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EXTRACELLULAR MATRIX (ECM) SYNTHESIS IN MUSCLE CELL CULTURES: QUANTITATIVE AND QUALITATIVE STUDIES DURING MYOGENESIS

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When the synthesis of extracellular matrix components was examined in G8-1 murine skeletal muscle cells as a function of differentiation, non-collagen and to an even greater extent collagen synthesis was increased. Specifically, collagen types I, III, IV, laminin and fibronectin were identified by SDS-PAGE. Immunoprecipitation, with specific antibodies revealed that both the cell layer and medium of differentiated multinucleated myotubes contained increased levels of type IV collagen and laminin, decreased levels of type III collagen and fibronectin and equivalent levels of type I collagen compared to mononuclear myoblasts.

Each muscle fiber in vertebrate skeletal muscle is ensheathed by a basement membrane (BM) that includes both basal lamina and a reticular layer of collagenous fibrils (1,2). The basal lamina is composed of type IV collagen, the glycoproteins laminin, fibronectin and entactin and heparin sulfate proteoglycan (3-5). It has been reported that myogenic cells are capable of synthesizing, secreting and depositing basal lamina (BL) and other extracellular matrix (ECM) proteins but lose this capability after fusion (6). It is well known that the synthesis of a variety of skeletal muscle cellular components changes dramatically during the fusion of mononucleated myoblasts into multinucleared myotubes (7). In regard to ECM components, the BM only appears postfusionally, since neither myoblasts nor contaminating muscle fibroblasts have a BM (8). We have previously reported that G8-1 clonal mouse myoblasts synthesize and secrete collagens and other extracellular proteins and incorporate these macromolecules into an insoluble and organized matrix (9). Recently we have shown that G8-1

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myoblasts secrete collagen types I, III, IV and V (10). The study presented here extends these observations to multinucleated myotubes. The results demonstrate that while overall protein synthesis in general and collagen synthesis in particular increases with myotube formation, that the synthesis of basal lamina specific proteins (type IV collagen and laminin) are increased to a relatively greater extent during myogenesis. This suggests a developmental regulation of the synthesis of ECM macromolecules during skeletal muscle differentiation in culture.

MATERIALS AND METHODS

Cell Culture and Labeling

G8-1 cells were grown in 60 mm dishes to obtain a high percentage of myotubes as previously described (9). The cell cultures were labeled at day 3 (myoblasts) and 10 (myotubes) in serum-free media containing 25 μCi/ml [2,3,4,5 ^{3}H]-proline (81 Ci/mmole, New England Nuclear, Boston, MA). 50 μg/ml ascorbic acid and 100 μg/ml BAPN (Sigma, St. Louis) for 5 hr at 37°C. Cultures to be labeled were preincubated 45 minutes in fresh media containing ascorbic acid and BAPN without serum prior to adding the [^{3}H]-proline. The samples for immunoprecipitation were labeled under the same conditions containing 100 μCi/ml proline.

Incorporation of [^{3}H]-Proline into Collagen and Non-Collagen Protein

The cultures were labeled as described above and the medium was removed and treated separately. The cell layer was scraped from the dishes directly into Tris buffered saline (TBS) using a rubber policeman. The samples were boiled for 10 min and frozen. For analysis, the samples were thawed and precipitated with 50% TCA to a final concentration of 5%. The samples were centrifuged at 10,000 RPM for 15 min at 4°C and washed 3 times with 5% TCA to remove unincorporated [^{3}H]-proline. The pellet was dissolved in 0.2 N NaOH, and the collagenase digestable protein (CDP) and non-collagen protein (NCP) were quantitated as described previously (11) using purified bacterial collagenase (Drs. Dieglemann, Lindblad, Richmond, VA). Protein was estimated as described previously (12).

Analysis of Medium and Cell-Layer Proteins by Proteinase Digestions

The samples from media and cell layer was treated with collagenase and pepsin as described previously (13). The digested products were resolved on 7% SDS-PAGE electrophoresis (14) and the fluorograms were prepared as described previously (15).

Immunoprecipitation

G8-1 cultures were labeled as described above. The media were removed and combined with protease inhibitors (final concentrations: EDTA 25 mM, PMSF, 0.2 mM, NEM 10 mM) 2% sodium deoxycholate, 10 mM Tris/HCl pH 7.4. The cells from each dish was scraped and homogenized in Tris-HCl buffer pH 7.4 containing 2% Triton X-100, 2% sodium deoxycholate and protease inhibitors. To determine the amounts of radiolabeled collagen types I, III, IV, laminin and fibronectin, media and cell layer (5 mg of protein) were combined with optimal amounts of antibodies to collagen types I, III, IV, laminin and

fibronectin (Dr. Hynda Kleinmann, NIH) and incubated for 60 min at 4°C. The antibody antigen complexes were then co-precipitated by the addition of protein A-agarose and incubated another 45 min at 4°C. The reaction mixtures was then centrifuged in a clinical centrifuge for 2 min. The pellets were resuspended in wash buffer containing 50 mM Tris-HCl (pH 7.4), 2% Triton X-100, 2% sodium deoxycholate, protease inhibitors and centrifuged for 2 min. The pellets were washed two more times. The final pellet was taken up in 2% sodium dodecylsulfate, 10 mM dithiotrietol, 5% β-mercuptoethanol, and 5 mM sodium phosphate (pH 7.0) and boiled for 5 min and the supernatant counted in liquid scintillation counter. Parallel controls without the antisera were substracted as blanks for each immunoprecipitation.

