Extracellular matrix proteins produced by a mouse skeletal muscle cell line, G8-1, were isolated and characterized. Cultures were incubated with $[^{35}S]$methionine or $[^{3}H]$glycine and $[^{3}H]$proline, and the labeled, substrate-attached proteins were obtained after cellular proteins were extracted by deoxycholate in neutral salt. The labeled matrix was analyzed by gel electrophoresis and fluorography before and after enzymatic digestion. Of the nine major bands present in the matrix, four were identified. Fibronectin and collagen were detected on the bases of their relative mobilities, differential labeling with $^{3}H$-versus $^{35}S$-labeled amino acids and their solubilization by protease free collagenase. High molecular weight material which was present in the matrix was also sensitive to collagenase and probably included cross-linked collagen and laminin. Proteins co-migrating with actin and myosin were also present in the extracellular matrix. These results are novel in that they demonstrate that the skeletal muscle phenotype, not contaminated with fibroblastic elements, is able to synthesize basal lamina-type macromolecules and incorporate them into an insoluble, extracellular matrix. Since this cell line is able to form functional synaptic contacts with neuronal cells, the influence of nerve on basal lamina production by muscle in vitro is possible.

The importance of the ECM in both in muscle cell development and maintenance of the differentiated state of the skeletal muscle fiber and its neuromuscular junction is well established. Earlier work (Hauschka and Konigsberg, 1966; Konigsberg, 1970) established a requirement for collagen as a substrate in the development of myoblasts into myotubes in primary cultures. In other studies using adult, innervated muscle, morphologic specialization of the muscle fiber basement membrane in synaptic regions was demonstrated by Zacks et al. (1961, 1973a, 1973b). Some evidence is available suggesting that muscle cells, in order to produce a highly specialized and developed ECM must interact with other cells in the environment (Lipton, 1977). Other studies have shown that treatment with collagenase or protease can release an extracellular, presumably muscle-elaborated protein, acetylcholinesterase, from the neuromuscular junction (Hall and Kelly, 1971; Betz and Sakman, 1973). Thus, at least some muscle proteins interact directly with the basal lamina at the neuromuscular junction.

Recent elegant experiments (Sanes et al., 1978; Marshall et al., 1977) have demonstrated that the basal lamina of the neuromuscular junction directs the site of reinnervation by the nerve. Further studies showed that acetylcholine receptors in regenerating muscle fibers accumulate at sites which correspond to regions of the basal lamina where the original synapse was present (Burdan et al., 1979). In addition, a number of clinical observations in neuromuscular diseases have suggested that some alternations in the ECM may be primary defects in pathogenesis (Elson et al., 1981; Duance et al., 1980a, 1980b; Stephens et al., 1982). Taken together, these reports serve to demonstrate the importance of studying the metabolism and production of ECM by skeletal muscle cells.

In this regard, several studies have shown that smooth muscle cells produce an extensive ECM (Burke and Ross, 1979; Jones et al., 1979). However, one of the primary difficulties experienced in studying skeletal muscle ECM production in tissue culture is the presence of multiple cellular types. The G-8 mouse myoblast cell line (Christian et al., 1977) offers a potential system in which to study the specific skeletal muscle cell phenotype. This muscle cell line is capable of nicotinic, cholinoergic synapse formation (Christian et al., 1977) and exhibits a variety of skeletal muscle cell-specific proteins and properties including acetylcholine receptors (Noble et al., 1978; Sugiyama, 1977), 16 S asymmetric, basal lamina-associated acetylcholinesterase (Sugiyama, 1977), contractile proteins (Moore et al., 1981), and striations (Noble et al., 1978). We felt this cell line would be particularly useful in exploring the components of the ECM synthesized by muscle cells. A preliminary report of ECM protein production by G-8 muscle cells during myogenesis has appeared (Beach et al., 1981b).

