Extracellular-matrix synthesis by skeletal muscle in culture

Major secreted collagenous proteins of clonal myoblasts

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We have previously shown that G8-1, a murine clonal skeletal-muscle cell line, produces a substrate-attached extracellular matrix [Beach, Burton, Hendricks & Festoff (1982) *J. Biol. Chem.* **257**, 11437–11442]. To examine further the expression of extracellular-matrix proteins by muscle cells, we have analysed the collagenous proteins secreted by G8-1 myoblasts. We have found that collagens and/or procollagens, corresponding to genetic types I, III and IV (and possibly V), are produced and secreted by G8-1 myoblasts. The major secreted collagenous polypeptides were identified as $\alpha 1$ type I and its precursors by using pulse-chase studies, pepsin and collagenase digestions and CNBr fragmentation. The presence of lesser amounts of the other collagens was determined by immunoprecipitation. These results demonstrate that clonal skeletal-muscle cells, in the absence of fibroblasts and an exogenous collagen substrate, are able to synthesize and secrete several extracellular-matrix collagenous proteins in proportions similar to those which are commonly found in muscle tissue and mixed cultures of muscle cells and fibroblasts.

The importance of the extracellular matrix in skeletal-muscle development has been well established (Hauschka & Konigsberg, 1966; Konigsberg, 1970; Hauschka, 1972; Hauschka & White, 1972; De La Haba *et al.*, 1975; Lipton, 1977; Puri *et al.*, 1980; Chiquet *et al.*, 1981; Ehrisman *et al.*, 1981; Popiela *et al.*, 1982; Beach *et al.*, 1983).

The cells in muscle tissue synthesizing collagen have usually been assumed to be fibroblasts or connective-tissue cells. However, little direct evidence supports this assumption. Recent efforts in analysis of muscle collagen metabolism have been directed towards identifying the cell responsible for collagen synthesis. Such studies have used tissue culture, but cell identity has proved difficult in primary cultures of skeletal muscle because of inevitable contamination with fibroblastic cells, which are known to synthesize collagens. Several

previous reports have documented secretion of collagenous proteins by the clonal rat myoblast line L-6 (Schubert *et al.*, 1973; Garrels, 1979; Sasse *et al.*, 1981), by a human rhabdomyosarcoma (Krieg *et al.*, 1979), and also by primary muscle cell cultures (Sasse *et al.*, 1981; Kühl *et al.*, 1982; Duance *et al.*, 1979a; Kundu *et al.*, 1981a,b; von der Mark *et al.*, 1981). Types I, III, IV and V collagens have also been detected in muscle tissue (Duance *et al.*, 1977; Bailey *et al.*, 1979a,b; Ketley *et al.*, 1976). The cellular origin of collagens in these studies is ambiguous, owing to recent observations that fibroblasts synthesize and secrete collagens types I, III, V (Sasse *et al.*, 1981) as well as type IV collagen and laminin (Kühl *et al.*, 1982).

We have shown that G8-1 clonal mouse myoblasts synthesize and secrete collagens and other extracellular proteins and incorporate these macromolecules into an insoluble matrix (Beach *et al.*, 1982a,b). In the present work we have studied, in more detail, the secreted collagenous proteins of this clonal murine line, which is capable of differentiating and expressing an extensive array of phenotypic skeletal-muscle-cell properties in

Abbreviations used: BAPN, β -aminopropionitrile; NEM, *N*-ethylmaleimide; phosphate buffered saline, 137 mM-NaCl/1.47 mM-KH₂PO₄/8.06 mM-Na₂HPO₄/2.68 mM-KCl; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; DME, Dulbecco's modified Eagle's medium.

vitro (Sugiyama, 1977; Christian *et al.*, 1977; Noble *et al.*, 1978; Moore *et al.*, 1981; Walsh & Phillips, 1981; Beach *et al.*, 1982a,b; Sugiyama *et al.*, 1982; Romstedt *et al.*, 1983).

