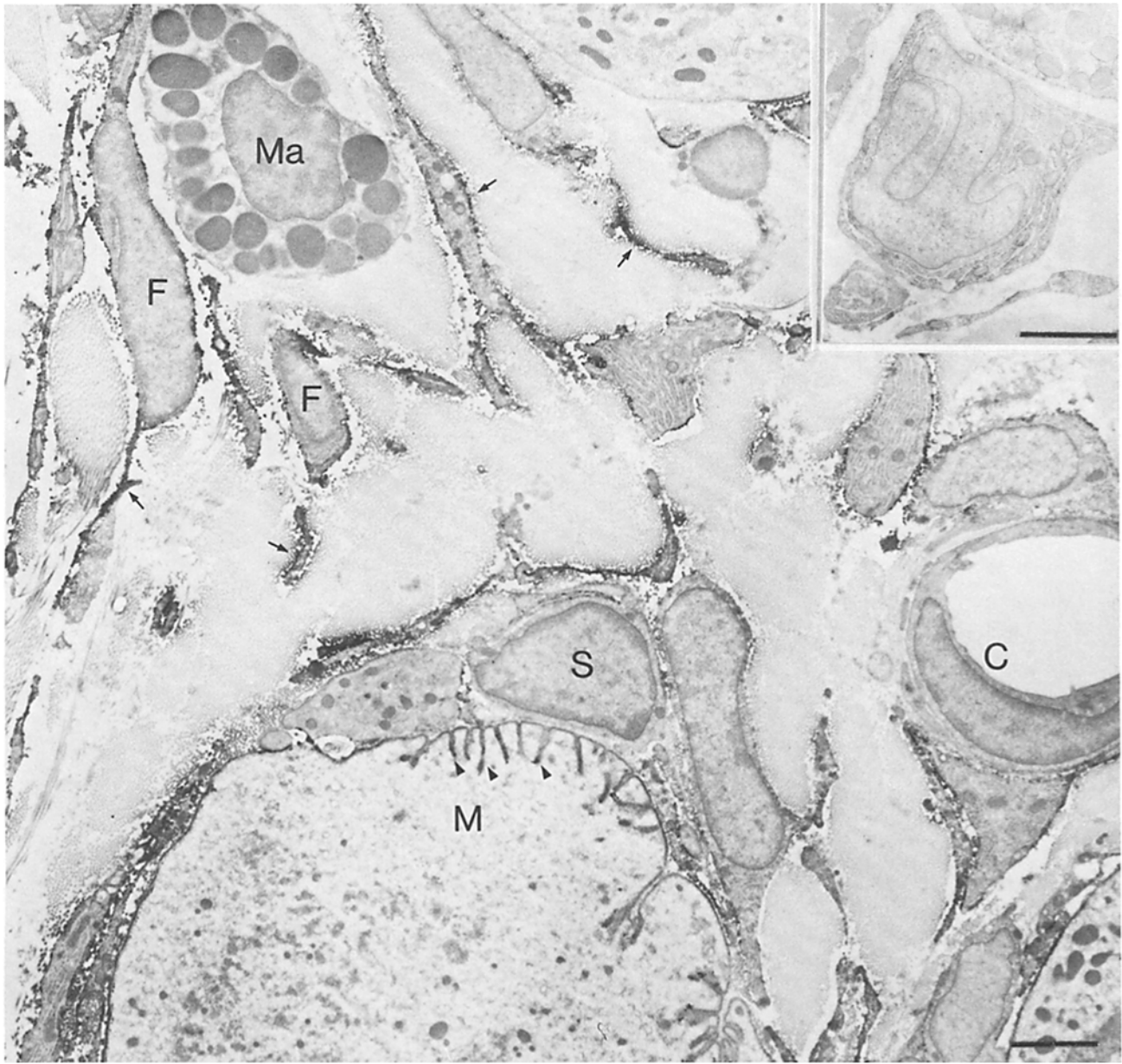
**Synthesis of Adhesion Molecules by Muscle Fibroblasts In Vitro.** The presence of N-CAM, tenascin(J1), fibronectin, and M-HSPG in fibroblast-rich interstitial spaces, and the association of N-CAM with fibroblast surfaces suggested that fibroblasts are the cellular source of the interstitial deposits of these adhesive macromolecules. However, it was also possible that these molecules were synthesized by other cells (e.g., muscle fibers) and bound selectively to sites provided by the fibroblasts. To distinguish between these alternatives, we cultured fibroblasts from denervated muscle and assessed their ability to synthesize N-CAM, tenascin(J1), fibronectin, and M-HSPG in vitro.

Fibroblast-rich cultures were prepared by explanting small pieces of endplate-rich regions of denervated muscle. The cultures were maintained for 3–5 d during which time cells migrated out of the explants onto the collagen substrata. Greater than 80% of the cells that migrated from these explants were identifiable as fibroblasts in that they expressed fibronectin, were flat, and bore multiple broad lamellipodia. A small percentage (<2%) of the cells were spindle shaped, and expressed embryonic myosin (not shown), and were thus likely to be myoblastic satellite cells. The remainder of the population consisted of small, round cells; many of these are likely to be fibroblasts in mitosis. We did not observe Schwann cells, as identified by an antibody to the 217c antigen (Fields and Dammerman, 1985), in these cultures. Thus, the explant method provides a way of preparing fibroblast-enriched cultures from small, defined regions of muscle.

We used immunofluorescence and immunoprecipitation methods to determine if fibroblasts synthesize adhesive molecules. First, the cultures were fixed and stained with antibodies to N-CAM, tenascin(J1), fibronectin, and M-HSPG followed by appropriate second antibodies. Anti-N-CAM stained the surface of fibroblasts, whereas antibodies to tenascin(J1), fibronectin, and M-HSPG stained the perinuclear cytoplasm of fibroblasts as well as the matrix surrounding these cells (Fig. 9, a–d). These results indicated that fibroblasts are synthesizing these adhesive molecules. To rule out the possibility that the molecules had merely been carried into culture by the fibroblasts, some cultures were treated with monensin, a carboxylic ionophore that inhibits glycoprotein secretion, thereby trapping newly synthesized glycoproteins in intracellular vacuoles (Tartakoff, 1983). In monensin-treated fibroblasts, all of the adhesive molecules accumulated inside the cells (Fig. 9, e–h). Thus, N-CAM, tenascin(J1), fibronectin, and M-HSPG are synthesized by muscle-derived fibroblasts.