**MATERIALS AND METHODS**

**Cell Culture**—G8-1 cells, originally described by Christian et al. (1977) were obtained after subcloning from Dr. M. Nirenberg, National Institutes of Health, Bethesda, MD. The experiments reported here utilized cells which were of passage 13 to 17 at the time of labeling or staining. G8-1 cells were plated from liquid $N_{2}$ frozen stocks into 100-mm tissue culture dishes (Corning). After 3 days of growth in DMEM (Gibco, Grand Island, NY), containing 50 units/ml of penicillin and 50 $\mu$g/ml of streptomycin, supplemented with 10% FBS (Flow Laboratories, Lot No. 29101429, Rockville, MD), cells were trypsinized and passed to 250-mm Petri dishes (Falcon, Lincoln, NE) and grown to 80-90% confluence. For acute experiments, cells from roller bottle cultures were trypsinized and plated into 60-mm (6 x 10^6 cells/dish) or 35-mm (1.75 x 10^6 cells/dish) tissue culture dishes (Falcon) in DMEM with 20% FBS. In previous studies (Festoff et al., 1982) we found G8-1 cells differentiated fully when grown directly on plastic. Fusion of G8-1 myoblasts was enhanced by follow-
ing a protocol described by Walsh and Phillips (1980). After myoblasts reached confluence (72 h) the medium was changed to DME supplemented with 5% horse serum (K. C. Biologicals, Lenexa, KS, Lot No. 26990) and 0.5% FBS. After 24 h, uridine and 5-fluorodeoxyuridine (Sigma) were added (10^-4 and 10^-5 M, respectively). On day 6 (48 h later) the medium was changed back to DME with 5% FBS. To avoid onset of fusion in cultures which were to be labeled as myoblasts, these cultures were plated (5 x 10^5 cells/60-mm dish), allowed to attach for 6 h, and then fed DME with 10% FBS and labeled compounds.

Cell-labeling Procedures—Proteins synthesized by the G8-1 muscle cells were detected after metabolic labeling with L-[35S]methionine (905 Ci/mmol), or [2-3H]glycine (14 Ci/mmol) and L-[2,4,5-3H]proline (139 or 160 Ci/mmol), all from New England Nuclear. 35S- or 3H-labeled amino acids were added at 10 Ci/µl on day 7 or day 11 for labeling myotubes or after attachment on day 1 for labeling myoblasts. Media were not changed until cells were processed 3 days later. Radiolabeled samples were counted in Aquasol (New England Nuclear) counter.

ECM Isolation—Matrices were isolated as described previously by Hedman et al. (1979). Briefly, cultures were rinsed 3 times with PBS (pH 7.4), extracted 3 times with 0.5% sodium deoxycholate (Sigma) in 10 mM Tris-HCl buffer, pH 8.0, with 150 mM NaCl, and again rinsed 3 times with 10 mM Tris buffer, pH 8.0. All steps were performed gently on ice (0-4 °C) in the presence of 1 mM phenylmethylsulfonyl fluoride (Sigma). Insoluble material remaining attached to the dish was solubilized at 37 °C with sample buffer (see below) by scraping the bottom of the dish with a Teflon scraper. Extraction at 80 °C did not increase the radioactivity extracted or change the composition observed on polyacrylamide gels.

Digestion of ECM—In experiments designed to determine the susceptibility of the ECM to neutral proteolytic attack, matrices were prepared as above. Following the 10 mM Tris-rinses, various exogenous enzymes were added at 0 °C in 0.1 M Tris, pH 8.0, containing 10 mM CaCl2. The incubations were initiated by transferring the dishes to 37 °C. The following enzymes were used at 10 µg/ml: trypsin (Type III, bovine pancreas, Sigma) and elastase (from porcine pancreas 177 units/mg of protein; Calbiochem-Behring, San Diego, CA). Protease-free collagenase (Form III, Advance Biofactures, Lymphook, NY) was used at 10 units/ml. Urokinase (from human urine, 0.6 units/mg, Sigma) was used at 0.1 mg/ml. Plasminogen was isolated from fresh human plasma as described elsewhere (Festoff et al., 1982), and was used at 4 µg/ml.