RESULTS AND DISCUSSION

As shown in Table I, skeletal muscle cells in culture incorporate [^{3}H]-proline into collagenase digestable protein (CDP) as well as non-collagen protein (NCP). Myotubes incorporate an increased level of [^{3}H]-proline into CDP and NCP in both medium and cell layer when compared to myoblasts. However, when the medium and cell layer are considered together, myotubes show a 25% increase in collagen synthesis.

To identify the collagenous proteins synthesized by G8-1 myoblasts and myotubes, the [^{3}H]-proline labeled medium and cell layers were analyzed by SDS-PAGE. As seen in Figure 1, six major collagenase sensitive (1a) and five pepsin resistant (1b) bands are detected ranging in molecular weight from

TABLE 1
The incorporation of [^{3}H]proline into collagenase digestable protein and non-collagen protein in myoblasts and myotubes[a]

	CDP	NCP	
	(dpm/μg protein)		% Collagen
Myoblasts:			
Media	1863 ± 103	1792 ± 99	16.14 ± 0.05
Cell layer	1308 ± 59	5989 ± 41	3.88 ± 0.14
Myotubes			
Media	2549 ± 40*	2166 ± 52*	17.78 ± 0.20*
Cell layer	1677 ± 42*	6936 ± 101*	4.26 ± 0.04**

[a]The cells were labeled with 25 μCi/ml of [^{3}H]proline for 5 hours. The incorporation of radioactive proline into medium and cell layer CDP and NCP was determined by collagenase digestion (11). Values were normalized to total protein present in samples. Each determination represents the average of six culture dishes ± SE.
*Statistically significant for values at $P \leq 0.01$.
**Statistically significant for values at $P < 0.05$.

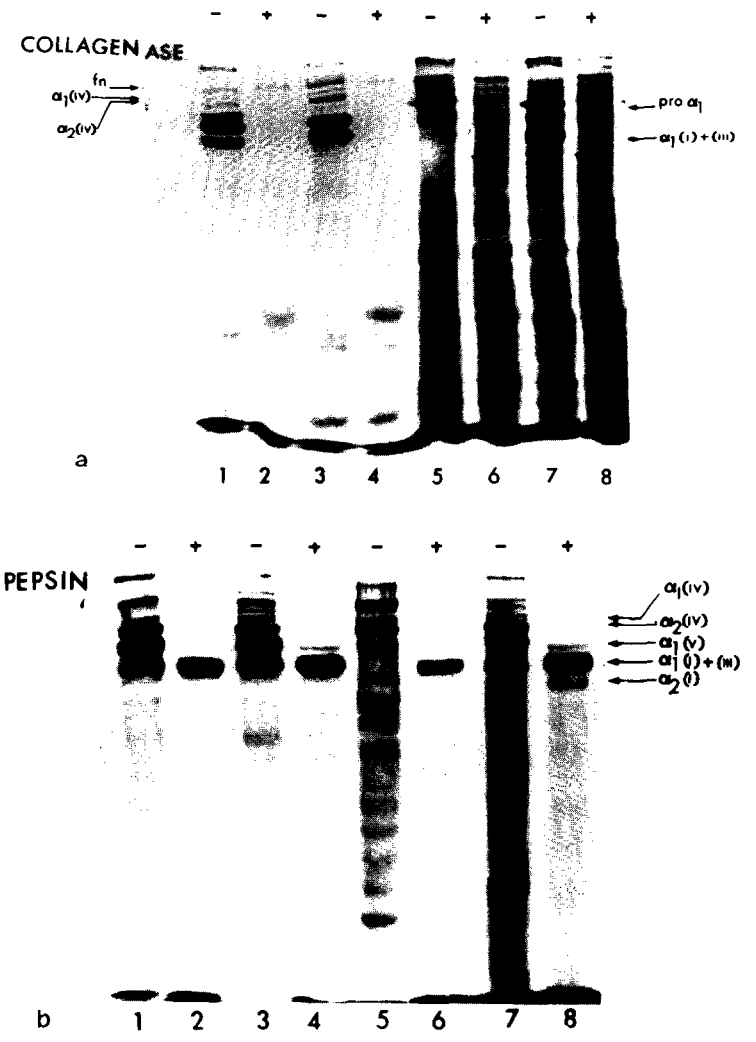

Fig. 1. SDS-PAGE analysis of [^{3}H]-proline labeled proteins synthesized by G8-1 muscle cells. Effect of (a) collagenase or (b) pepsin digestion. Myoblasts (lanes 1,2,5 and 6) and myotubes (lanes 3,4,7 and 8) were incubated with [^{3}H]-proline and the media (lanes 1-4) and cell layer (lanes 5-8) were processed and digested with (+) or without (-) the indicated enzyme as described in Materials and Methods.