Second, we immunoprecipitated metabolically labeled material from fibroblast-enriched cultures using antibodies to N-CAM, J1, and fibronectin. Fig. 10 demonstrates that fibroblasts synthesize a  $\sim 140$ -kD form of N-CAM, a  $\sim 220$ -kD form of tenascin(J1), and a  $\sim 210$ -kD form of fibronectin. The apparent molecular mass of fibronectin matches that reported for other tissues (Akiyama and Yamada, 1987); the form of N-CAM is similar to that previously seen in denervated muscle (e.g., Covault et al., 1986), and the form of tenascin(J1) is that expected from the reactivities of monoclonal antibodies described above (see Fig. 1). Although an immunoprecipitation for M-HSPG was not performed, the intracellular staining of fibroblasts with two well-characterized monoclonal antibodies provides strong evidence that fibroblasts synthesize this molecule. Together, the immunofluo-





**Figure 7.** Electron micrograph of a synaptic site in 1-wk denervated muscle, stained with anti-N-CAM and peroxidase-conjugated second antibodies. N-CAM is associated with the surface of fibroblast somata (*F*) and processes (*arrows*) but not with other interstitial cells such as mast cells (*Ma*) or capillary endothelial cells (*C*). The Schwann cell (*S*) that caps the infolded postsynaptic membrane (*arrowheads*) is also N-CAM poor. However, as shown previously, anti-N-CAM also stains the surface of denervated muscle fibers (*M*). Inset shows a fibroblast from a denervated muscle that had been incubated with nonimmune IgG instead of anti-N-CAM. Bars, 2  $\mu\text{m}$ .

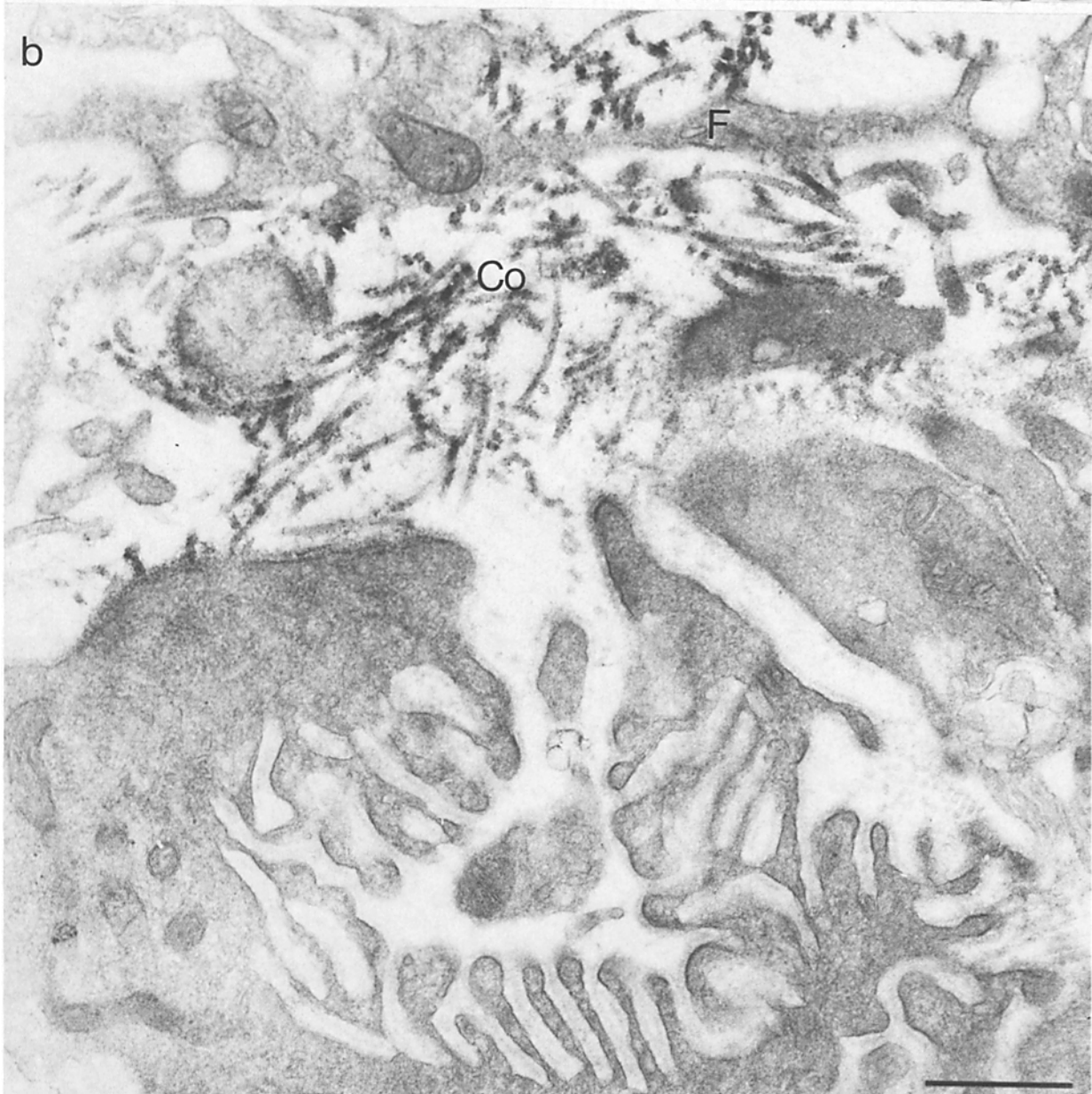
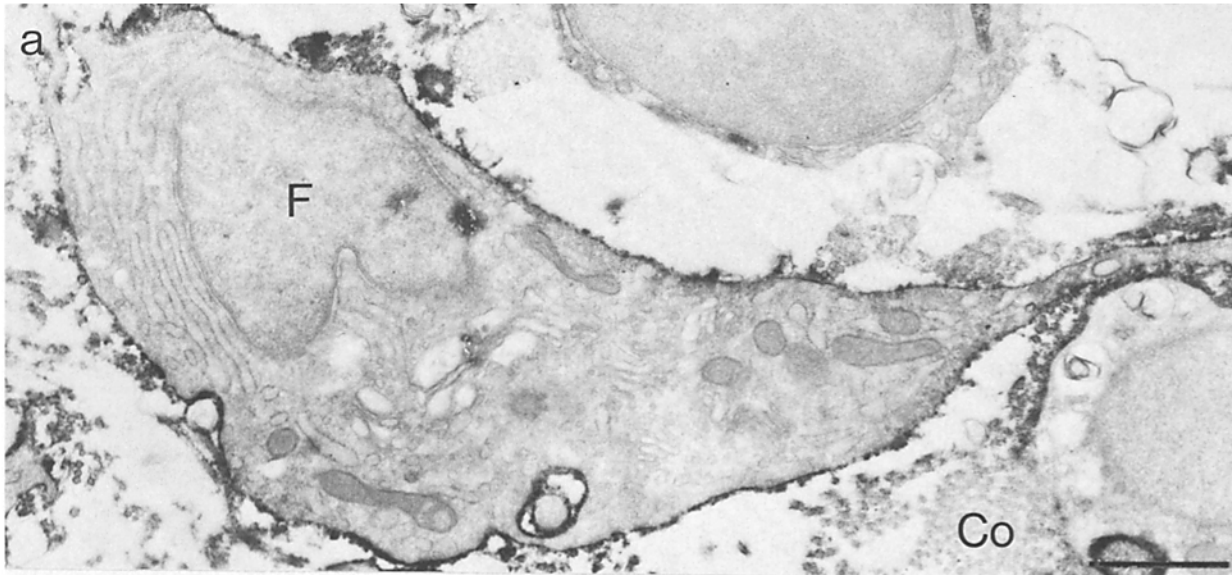
rescence and immunoprecipitation results show that fibroblasts from denervated muscle can synthesize the group of adhesive molecules that accumulates perisynaptically *in vivo*.