Analysis of Cells and Matrices—ECM samples were applied to gels as isolated in sample buffer. Samples of cellular proteins in deoxycholate or Ca++ and Mg++-free PBS were solubilized in sample buffer (5% β-mercaptoethanol, 10% glycerol, 1.0% sodium dodecyl sulfate and 62.5 mM Tris-HCl, pH 6.8). Soluble samples which required concentration were precipitated by addition of an equal volume of 10% trichloroacetic acid (v/v). These precipitates were then rinsed at 0 °C with acetone 3 times, dried, and solubilized in sample buffer. All gel samples were heated to 95 °C for 3 min after addition of sample buffer.

Solubilized samples were analyzed on 4-15% linear gradient sodium dodecyl sulfate-polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970). After electrophoresis, gels were fixed and stained in 0.1% Coomassie brilliant blue R-250 (Bio-Rad Labs, Richmond, CA) in 45% methanol, 10% acetic acid. After destaining, gels containing 3H or 35S were impregnated with ENHANCE (New England Nuclear), dried, and exposed at -80 °C for 1-5 weeks. Molecular weight markers and protein standards were visualized after Coomassie blue staining and the positions of these bands were marked in ink on the fluorographs. The following molecular weight markers (Bio-Rad) were used: myosin (M × 200,000), β-galactosidase (M × 116,250), phosphorylase B (M × 92,500), bovine serum albumin (M × 66,200), and ovalbumin (M × 45,000).

Actin was purified from rabbit leg and back muscles by the method of Spudich and Watt (1971). Fibronection was purified from human plasma by the method of Ruoslahti et al. (1978). Myosin was purified from chicken gizzards as previously described (Beach et al., 1981a). All other reagents were of the highest purity commercially available.
the fusion index was 0.5 and mature myotubes were seen (Fig. 1C). The fusion index remained high through the 10th day of culture and myotubes matured further (Fig. 1D). This correlated with morphological observations that cells labeled from day 7 to day 10 were predominantly myotubes. These cells were mature-appearing, multinucleated myotubes, often several hundred micrometers long, with infrequent branches (Fig. 1D).

$^{[35]S}]$Methionine Labeling—Myotube cultures labeled with $^{[35]S}$methionine from day 7 to day 10 or from day 11 to 14 incorporated approximately 10,000 cpm/60-mm dish into material extracted as ECM. Characterization of molecular composition of the labeled ECM was accomplished following separation by 4-15% SDS-polyacrylamide gel electrophoresis and detection by fluorography. Fig. 2 shows the composition of $^{35}$S-labeled material found in the cell extracts and the matrix produced by myotube cultures. The composition of the labeled ECM was relatively simple (Fig. 2, lane 5); only 8-10 bands were seen. This gel photograph was intentionally underexposed as not to obscure the numerous bands in the deoxycholate extract. The bands in the ECM at $M_r$ ~ 240,000, 200,000, 45,000, and 30,000 were also evident in the deoxycholate cell extracts (Fig. 2, lanes 2 and 3) and in cells released from the dish by Ca$^{2+}$- and Mg$^{2+}$-free PBS (Fig. 2, lane 1). High molecular weight material (≥400,000) barely able to penetrate the gel was enriched in the ECM relative to the deoxycholate extract.

$^{[3]H}]$Glycine and Proline Labeling—Myotube ECM labeled by incorporation of $[^3]$H]proline and $[^3]$H]glycine was similar in many respects to the $^{[35]}$S-methionine-labeled matrix. The composition of $^3$H-labeled ECM was also relatively simple (about 8-10 bands were apparent). Most of these bands co-migrated with those seen in $^{35}$S-ECM (Fig. 3). $^3$H-ECM was also enriched in high molecular weight material, relative to $[^3]$H]deoxycholate extracts. However in $^3$H-labeled ECM, the band at $M_r$ ~ 240,000 was the most prominent band, while in $^{35}$S-ECM, the bands at 200,000 and 45,000 were more prominent (cf. Fig. 3, lanes 3 and 6 versus lane 1). In addition, a protein band at $M_r$ ~ 170,000 was clearly visualized in $^3$H-labeled ECM. This band was not seen in $^{35}$S-ECM (cf. Fig. 3, lanes 3 and 6 versus lane 1). A faint band at this $M_r$ appeared in $[^3]$H]deoxycholate extracts (Fig. 3, lane 5). Although most of the bands observed in isolated ECM could be identified in deoxycholate extracts, a comparison of lanes 3 and 5 in Fig. 3 demonstrate marked differences in the protein composition of these fractions. For example, the band at $M_r$ ~ 53,000 was enriched in $^3$H-ECM compared to $[^3]$H]deoxycholate extracts. Additionally, high molecular weight material (≥400,000) was enhanced in $^3$H-ECM relative to $[^3]$H-labeled cell extracts (Fig. 3, lane 3 versus lane 5). Similar differences between ECM and deoxycholate extracts were observed when muscle cells were labeled with $^{35}$S-methionine.