Experimental

Cell culture and labelling

G8-1 cells were grown as previously described (Beach *et al.*, 1982a). Osteoblastic cells were dissociated from newborn-mouse calvaria by sequential digestion as previously described (Wong & Cohn, 1975), and were kindly provided by Dr. Robert Jilka (Calcium Research Laboratories, Veterans Administration Medical Center, Kansas City, MO, U.S.A.). Secreted proteins were labelled by incubating confluent 3-day-old cultures in tissue-culture dishes (Falcon, Cockeysville, MD, U.S.A.) at 37°C in serum-free media containing [2,3,4,5-³H]proline (81 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.), 50 µg of ascorbic acid (Sigma, St. Louis, MO, U.S.A.)/ml and 100 µg of BAPN (Sigma)/ml. Cultures to be labelled were preincubated for 1 h in fresh media containing these additives before adding the [³H]-proline. For demonstration of the labelled proteins in media from osteoblasts or myoblasts, [2,3,4,5-³H]proline (10 µCi/ml) was incubated 16 h with the cells in a total volume of 5 ml in 60 mm-diameter dishes.

Media collagen analysis

To obtain newly synthesized labelled media proteins with minimal degradation, labelled media were removed from the culture dishes and added to a concentrated mixture of inhibitors to obtain the final concentrations of 0.5 mM-PMSF, 20 mM-EDTA and 10 mM-NEM. The cells were rinsed once with phosphate-buffered saline containing the same concentrations of the inhibitors. The media and rinse were combined, chilled on ice and centrifuged for 10 min at 300g to remove cellular debris. Cells were scraped into 2.0 ml of phosphate-buffered saline containing inhibitors and homogenized. Collagenous proteins were enriched by (NH₄)₂SO₄ precipitation as previously described (Sage & Bornstein, 1982). Gels were run as previously described (Beach *et al.*, 1981), a discontinuous buffer system (Laemmli, 1970) being used.

Pulse-labelling

Pulse-labelling was accomplished by incubation for 1 h with 100 µCi of [2,3,4,5-³H]proline/ml in a total volume of 2 ml in 60 mm-diameter dishes, followed by three rinses with unlabelled medium containing 1 mM-L-proline, and subsequent incubation in 2.0 ml of unlabelled medium with 1 mM-proline. Samples (500 µl) were removed for analy-

sis at 2, 5, 12 and 26 h. Inhibitors were added and media collagens were precipitated by 20% (w/v) (NH₄)₂SO₄, dialysed against 0.5 M-acetic acid and analysed on 5% (w/v)-polyacrylamide gels.

Pepsin and collagenase digestions

For pepsin digestion, the labelled media and cell layers were dialysed against 0.5 M-acetic acid with PMSF, EDTA and NEM as described above for 16 h, and then dialysed against acetic acid without inhibitors for an additional 24 h. Aliquots (200 µl) of the dialysed media were added to 0.1 vol. of pepsin (1 mg/ml; Worthington type A) in 0.9% NaCl/0.5 M-acetic acid (1:1, v/v), mixed, and incubated 24 h at 4°C. The pepsin digests were neutralized with NaOH and samples were mixed with concentrated sample buffer before electrophoresis on 5%-polyacrylamide gels.

For collagenase digestion, labelled media were centrifuged at 300g for 5 min to remove cellular debris, both media and labelled cell fractions were dialysed against collagenase buffer (100 mM-Tris/HCl/10 mM-CaCl₂, pH 8.0) containing inhibitors. Cell and media samples were then dialysed against collagenase buffer without the inhibitors overnight, divided into aliquots and incubated for 4 h at 37°C in the presence or absence of collagenase (10 units/ml; type III; Advance Bio-factures, Lynbrook, NY, U.S.A.). Samples were then dialysed against 10 mM-NH₄HCO₃, freeze-dried and reconstituted in sample buffer, heated to 95°C for 3 min and analysed on 5%-polyacrylamide gels.

CNBr-fragment analysis

Labelled samples of G8-1 media collagenous proteins were prepared as described above from cultures labelled for 16 h with 100 µCi of [2,3,4,5-³H]proline in 0.5 ml in 35 mm-diameter dishes. Samples (200 000 c.p.m.) of these labelled proteins were combined with 40 µg of mouse type I collagen, solubilized in sample buffer and electrophoresed on 0.75 mm-thick 5%-polyacrylamide gels in the presence of 0.1% SDS. After electrophoresis, the gels were briefly stained and destained; the α1 bands were identified, cut out and equilibrated in 70% (w/v) formic acid. The gel slices were then allowed to react for 16 h at 25°C with CNBr (50 mg/ml in 70% formic acid) in sealed tubes under N₂. The slices were rinsed five times with water and equilibrated with 30% (v/v) glycerol/0.1 M-Tris/0.02% Bromophenol Blue, pH 6.8, for over 1 h. The slices were homogenized in sample buffer and electrophoresed on 12.5%-polyacrylamide gels.