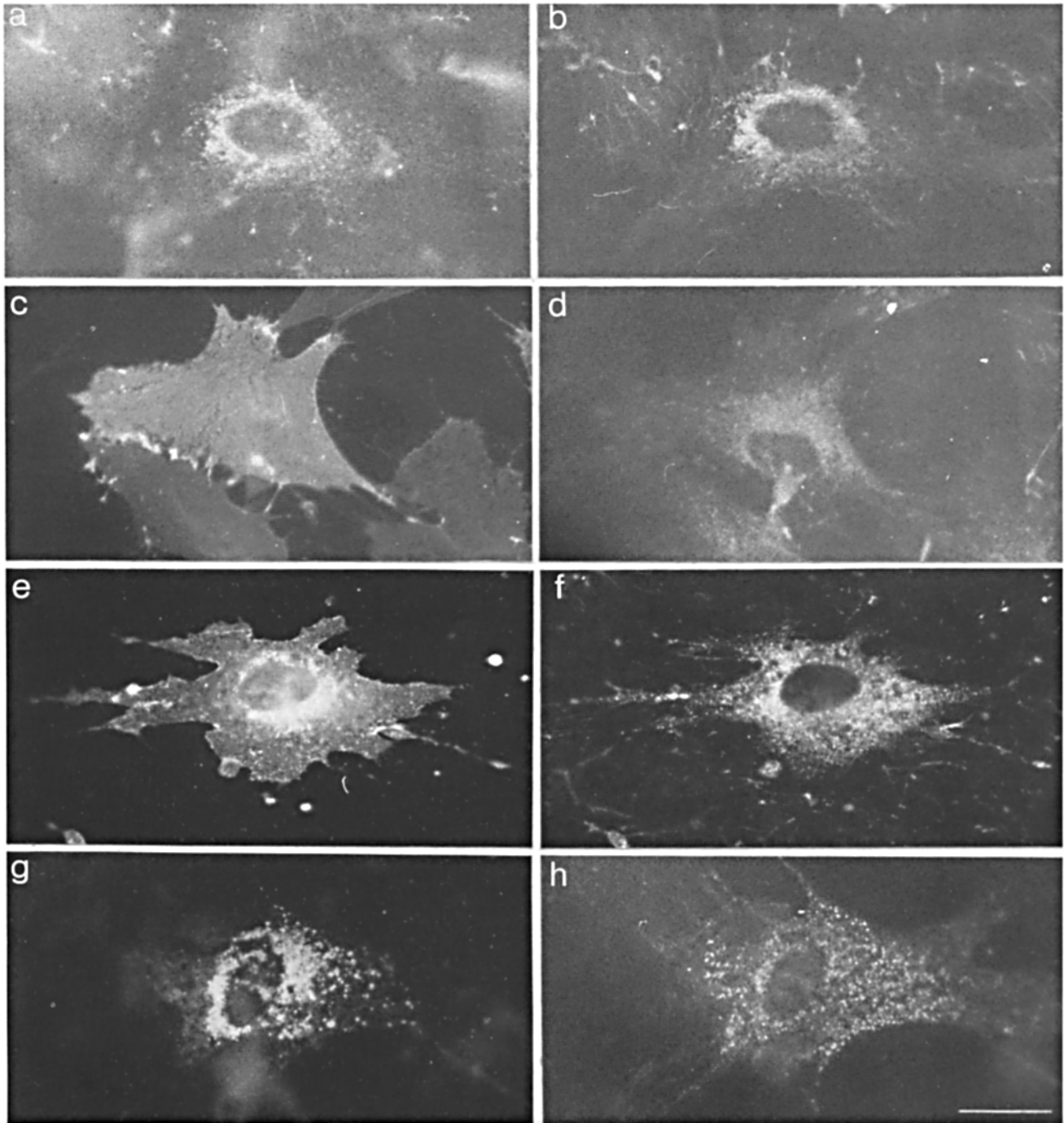
#### ***Fibroblasts Differentially Regulate Expression of Tenascin(J1) and Fibronectin***

The result that perisynaptic fibroblasts are responsible for the interstitial accumulation of four adhesive molecules after denervation raises a number of new questions. For example, are perisynaptic fibroblasts a unique class of cells, or are they ordinary fibroblasts responding to a local signal? Does the

perisynaptic accumulation of adhesive molecules reflect an increase in synthesis per cell or simply an increase in fibroblast number? Is the expression of all four molecules coordinately regulated? The result that muscle-derived fibroblasts synthesize adhesive molecules *in vitro* gave us a way to begin addressing these questions. Focusing on tenascin(J1) and fibronectin, we monitored the ability of fibroblasts to accumulate these molecules under a variety of conditions.

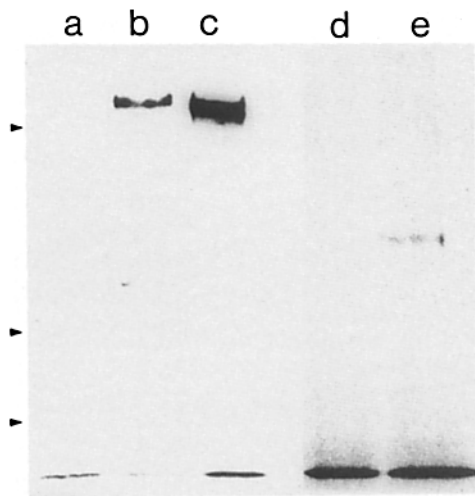
***Comparison of Fibroblasts from Normal and Denervated Muscle.*** We first asked whether fibroblasts from innervated and denervated muscle differ in their ability to accumulate tenascin(J1). Because fibroblasts do not migrate from the





**Figure 9.** Fibroblasts cultured from denervated muscle synthesize tenascin(JI), N-CAM, fibronectin, and M-HSPG: immunohistochemical evidence. (a-d) Cells from 5-d-old explant cultures were fixed, permeabilized, and doubly stained with anti-tenascin(JI) (a) or anti-N-CAM (c) plus antifibronectin (b and d). Anti-tenascin(JI) stains the perinuclear cytoplasm of fibronectin-positive fibroblasts as well as extracellular fibrils surrounding these cells. Anti-N-CAM stains only the surface of fibroblasts. (e-h) Sister cultures were incubated with 1  $\mu$ M monensin for 5 h before fixation, to trap newly synthesized proteins in intracellular vacuoles. Accumulation of N-CAM (e), fibronectin (f), tenascin(JI) (g), and M-HSPG (h) is now pronounced. Bar, 20  $\mu$ m.