In Fig. 3, lane 4, the Coomassie blue bands from unlabeled human plasma fibronectin, chicken gizzard myosin, and rabbit skeletal muscle actin were represented by the doublet at $M_r$ ~ 240,000, the band at $M_r$ ~ 200,000, and the band at $M_r$ ~ 45,000. These proteins were seen to co-migrate with the previously mentioned bands in the $^3$H- and $^{35}$S-labeled ECM.
These three proteins, actin, myosin, and fibronectin have previously been found to be associated with detergent-insoluble matrix produced by a variety of cell types (Hedman et al., 1979; Birdwell et al., 1978; Culp et al., 1980).

Comparison of the ECM produced by myoblasts and myotubes under these experimental conditions revealed no clearly discernible differences. In Fig. 3, lane 6 (10-day myotube ECM) contained relatively less myosin and more actin than lane 3 (3-day myotube ECM); however, this was somewhat variable in other preparations. Likewise, 35S-labeled matrices of myoblasts and myotubes were similar (not shown). ECM from 10-day myotubes (Fig. 2, lane 6) was not distinguishable from ECM from 14-day myotubes (Fig. 2, lane 5).

Enzymatic Digestion of ECM—We next assessed the susceptibility of G8-1 myotube ECM to several extracellularly active enzymes. Fig. 4 shows the fraction of 35S-labeled ECM which is solubilized during a 4-h incubation with 2 extracellular proteases at neutral pH. Trypsin and activated plasminogen (plasmin) released 50% or more of the insoluble matrix by 2 h. The release approached a plateau around 4 h. Elastase released radioactivity with a similar time course to trypsin (not shown) and by 4 h released nearly 50% of 3H- or 35S-labeled matrix material (Table I). This table also shows that the fraction of counts released by urokinase-activated plasminogen and trypsin are independent of the particular label. Protease-free bacterial collagenase did not quantitatively release radioactivity above buffer controls (Table I).

The insoluble material remaining after enzymatic digestion was subsequently analyzed by sodium dodecyl sulfate-gel electrophoresis and fluorography (Fig. 5). Trypsin (lane 2) eliminated most of the bands present in the 3H-glycine- and 3H-proline-labeled matrix. All that remained was labeled material running at the dye front and some low molecular weight bands. Protease-free collagenase (lane 2) eliminated the 3H-labeled band (arrow) at M, ~170,000 and reduced the intensities of the fibronectin band and of the high molecular weight material that barely entered the gel.

35S-Methionine-labeled matrix was also efficiently digested by trypsin although some material of Mr = 45,000 and lower molecular weight remained. Collagenase reduced the fibronectin band but had no clear effect on the material at the top of the gel when 35S-labeled matrices were analyzed by SDS-gel electrophoresis after enzymatic digestions (not shown).

We have assigned apparent molecular weights of the labeled species in 3H and 35S-labeled skeletal muscle ECM by comparison with molecular weight standards on semilogarithmic plots. Table II shows the molecular weights and tentative identification of several ECM polypeptides, based on data presented above.

**DISCUSSION**

Hedman *et al.* (1979) described a simple procedure for isolation of the extracellular matrix produced by fibroblasts.
in culture. By combined morphological and biochemical techniques, these authors identified fibronectin, collagen, and other glycoproteins in ECM produced by 3T3 cells. We have characterized the extracellular proteins incorporated into a similar deoxycholate insoluble matrix produced by clonal skeletal muscle cells in vitro. Analysis of the ECM by gel electrophoresis and enzymatic digestion has enabled us to identify some of the proteins which are deposited on the substratum. As an indication of possible developmental regulation of these proteins we have also compared the ECM produced by myoblasts and myotube cultures.