Immunoprecipitation

For immunoprecipitation of type IV collagen,

confluent 3- or 10-day G8-1 cultures were incubated for 15h in 0.5ml of medium containing 100 μ Ci of [2,3,4,5- 3 H]proline. The media were removed and combined with inhibitors (final concentrations: EDTA, 25mM; PMSF, 0.2mM; NEM, 10mM) and then adjusted to 0.3% Nonidet P40 (Gallard-Schlessinger, Coyle Place, NY, U.S.A.), 0.3% deoxycholate, 0.1% bovine serum albumin, 0.1M-Tris/HCl, 0.9% NaCl, pH 7.4, and rabbit anti-(mouse type IV collagen) antibody (10 μ g, affinity-purified) was added to 500 μ l of labelled myoblast media proteins.

For immunoprecipitation of type I and type III procollagens, confluent 3-day cultures of G8-1 myoblasts were rinsed twice with DME, preincubated for 30min in 2.0ml of DME, 50 μ g of ascorbate/ml and 0.3mM- α -dipyridyl at 37°C and labelled in 2.0ml of the same medium containing 25 μ Ci of 3 H-proline/ml for 4 or 18h. Media were removed at these times and adjusted to 1% Triton X-100, 0.5% deoxycholate, 1mM-*p*-chloromercuribenzoate, 20mM-EDTA, 1mg of bovine serum albumin/ml, 10mM-Tris/HCl, pH 7.4, and 0.1mM-PMSF. Then 50 μ l of rabbit anti-[rat pro-(α 1 type I collagen)] serum or 10 μ l of sheep anti-[human pro-(α 1 type III collagen)] serum were added to 1.0ml of the Triton/deoxycholate supernatant. The antibody and labelled supernatant solutions were mixed and incubated for 1.5h at 4°C and precipitated with Protein A-agarose (Sigma) and then analysed by SDS/polyacrylamide-gel electrophoresis.

Results

Collagenase-sensitive proteins of myoblasts

In order to identify the collagenous proteins secreted by G8-1 myoblasts, we digested cellular and media proteins with bacterial collagenase as described in the Experimental section. Analysis on SDS/5%-polyacrylamide gels revealed three major collagenase-sensitive bands in media (cf. lanes 3 and 4, Fig. 1a). For comparison, one major and two minor collagenase-sensitive bands were observed in the cells (cf. lanes 5 and 6, Fig. 1a). The lowest- M_r (95000) major collagenase-sensitive protein, which was more apparent in media than in cells, co-migrated with the α 1 chain of purified rat tail collagen (Fig. 1a). The higher- M_r (150000) collagenase-sensitive component evident in both cell and media preparations was consistent with it being procollagen α 1(I). The broad intermediate- M_r collagenase-sensitive band, which was also more apparent in media than in cells, migrated in the region where the *N*-terminal (pN) and *C*-terminal (pC) fragments of procollagen α 1(I) would be expected to be located. No collagenase-sensitive band was seen in the M_r range of α 2(I) in

either cell or media preparations. Other major bands, including a prominent one co-migrating with fibronectin, seen in cells and media, were not eliminated or reduced by collagenase treatment.

Effects of disulphide reduction and pepsin digestion

When proteins from the medium were analysed on SDS/5%-polyacrylamide gels in the presence or absence of the reducing agent β -mercaptoethanol, several bands were seen (Fig. 1b). A diffuse high- M_r (300000) protein was seen only in the unreduced preparation. On reduction with β -mercaptoethanol, this band disappeared, and the intensity of a band at M_r 130000 and a band of M_r 150000 increased relative to the other major bands (arrows, Fig. 1b). The band at M_r 150000 co-migrated with procollagen α 1(I). In this gel, the band at M_r 130000, which corresponds to the pC and pN collagen intermediates of Fig. 1(a), was resolved as a doublet. The higher- M_r band of these two increased on reduction. This band corresponds to the pC α 1(I) collagen. Type III collagens and procollagens, as well as procollagen α 1(I) and pC α 1(I), are known to be disulphide-linked, whereas pN α 1(I) and α 1(I) are not (Bornstein & Traub, 1979). When cell or media preparations were treated with pepsin to digest non-collagenous proteins, only one band (M_r 95000) remained (Fig. 1c). This band co-migrated with the α 1(I) and α 1(III) chains on these gels. Again, no band co-migrating with the α 2(I) collagen observed in osteoblast media (lanes 1 and 4) was observed. The removal of non-collagenous procollagen peptides would cause the procollagens, pC and pN intermediates to migrate with the processed α -chains (Bornstein & Traub, 1979).