**Figure 8.** N-CAM and tenascin(JI) have different associations with fibroblasts. 1-wk denervated muscles were stained with anti-N-CAM (a) or anti-tenascin(JI) (b), and electron micrographs prepared from perisynaptic interstitial regions like that shown in Fig. 6. Anti-N-CAM stains the surface of fibroblasts (F) intensely, but nearby collagen fibrils (Co) poorly, while anti-tenascin(JI) stains collagen fibrils that abut fibroblast processes, but not the fibroblast surface itself. A denervated postsynaptic site is visible in b. Bars, 1  $\mu$ m.

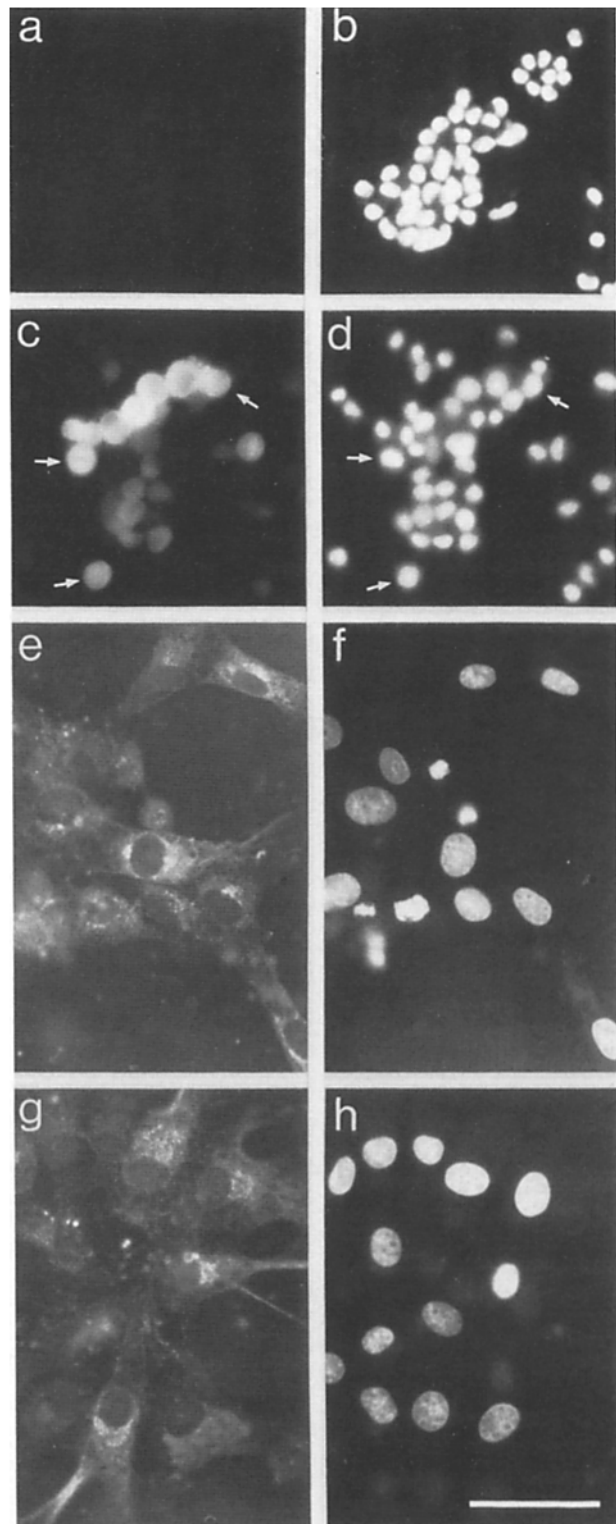


**Figure 10.** Fibroblasts cultured from denervated muscle synthesize tenascin(J1), N-CAM, and fibronectin: biochemical evidence. Cultures were incubated overnight with [<sup>35</sup>S]methionine, to label newly synthesized proteins. Aliquots of the medium (lanes *a-c*) or of a cell layer extract (lanes *d* and *e*) were then incubated with J1 antibodies (lane *b*), antifibronectin (lane *c*), anti-N-CAM (lane *e*), or nonimmune IgG (lanes *a* and *d*) as indicated, followed by protein A-Sepharose. Finally, the immunoprecipitates were analyzed by gel electrophoresis and fluorography. Arrowheads indicate positions of molecular mass standards, myosin (200 kD), phosphorylase b (97 kD), and BSA (68 kD).

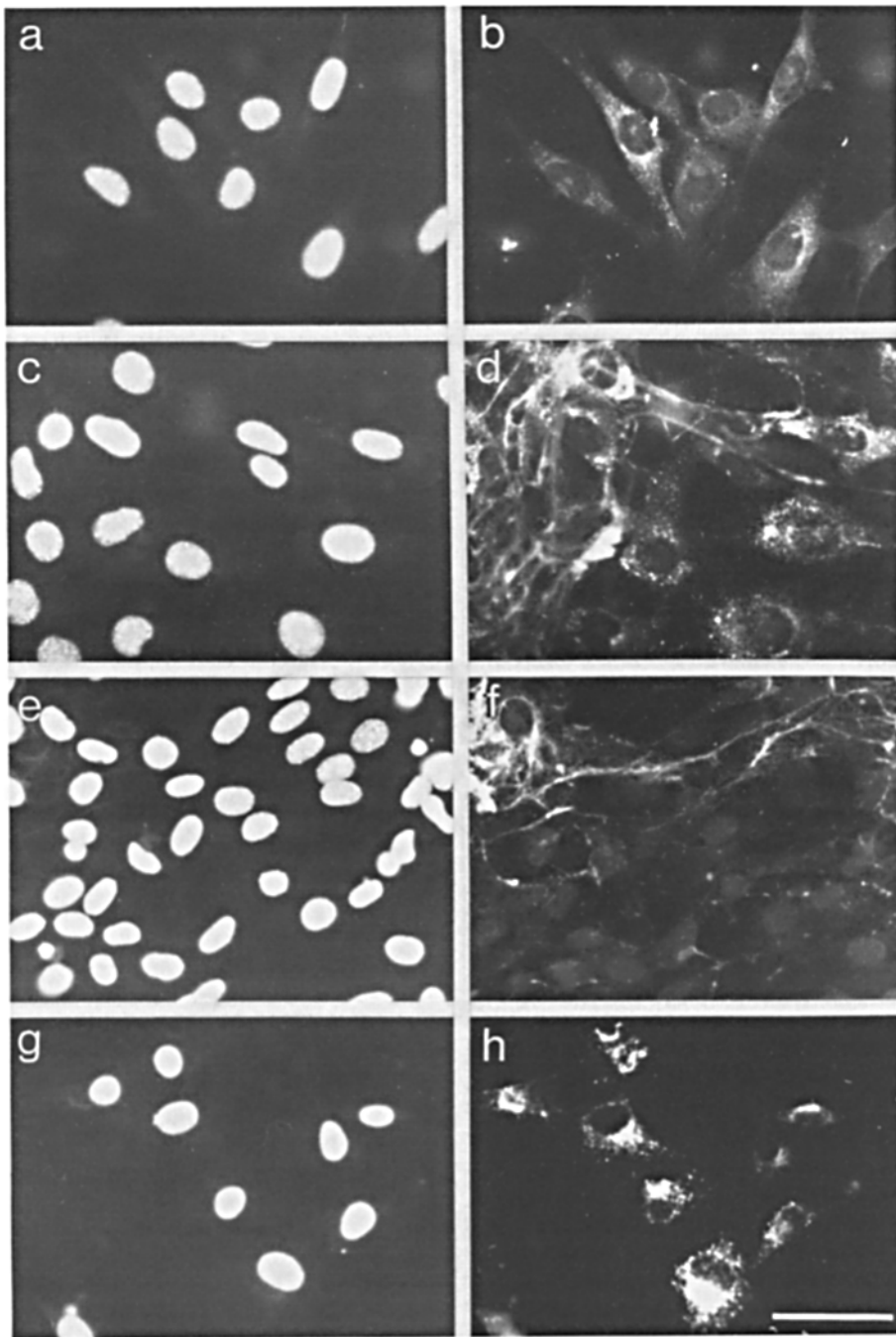
explant cultures described above until 1–2 d *in vitro*, we used an enzymatic method to dissociate mononucleated cells from endplate-containing areas of diaphragm, and plated the cells that were liberated for examination at shorter times in culture. 4 h after plating, the percentage of tenascin(J1)-positive fibroblasts was three- to fourfold greater in cultures from denervated muscles ( $20.3\% \pm 2.5$ ; mean  $\pm$  SEM from four cultures) than in cultures from innervated muscle ( $5.8\% \pm 0.2$ ,  $n = 4$ ; Fig. 11, *a-d*). After 5 d in culture, however, the percentage of tenascin(J1)-positive fibroblasts in both cultures was higher but similar ( $69.0\% \pm 3.2$  and  $69.8\% \pm 3.1$ ,  $n = 4$  each; Fig. 11, *e-h*). Thus, fibroblasts from innervated and denervated muscle differ initially in their content of tenascin(J1). However, when fibroblasts from innervated muscle are placed in culture, they come to resemble their counterparts from denervated muscle.