Previous studies employing biochemical and immunological probes have established that fibronectin is present in the detergent-insoluble matrix produced by a variety of cells (Birdwell et al., 1978; Hedman et al., 1979; Culp, 1980). Rat muscle cell lines express fibronectin during myogenesis in vitro (Chen, 1977; Furcht et al., 1978) and the G8-1 mouse cell line does as well (Walsh and Phillips, 1981). We have demonstrated here that G8-1 skeletal muscle cells synthesize a protein similar to fibronectin and incorporate it into a substrate-attached ECM. Our identification of this protein as fibronectin is based on (a) its co-migration with purified fibronectin and (b) its release from the matrix when collagen is digested with protease-free collagenase. Further confirmation of the identity of the M, ~ 240,000 band as fibronectin is provided by comparison of the relative intensity of this band in [3H] and [S35] labeled ECM. Fibronectin contains about 160 glycines and prolines but only 11 methionines/1000 residues (Yamada et al., 1978). Thus, the markedly enhanced labeling of this band seen in [3H]-labeled preparations is consistent with its identification as fibronectin.

Proteins which co-migrate with actin and myosin are also components of the matrix. Nonspecific absorption of these contractile proteins is unlikely for two reasons. First, while cellular actin and myosin are synthesized far more actively by clonal muscle cell myotubes than myoblasts (Moore et al., 1981; Garrels, 1978), they are not enriched in myotube ECM relative to myoblast ECM (Fig. 3). Second, several groups (Culp et al., 1980; Letourneau, 1981; Schubert, 1978) have shown that these proteins are present at attachment points for cells in culture even after detergent solubilization (Birdwell et al., 1978). Therefore, we feel these two proteins represent cross-linked collagen and associated proteins such as laminin, recently shown to be co-distributed with acetylcholine receptor clusters on myotube surfaces (Daniels et al., 1985). Presumably, the release of labeled fibronectin by protease-free collagenase is due to the solubilization of collagen to which it was bound in the ECM.

Several cell types in addition to fibroblasts (Hedman et al., 1979) have been shown to produce ECM in culture. Smooth muscle cells produced an extensive ECM which has been isolated and characterized (Jones et al., 1979; Burke and Ross, 1979). Other authors have examined the collagen components of matrix produced by endothelial cells (Tseng et al., 1981). In addition, Culp et al. (1980) have studied some of the extracellular proteins which were deposited by neurons in culture. The L6 rat skeletal muscle cell line has also been shown to produce some extracellular proteins or substrate-attached material including a major band at M, ~ 55,000 (Schubert 1977). We found a similar ECM band at M, ~ 31,000. However, collagen or collagenous proteins were not identified in the earlier study (Schubert 1977). In another previous study using clonal rat myotubes it was suggested that muscle cells were capable of synthesizing soluble collagenous proteins but were unable to incorporate them into an organized insoluble matrix (Lipton, 1977). In the present study, we have used gel electrophoresis and enzymatic degradation to identify the major components incorporated into ECM by myoblasts and myotubes in cultures free of nonmyogenic cells. These ECM preparations have features similar to these described in previous studies with connective tissue cells, such as the presence of fibronectin, collagen, and high molecular weight material. In addition, we show evidence that in the absence of fibroblast elements skeletal muscle cells produce one or more genetic types of collagen. Experiments to examine further the collagen types, the procollagens synthesized and released, effects of nerve extracts, vitamin C, and neuronal co-culture on ECM production during in vitro myogenesis are in progress. Such studies may further elucidate mechanisms of interaction between nerve and muscle and help identify aberrations that may exist in some neuromuscular diseases.

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REFERENCES

Betz, W., and Sklman, B. (1973) J. Physiol. (Lond.) 230, 673-688
Skeletal Muscle Matrix Production in Vitro

11442


Lipton, B. H. (1977) Dev. Biol. 61, 153-165


Zacks, S. I., Sheff, M. F., and Saito, A. (1973b) J. Histochem. Cytochem. 21, 703-714