Pulse-labelling studies

Since the foregoing results suggested that the major collagenous proteins present in G8-1 muscle-cell media corresponded to an α 1 collagen chain, we pulse-labelled these cells and analysed the media to determine whether the higher- M_r bands observed were related to the α -chain by a precursor-product relationship. As shown in Fig. 2, there was a stepwise quantitative processing of the labelled bands appearing in the media to stable lower- M_r forms during the post-pulse incubation period. The band trailing the pro- α 1(I) migrates in the region where procollagen type III would appear. Since the α -chains of type I and III collagens co-migrate in these gels, the type identity of the α -chain observed in Figs. 1, 2 and 3 was not clear.

CNB, peptide fragment analysis

We also compared the CNBr digests of the labelled α -band from pepsin-treated G8-1 media,

Fig. 1. SDS/polyacrylamide-gel electrophoresis analysis of [3H]proline-labelled proteins secreted by G8-1 muscle cells (a) Effect of collagenase treatment on media (lanes 3 and 4) and cellular (lanes 5 and 6) [3H]proline-labelled proteins. Dialysed extracts were incubated for 4 h at 37°C with (lanes 3 and 5) or without (lanes 4 and 6) 10 μ g of purified bacterial collagenase/ml. Radioactive (5×10^4 c.p.m.) non-dialysable proteins were loaded in each lane. Lane 1 is [3H]succinimidylated type I collagen from rat tail (10^4 c.p.m.). Lane 2 shows ^{14}C -labelled M_r marker proteins (2000 c.p.m.; Amersham, Clearbrook, IL, U.S.A.: a, myosin, 200 000; b, β -galactosidase, 116 000; c, phosphorylase a, 96 000; d, bovine serum albumin, 65 000). Gels contained 5% (w/v) acrylamide. Arrows indicate minor collagenase-sensitive components seen in cells, and discussed in the Results section. (b) Effect of reduction on [3H]proline-labelled media proteins. Unfractionated media were dialysed against 0.5M-acetic acid, freeze-dried, and reconstituted in sample buffer without β -mercaptoethanol. β -Mercaptoethanol was added to a portion (lane 2) before loading 10^5 c.p.m./well and running the sample on a 3.5–10% polyacrylamide gradient gel (lane 1 is media without β -mercaptoethanol). The positions where collagen chains (rat tail, type I) and rat fibronectin (fn) migrate are indicated. The arrows indicate the two bands, discussed in the Results section, that are increased following reduction. (c) Effect of pepsin treatment on [3H]proline-labelled media (lanes 2 and 3) and cellular (lanes 5 and 6) proteins. Dialysed extracts in 0.5M-acetic acid were incubated in the presence (lanes 3 and 6) or absence (lanes 4 and 5) of pepsin (100 μ g/ml; 20 h at 4°C). Samples were then neutralized, adjusted to 0.1% SDS, 5% (v/v) β -mercaptoethanol and 62.5M-Tris/20% (v/v) glycerol, pH 6.8, and 5×10^4 c.p.m. were run on 5–12% polyacrylamide gels. Lanes 1 and 4 are 20%-(w/v)- $(NH_4)_2SO_4$ precipitates of osteoblast media. The positions where standards of fibronectin (fn), procollagen and collagen chains migrated are indicated.

with the fragments obtained from similar digests of purified unlabelled mouse $\alpha 1$ type I collagen chains. Samples of each were run in the same well on SDS/5%-polyacrylamide gels, the $\alpha 1$ band was cut out and digested with CNBr and the fragments

were subsequently separated on a 12.5%-polyacrylamide gel. The labelled media collagen $\alpha 1$ band was not detectable by staining with Coomassie Blue. As shown in Fig. 3, no radioactivity was found in the $\alpha 1$ -chain location, indicating complete