**Comparison of Perisynaptic and Other Fibroblasts.** We next asked whether perisynaptic fibroblasts are unique in their ability to synthesize tenascin(J1). Explants of extrasynaptic regions of denervated muscle were maintained in culture for 3–5 d, during which time fibroblasts migrated from the explants. Staining of such cultures revealed that fibroblasts from extrasynaptic and perisynaptic areas accumulate tenascin(J1) to similar levels (not shown). In addition, as expected from recent studies on tenascin (see Discussion) fibroblasts from adult skin and lung, and 3T3 fibroblasts were tenascin(J1) positive. These results indicate that the ability to synthesize tenascin(J1) is a property of fibroblasts from many sources.

**Comparison of Proliferating and Quiescent Fibroblasts.** *In vivo*, the perisynaptic accumulation of tenascin(J1) that follows denervation is roughly coincident with the activation



**Figure 11.** Fibroblasts cultured from innervated muscle are transiently tenascin(J1) negative. Fibroblasts isolated by enzymatic dissociation of innervated (*a, b, e, and f*) and denervated (*c, d, g, and h*) diaphragms were maintained in culture for 4 h (*a-d*) or 5 d (*e-h*) and then stained with J1 antibodies (*a, c, e, and g*) and a nuclear dye (*b, d, f, and h*). Arrows mark corresponding cells in each micrograph. *a* and *c* were photographed and printed using identical exposure times, as were *e* and *g*. Bar, 50  $\mu$ m.



**Figure 12.** Modulation of tenascin(J1) expression in growing 3T3 cultures. Fibroblasts were plated at low (*a* and *b*), intermediate (*c* and *d*), and high (*e* and *f*) cell densities. High density cultures were replated at low density and examined 24 h later (*g* and *h*). All cultures were treated with monensin for 5 h, then fixed and doubly stained with a nuclear dye (*left column*) and J1 antibodies (*right column*). The cytoplasmic accumulations of tenascin(J1) decline with increasing cell density, but reappear after replating of high density cultures. Matrix-associated tenascin(J1) increases as cells reach confluence, but declines in postconfluent cultures. The cultures were ~10% confluent (*a*, *b*, *g*, and *h*), ~90% confluent (*c* and *d*), or 1-d postconfluent (*e* and *f*) when they were fixed. Bar, 50  $\mu$ m.

(proliferation) of perisynaptic fibroblasts. This correlation suggests that the ability of perisynaptic fibroblasts to accumulate tenascin(J1) is repressed in quiescent fibroblasts and/or stimulated in proliferating fibroblasts. To test this idea, we used 3T3 fibroblasts because their mitotic activity is easily manipulated: 3T3 cells divide until they form a confluent monolayer, at which point proliferation ceases (Todaro and Green, 1963). For this and other reasons, 3T3 cells have frequently been used to study metabolic differences between quiescent and proliferating cells (Todaro et al., 1965; Dulbecco and Stoker, 1970; Herman and Pledger, 1985; for reviews, see Boynton and Leffert, 1985; Pledger, 1985).

The ability of quiescent and proliferating 3T3 cells to accumulate tenascin(J1) was assayed in three ways: by immunofluorescence, immunoblotting, and immunoprecipitation. For immunofluorescence, 3T3 cells were plated at different densities and cultured for 48 h, then treated with monensin to trap newly synthesized tenascin(J1), and stained with antibodies. In subconfluent cultures, high levels of tenascin(J1) were detectable in the perinuclear cytoplasm (Fig. 12, *a* and *b*). As cells became confluent, tenascin(J1) accumulated in the extracellular matrix but intracellular staining was dimmer (Fig. 12, *c* and *d*). In postconfluent cultures, levels of matrix-associated tenascin(J1) had decreased and tenascin(J1) was barely detectable in the cytoplasm (Fig. 12, *e* and *f*).