95 to 185 Kd. Using molecular weight and known collagen standards the following designations have been made: Mr 95,000 = α_1(I); Mr 135,000 = N terminal (PN) and C-terminal (PC) fragments of procollagen α_1(I); Mr 150,000 = procollagen α_1(I); Mr 170,000 = procollagen α_1(V); Mr 180,000 = α_2(IV) and Mr 185,000 = α_1(IV). Another major band, Mr = 220,000, comigrating with fibronectin was unaltered by collagenase (1a)

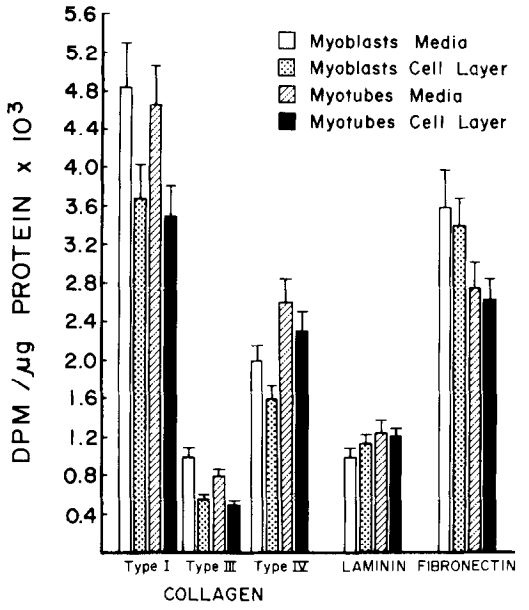

Fig. 2. Quantitation of collagens during myogenesis by immunoprecipitation: Myoblasts and myotubes were incubated with [^{3}H]-proline and the medium and cell layer proteins were immunoprecipitated with specific antibodies to collagen types I, III, IV, laminin and fibronectin as described in Materials and Methods. The values represent the mean ± S.E. of the data obtained from 5 experiments.

but completely digested by pepsin (1b). The $\alpha 2$(I) chain was apparent only in the cell layer of myotubes and very faintly in myoblasts.

It is evident from Figure 1 that the proportions of collagen types change during myogenesis. To quantitate these changes in collagen as well as laminin and fibronectin, the medium and cell layer were immunoprecipitated with antibodies to collagen types I, III, IV, laminin and fibronectin. Specificity of these antibodies were confirmed by SDS-PAGE electrophoresis (data not shown). As shown in Figure 2, while more type I collagen is secreted than is associated with cells of both myoblasts and myotubes, there is no significant difference in type I collagen levels during myogenesis. Type III collagen is decreased (18%) in myotubes realtive to myoblasts as is fibronectin (23%). On the other hand, type IV collagen is significantly increased (36%) in myotube cultures consistent with the observations on SDS-PAGE (Fig. 1). Finally the non-collagenous protein laminin is also increased (14%) in myotube cultures.

The results presented here confirm our earlier study demonstrating that collagen and/or procollagen types I, III, IV and V are produced and secreted by G8-1 myoblasts (10). Here we have extended these observations to G8-1 myotubes and have shown that they also produce these collagen types as well as laminin and fibronectin. Although a concensus has developed in recent years that myogenic cells are capable of collagen synthesis (8,16-18), controversy exists regarding which genetic types are made by each cell type and how such synthesis might be regulated. Recently it has been proposed that mononuclear myoblasts secrete the proteins of the basal lamina, such as type IV collagen and laminin and that these proteins subsequently associate with or bind to the myotube surface through specific cell surface receptors (19). While it is possible that these molecules do associate with myotubes through specific receptors, the results presented here clearly demonstrate that both G8-1 myotubes as well as myoblasts are capable of synthesizing and secreting these basal lamina molecules. Thus G8-1 myotubes are capable of elaborating their own basal lamina as seen in other cells (20-22).

The data presented here also demonstrate that the levels of these basal lamina molecules change during myogenesis, with an increase after myoblast fusion in specific basal lamina associated collagen (type IV) and non-collagen proteins (laminin) at the expense of fibrillar collagen (type I). This suggests a developmental regulation of synthesis such as has been proposed in an earlier study with enriched L-6 rat myotubes (23). Studies are currently in progress to examine the levels of mRNA encoding the interstatial collagen (Type I) and basement membrane collagen (type IV) to determine if this regulation is at pre- or post-translational level.

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