Fig. 2. Pulse-labelling of G8-1-muscle-cell secreted proteins

Cells were labelled for 1 h with [^3H]proline as described in the Experimental section, rinsed, and incubated for 26 h in unlabelled media as described in the Experimental section. At the indicated times, 500 μl aliquots were taken and 20%-(w/v)- $(\text{NH}_4)_2\text{SO}_4$ precipitates were obtained and prepared for electrophoresis as described in the Experimental section. Identification of the labelled proteins is based on co-migration with standards and is detailed in the text referring to Fig. 5. Differences in load from lane to lane are due to incomplete recovery of $(\text{NH}_4)_2\text{SO}_4$ precipitates.

digestion, and the labelled media fragments (lane 4) and unlabelled standard $\alpha 1(\text{I})$ fragments (lane 2) co-migrated. The relative intensities of the peptide bands in standard and labelled digests were quite similar. Other than these peptides, no additional bands corresponding to type III collagen fragments were observed.

Immunoprecipitation of collagenous proteins

To clarify further the identity of the procollagen chains observed previously (Figs. 1 and 2), we immunoprecipitated labelled procollagens from cells and media with antibodies to procollagen $\alpha 1(\text{I})$ and collagen $\alpha 1(\text{III})$. As shown in Fig. 4(a), the antibody to pro- $\alpha 1(\text{I})$ precipitated a protein (M_r 150000) corresponding to pro- $\alpha 1(\text{I})$ from both media and cells. The antibody to collagen $\alpha 1(\text{III})$ precipitated bands (M_r 155000 and 150000) corresponding to pro- $\alpha 1(\text{III})$ and pro- $\alpha 1(\text{I})$. This antibody also precipitated pro- $\alpha 1(\text{I})$ chains from cell extracts as well as an additional band (M_r 170000). The antibody to collagen type III was known to cross-react with procollagen type I and procollagen type V (H. Kleinman, personal

Fig. 3. Analysis of CNBr fragments of pepsin-treated unlabelled $\alpha 1(\text{I})$ mouse collagen and [^3H]proline-labelled $\alpha 1$ chain from pepsin-treated G8-1 media

The unlabelled (30 μg) and labelled (10^5 c.p.m.) proteins were run in the same lane on 5%-polyacrylamide gels and the $\alpha 1$ band was cut out and digested with CNBr as described in the Experimental section. Digested samples were then run on 12.5%-polyacrylamide gels with standards. The Coomassie Blue-stained gel (lanes 1–3) shows M_r standards (lane 1), stained CNBr fragments of the unlabelled standard $\alpha 1(\text{I})$ fragments (lane 2), and the undigested unlabelled type I collagen standard (lane 3). The fluorograph of lanes 2 and 3 shows the CNBr fragments of the [^3H]proline-labelled G8-1 cell $\alpha 1$ chain (lane 4) and the undigested [^3H]proline-labelled G8-1 cell $\alpha 1$ chain (lane 5) from G8-1 media.

communication). Thus the M_r -170000 band seen in cell precipitates may be procollagen type V, which migrates at about this M_r (Fessler *et al.*, 1981). The absence of type III procollagen in cell-extract immunoprecipitates may be due to a relative predominance of $\alpha 1$ type I procollagen in the cells (see the Discussion section). Nevertheless, the present studies demonstrate that immunoprecipitable procollagens of $\alpha 1(\text{I})$ and $\alpha 1(\text{III})$, and probably type V collagens, are secreted by these clonal myoblasts. Fibronectin is known to bind to protein A, and this explains the presence of a band co-migrating with fibronectin in the immunoprecipitates shown here and below (in Fig. 4b), as well as in controls, where antibody was omitted (results not shown).