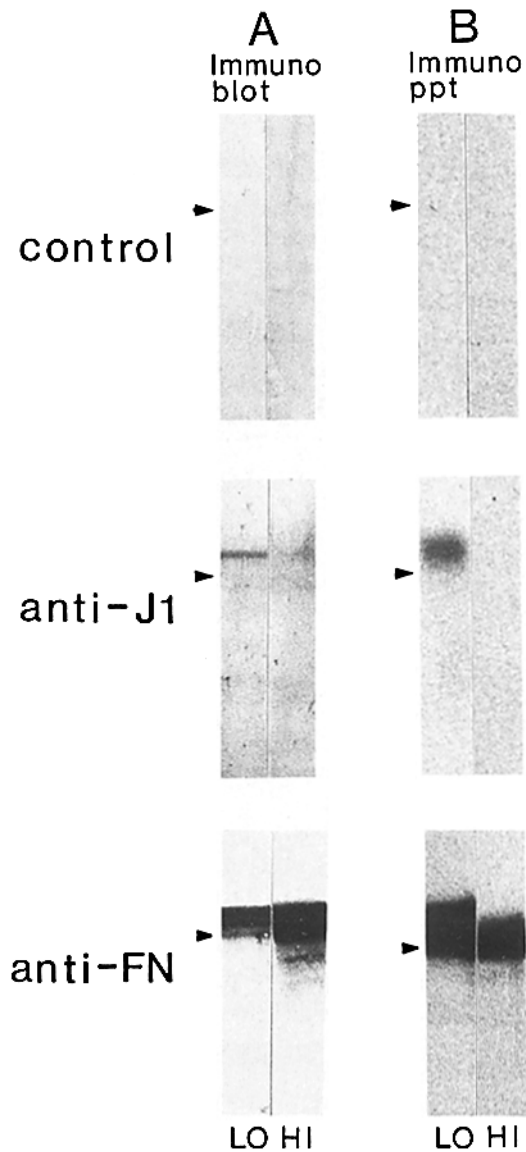
However, when these cultures were replated at low density and examined 24 h later, the cells once again accumulated intracellular tenascin(J1) (Fig. 12, *g* and *h*). These results suggest that the synthesis of tenascin(J1) by fibroblasts is regulated in parallel with proliferative activity.

To determine whether the immunofluorescence results reflected changes in the level of tenascin(J1) protein, we used immunoblotting. Growing and confluent cultures (corresponding roughly to those in Figs. 12, *a* and *b*, and *e* and *f*, respectively) were treated with monensin for 5 h, and aliquots of the cell layers corresponding to equal numbers of cells were fractionated by electrophoresis, transferred to nitrocellulose, and probed with antitenascin(J1). In confirmation of the immunofluorescence analysis, the resulting immunoblots showed that levels of tenascin(J1) per cell decreased >10-fold after cells became confluent (Fig. 13 *A*).

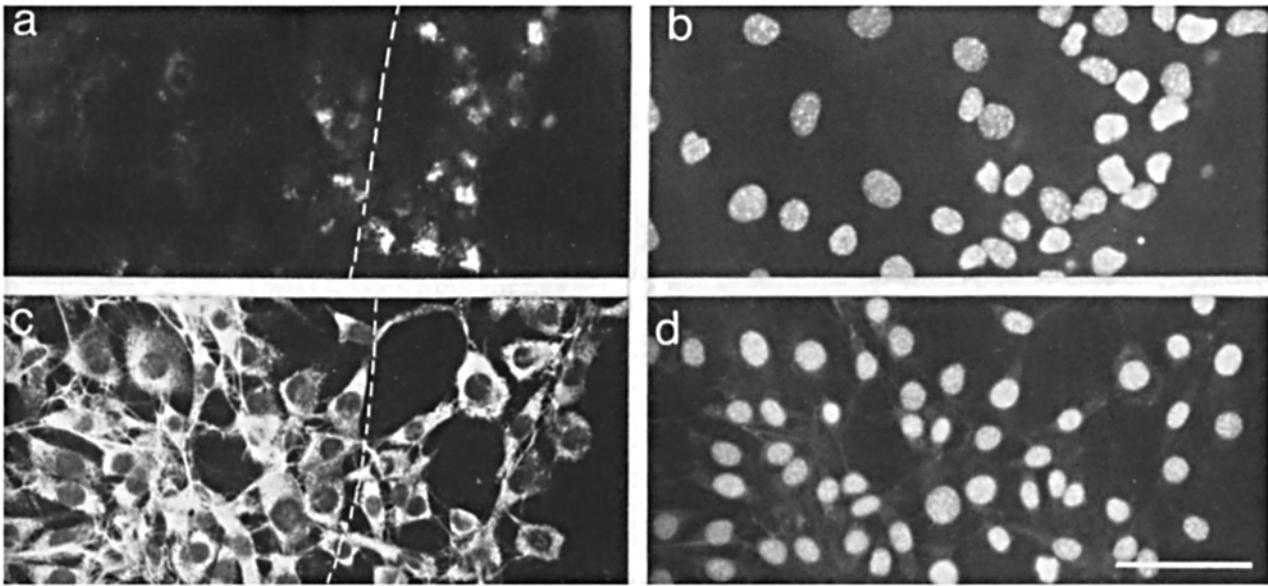
To test the ability of proliferating and quiescent cells to synthesize and secrete tenascin(J1), we labeled cells with [<sup>35</sup>S]methionine, then immunoprecipitated tenascin(J1) from aliquots of media that contained equal amounts of protein-associated (TCA-precipitable) radioactivity. The tenascin(J1) was detected by SDS gel electrophoresis and fluorography. Densitometric analysis of the fluorographs (Fig. 13 *B*) revealed that growing cells secreted ~30-fold more [<sup>35</sup>S]tenascin(J1) than confluent cells.

Finally, to compare quiescent and active fibroblasts in the same cultures, we took advantage of the fact that when a confluent monolayer of fibroblasts is "wounded" by scraping off part of the layer, cells at the wound's edge become activated, migrate into the cell-depleted space, and resume cell division; in contrast, cells distant from the wounded site remain quiescent and mitotically inactive (Todaro et al., 1965; Dulbecco and Stoker, 1970). Accordingly, portions of confluent monolayers were removed and the cultures were maintained for 20 h before they were processed for immunofluorescence; at this time, >90% of proliferating 3T3 cells are located in the wounded area (Dulbecco and Stoker, 1970). Some cultures were treated with monensin before staining to trap newly synthesized tenascin(J1) intracellularly, while other cultures were stained without monensin treatment to assess steady-state levels of tenascin(J1). Qualitatively similar results were obtained in both cases: tenascin(J1) was present in the perinuclear cytoplasm of cells that were adjacent to or in the wounded area, whereas cells distant from the wound contained little or no tenascin(J1) in their cytoplasm (Fig. 14, *a* and *b*). Thus, when quiescent fibroblasts are stimulated to proliferate, their capacity to accumulate tenascin(J1) increases.

**Comparison of Tenascin(J1) and Fibronectin Accumulation.** To ask whether the capacity of fibroblasts to accumulate other adhesive molecules is regulated in parallel with that of tenascin(J1), the experiments described above were repeated using antibodies to fibronectin. In each case, cells that differed greatly in their content of tenascin(J1) differed far less in their content of fibronectin. Thus, virtually all mononucleated cells freshly dissociated from innervated and denervated muscles were fibronectin positive (cf. Fig. 11). Furthermore, subconfluent and confluent cultures of 3T3 cells were stained at similar intensities by antifibronectin (cf. Fig. 12), accumulated similar levels of fibronectin as assessed by immunoblotting (Fig. 13 *A*), and secreted similar levels of fibronectin as assessed by immunoprecipitation



**Figure 13.** Biochemical evidence for modulation of tenascin(J1) and fibronectin levels in growing and confluent 3T3 fibroblasts. (*A*) Immunoblots of monensin-treated fibroblasts grown at low and high cell densities. Aliquots of cell layer corresponding to equal numbers of cells were fractionated by gel electrophoresis and transferred to nitrocellulose filters. The filters were probed with nonimmune IgG, J1 antibodies, or antifibronectin, then incubated with second antibody and reacted for alkaline phosphatase. The amount of tenascin(J1) declines precipitously with increasing cell density, while fibronectin levels increase slightly. (*B*) Immunoprecipitation analysis of media from metabolically labeled low-density (growing) and high density (confluent) 3T3 cultures. Aliquots of media containing equal numbers of TCA-precipitable counts were incubated with nonimmune IgG, J1 antibodies, or antifibronectin, then incubated with protein A-Sepharose. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Levels of newly synthesized tenascin(J1) decrease markedly with increasing cell density, while levels of fibronectin drop only two- to threefold. The ~180–250-kD regions of the blots and autoradiographs are shown. Arrowheads indicate position of the molecular mass marker myosin (200 kD).



**Figure 14.** Accumulation of tenascin(J1) and fibronectin expression in wounded 3T3 cultures. Monensin-treated 3T3 cultures were treated with monensin 15 h after wounding, then fixed 5 h later and stained with J1 antibodies (a), or antifibronectin (c), and a nuclear dye (b and d). The edge of the wound was located by phase contrast optics (dashed lines). Tenascin(J1) accumulates inside fibroblasts that have been activated by injury and invaded the bare area of the dish, but it is absent in cells far from this area. In contrast, fibronectin is associated with cells both near and distant from the wound. Similar results were obtained in cultures that had not been treated with monensin, although intracellular staining was dimmer. Bar, 100  $\mu\text{m}$ .

(Fig. 13 B). (In the experiments shown in Fig. 14, immunoblotting showed approximately threefold more fibronectin per cell in the cell layer of confluent than subconfluent cells, presumably reflecting the build-up of extracellular matrix. Immunoprecipitation, on the other hand showed approximately threefold more  $^{35}\text{S}$ -fibronectin per unit  $^{35}\text{S}$ -protein in subconfluent than postconfluent cells, consistent with the decline in fibronectin synthetic rate in quiescent cultures reported by Senger et al. [1983]. In both cases, however, changes in fibronectin were many times smaller than changes in tenascin[J1] levels.) Finally, in cultures that had been wounded 20 h earlier, antifibronectin stained migratory, mitotically active cells near the wound's edge and quiescent cells distant from the wound with similar intensity (Fig. 14, c and d). Together, these results indicate that fibronectin levels in fibroblasts are considerably less dependent than tenascin(J1) levels on the cells' level of proliferative activity.

## Discussion

We found previously that tenascin(J1), N-CAM, M-HSPG, and fibronectin accumulate near denervated synaptic sites in skeletal muscle (Covault and Sanes, 1985; Sanes et al., 1986). Here, we have investigated the cellular basis of this response. In the first section of this paper, we show that a cellular accumulation accompanies the perisynaptic appearance of the adhesive molecules, that the cells are fibroblasts, and that they arise by local proliferation. In the second section, we demonstrate that fibroblasts from denervated perisynaptic areas can synthesize tenascin(J1), N-CAM, M-HSPG, and fibronectin in vitro, and are thus likely to be a major cellular source of the perisynaptic deposits of these molecules. Finally, using cultured fibroblasts, we provide evidence that expression of one of the adhesive molecules, tenascin(J1), is

increased when quiescent fibroblasts are stimulated to proliferate. From these results, we conclude that fibroblasts near synaptic sites are activated by denervation to proliferate and to initiate a biosynthetic program that changes the molecular composition of spaces that abut denervated synaptic sites.

The phenomenon of interstitial cell proliferation after denervation of skeletal muscle is well-documented (e.g., Zak et al., 1969; McGeachie and Allbrook, 1978; Murray and Robbins, 1982a,b). For example, Murray and Robbins observed that 4-d denervated mouse leg muscles incorporate 20-fold more  $^3\text{H}$ thymidine into DNA than normal muscle, that most (80%) of the thymidine-labeled cells in 4-d denervated muscle are interstitial, and that the majority of these are fibroblasts. However, these authors found no difference in  $^3\text{H}$ thymidine uptake beneath synapse-rich and synapse-free regions of muscle. In contrast, Connor and McMahan (1987) recently reported a selective increase upon denervation in the number of fibroblastic cells in junctional regions as compared to extrajunctional regions of frog cutaneous pectoris and rat platysma muscles. Our results are consistent with those of Connor and McMahan. We found fourfold more interstitial cells in perisynaptic as compared to non-synaptic areas of denervated rat diaphragm. This difference results, at least in part, from a higher level of mitotic activity near denervated endplates. Furthermore, the cells that accumulated near denervated endplates of rat diaphragm are morphologically and immunohistochemically identifiable as fibroblasts. Thus, the selective accumulation of fibroblasts near synaptic sites appears to be a general response of skeletal muscle to denervation.

Three lines of evidence indicate that perisynaptic fibroblasts are the cellular source of the interstitial deposits of tenascin(J1), N-CAM, fibronectin, and M-HSPG that accumulate after denervation. (a) The accumulation of fibroblasts

after denervation is roughly coincident with the accumulation of the adhesive molecules. (b) N-CAM is associated with the surface of these interstitial fibroblasts, and tenascin(J1) is associated with collagen fibrils that abut fibroblast surfaces. (c) Fibroblasts cultured from perisynaptic areas of denervated muscle synthesize all four molecules *in vitro*. That fibronectin and M-HSPG are produced by these cells is unsurprising; both are well-documented as fibroblast products (e.g., Akiyama and Yamada, 1987; Gallagher et al., 1986). The association of the J1 antigen with fibroblasts had not heretofore been studied (although it is mentioned by Kruse et al., 1985), but during the course of our studies, it became apparent that tenascin(J1) is closely related to the myotendinous antigen (Chiquet and Fambrough, 1984), tenascin (Chiquet-Ehrismann et al., 1986; Faissner et al., 1988), hexabrachion (Erickson and Taylor, 1987), glioma mesenchymal extracellular matrix protein (Bourdon et al., 1985), and cytotoxin (Grumet et al., 1985; Hoffman et al., 1988), most of which are known to be synthesized by fibroblasts (see also footnote 2). Perhaps more surprising is the production of N-CAM by muscle fibroblasts. N-CAM is not generally considered to be expressed by fibroblasts, and we, too, find far lower levels of N-CAM-immunoreactivity on cultured fibroblasts than on cultured muscle cells (Covault and Sanes, 1986) or neurons (Covault et al., 1987). However, Martini and Schachner (1988) and Seilheimer and Schachner (1988) have recently shown that some fibroblasts in or derived from peripheral nerve are N-CAM positive, and Maier et al. (1986) demonstrated a population of N-CAM-positive mesenchymal cells, some of which might be related to fibroblasts, in regenerating newt limb. It will be important to determine whether fibroblasts from other sources can synthesize N-CAM, to compare the level of N-CAM in fibroblasts with that in other cells, and to learn which of several muscle-associated forms of N-CAM (Covault et al., 1986; Dickson et al., 1987) fibroblasts express.

Having concluded that fibroblasts are responsible for the perisynaptic accumulation of adhesive molecules *in vivo*, we monitored the expression of tenascin(J1) and fibronectin by fibroblasts cultured from several sources and under various conditions with the aim of learning how the accumulation that had been documented *in vivo* is regulated. From our results, we draw four conclusions that may be helpful in understanding this response of muscle to denervation.

First, fibroblasts dissociated from perisynaptic regions of innervated and denervated muscle differ in their capacity to accumulate tenascin(J1): during the first several hours after dissociation the fraction of fibroblasts that is detectably tenascin(J1) positive is severalfold higher in cultures from denervated muscle than in cultures from innervated muscle. Similar results were obtained for N-CAM, although the low levels of expression by both types of fibroblasts made this difference more difficult to document by the immunofluorescence method used (data not shown). Further studies will be required to learn whether differences in rates of synthesis and/or rates of degradation account for the differences in level that we have observed at short times in culture, or for the enhanced ability of fibroblasts from innervated muscle to accumulate tenascin(J1) after longer times in culture. In any case, however, it seems likely that the accumulation of tenascin(J1) seen *in vivo* is not accounted for solely by the increase

in fibroblast number that follows denervation, but also results from an increased level of tenascin(J1) per cell.

Second, fibroblasts from perisynaptic regions of muscle are not unique in their ability to accumulate tenascin(J1). Fibroblasts from nonsynaptic regions of denervated muscle, which are not detectably tenascin(J1) positive *in vivo*, accumulate tenascin(J1) after a few days *in vitro*. Fibroblasts from nonmuscle sources (skin and lung) and even fibroblasts of the 3T3 cell line synthesize tenascin(J1) *in vitro*; as noted above, this result is consistent with recent observations that the J1-200/220 antigens are closely related to tenascin and cytotoxin, which are known to be produced by fibroblasts. Thus, the localized accumulation of tenascin(J1) observed *in vivo* is likely to result from special properties of the perisynaptic environment. For example, cells in that area might generate a localized signal to which a variety of fibroblasts could potentially respond; candidate sources include muscle fibers, degenerating axons, endplate-associated Schwann cells, cells within denervated nerve trunks, and cells such as lymphocytes and macrophages that are known to infiltrate axotomized peripheral nerves. Candidate signals are polypeptide growth factors such as PDGF, fibroblast growth factor, EGF, and insulin-like growth factor, all of which are produced by many cells and are known to stimulate DNA synthesis in quiescent fibroblasts *in vitro* (for reviews, see Boynton and Leffert, 1985; Deuel, 1987). Alternatively, the increased extracellular space in perisynaptic compared to extrasynaptic areas (Fig. 2) might diminish contact inhibition and thereby favor cellular accumulation in these areas in response to a widely distributed signal.

Third, in 3T3 cells, tenascin(J1) expression and mitotic activity are regulated in parallel. This is seen when mitotically active (subconfluent) and quiescent (confluent) cultures are compared, when quiescent cells are replated at low density, and when confluent monolayers are wounded. It is well-established that fibroblast growth is density dependent: cells divide until they become "contact-inhibited," at which point polyribosomes disaggregate, protein synthetic rate decreases, and mitotic activity ceases. It is currently thought that mitogens, changes in cell shape, and intercellular contacts all affect a program of cell activation, in which a large number of genes are coordinately regulated (for reviews see Pledger, 1985; Deuel, 1987). Our results suggest that expression of tenascin(J1) is part of this program. Thus, when perisynaptic fibroblasts are stimulated to divide *in vivo*, increased tenascin(J1) expression may accompany the activation. It may therefore be unnecessary to postulate the existence of an "instructive" signal that induces tenascin(J1) synthesis in perisynaptic fibroblasts; instead, once they are activated, expression of this molecule may be a normal feature of their phenotype.

Finally, fibroblasts are able to discoordinately regulate their expression of different adhesive molecules: while tenascin(J1) accumulation greatly varies with mitotic rate, quiescent and active fibroblasts accumulate similar levels of fibronectin. Preliminary evidence suggests that N-CAM is regulated in parallel with tenascin(J1); we have not studied M-HSPG in this regard. Thus, while the perisynaptic accumulation of tenascin(J1) *in vivo* presumably results from both an increase in cell number and an increase in production per cell, the accumulation of fibronectin may result primar-



ily from an increase in cell number. This difference may explain an initially puzzling immunohistochemical result (Sanes et al., 1986): while tenascin(J1) is virtually undetectable in innervated muscle and appears only after denervation, interstitial fibronectin is readily detectable throughout innervated muscle, and its perisynaptic increase after denervation represents a quantitative rather than a qualitative change.

Our interest in the cellular and molecular changes that occur in perisynaptic areas upon denervation stems from the observation that regenerating motor axons preferentially reinnervate original synaptic sites (e.g., Ramón y Cajal, 1928; Gutmann and Young, 1944; Bennett and Pettigrew, 1975; Sanes et al., 1978). Since these sites occupy <0.1% of the muscle fiber surface, it seems unlikely that axons could find them by an unguided process of random search. One source of guidance is provided by denervated intramuscular nerve branches through which axons frequently regenerate to reach synaptic regions. However, axons can find their way to synaptic sites even when they grow outside of preexisting nerve sheaths (discussed in Sanes et al., 1978; Sanes and Covault, 1985), suggesting the existence of a second set of intramuscular navigational cues. In support of this idea, we recently found that when neurons are cultured on cryostat sections of denervated muscle, they extend longer neurites if the sections are cut from regions near synapses than if they are cut from synapse-free regions (Covault et al., 1987). The perisynaptic fibroblasts we have identified here, or the adhesive molecules they synthesize, could account for this neuritic preference. In fact, inhibitory effects of anti-N-CAM on limb regeneration in newt have been interpreted as indicating a functionally important interaction of growing axons with a population of N-CAM-positive mesenchymal cells (Maier et al., 1986) that could be related to fibroblasts. Furthermore, fibroblasts can clearly serve as a substrate for neurite extension in vitro (e.g., Noble et al., 1984; Fallon, 1985), and this extension is greatly inhibited by antibodies to integrins (Tomaselli et al., 1986), receptors for a set of extracellular matrix molecules that includes fibronectin (Ruoslahti, 1988), and may include tenascin(J1) (Chiquet-Ehrismann et al., 1988; Jones et al., 1988). It thus seems plausible that perisynaptic fibroblasts could use the adhesive molecules we have studied to influence regenerating axons.

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