We were also interested in whether G8-1 cells synthesized and secreted type IV collagen. As

Fig. 4. Immunoprecipitation of [3H]proline-labelled proteins synthesized by G8-1 myoblasts (a) Immunoprecipitation of media (lanes 1 and 2) and cellular (lanes 3 and 4) G8-1 proteins. Rabbit anti-(rat type I procollagen) antisera were used in lanes 1 and 3; sheep anti-(human type III collagen) antisera were used in lanes 2 and 4. The gel contained 5% acrylamide. Position of standard [3H]collagen (rat tail) chains are indicated on the left, the arrow indicating the position of presumptive type V procollagen. (b) Immunoprecipitation of type IV collagen from media by affinity-purified rabbit anti-(mouse type IV collagen) antibodies. Lane 1, [3H]proline-labelled media from 3-day G8-1 cultures; lane 2, [3H]proline-labelled media from 3-day osteoblast cultures; lane 3, [3H]proline-labelled media from 10-day G8-1 cultures. The position of $\alpha 1$ - and β -chains of rat tail collagen fibronectin (fn) and the two chains of EHS (Engelbreth-Holm-Swarm)-sarcoma type IV collagen standards are indicated. The gel contained 3.5–10% acrylamide. All immunoprecipitates contain a band corresponding to fibronectin, even when no antisera were added (results not shown). The additional lower- M_r [130 000 (*) and 110 000 (arrow)] bands observed in this Figure are probably proteolytic fragments of type IV collagen.

shown in Fig. 4(b), labelled $\alpha 1(IV)$ and $\alpha 2(IV)$ collagen chains were precipitated from the media of G8-1 cells by specific antibodies to type IV collagen (H. Kleinman, personal communication; see also Yaoita *et al.*, 1978). The relative amount of type IV collagen chains was greater in 10-day myotube cultures (lane 3) than in 3-day cultures (lane 1), where it was barely detectable. These results correlate with our findings of increased basal-lamina production in myotubes (Beach *et al.*, 1982b). In addition, fragments of M_r 130 000 in myoblasts (lane 1) and of 110 000 in myotubes (lane 3) were also precipitated. No type IV immunoreactivity label was precipitated from osteoblast media (lane 2).

Comparison with osteoblast cell type 1 collagen

We next compared the higher- M_r proteins, since we had demonstrated a precursor-product relationship with the α -chain (Fig. 2), with the precursors to type I collagen in cells previously shown to synthesize primarily type I collagen chains and their precursors. Autoradiographs of SDS/polyacrylamide-electrophoresis gels of the [3H]proline-labelled proteins secreted into the media by G8-1 mouse myogenic cells are shown in Fig. 5, lane 1. They are compared with the proline-labelled pro-

teins secreted by osteoblast cells derived from newborn-mouse calvaria (Fig. 5, lane 2). Fewer labelled proteins are seen in the media from G8-1 cells, although several of the bands are common to the media of these two different types of mouse cells. We next precipitated the collagens and procollagens of both murine cells with 20% (w/v) $(NH_4)_2SO_4$ to facilitate loading of sufficient radioactivity on the gels without an overload of protein. The resultant labelled collagenous proteins are compared as shown in Fig. 5, lanes 3 and 4. The pattern obtained from osteoblasts (lane 4) may be readily contrasted with that obtained from myoblasts (lane 3). The known proteins secreted by osteoblasts (Fessler & Fessler, 1978) are indicated in the Figure to facilitate comparison. It is apparent that several of the collagenous proteins secreted by osteoblasts were not secreted by G8-1 myoblasts. In particular, the $\alpha 2$ chain of type I collagen was not present in myoblast media. In addition, pro- $\alpha 2$, pC $\alpha 2$, and pN $\alpha 2$, the precursors to the $\alpha 2$ chain, were not seen. The major labelled proteins seen in myoblast media co-migrated with the $\alpha 1$ chain of type I collagen and its precursors [pro- $\alpha 1(I)$, pC $\alpha 1(I)$, and pN $\alpha 1(I)$]. A band corresponding to pro- $\alpha 1(III)$ was also seen in both osteoblast and myoblast media. A prominent band

Fig. 5. Gel-electrophoretic comparison of [^3H]proline-labelled proteins secreted by G8-1 myogenic cells and mouse osteoblasts

Unfractionated media from myoblasts (lane 1) and from osteoblasts (lane 2) and 20%-(w/v)- $(\text{NH}_4)_2\text{SO}_4$ precipitates of the media of myoblasts (lane 3) and of osteoblasts (lane 4) labelled 16h and prepared as described in the Experimental section are shown. The positions of the various collagens and procollagens are indicated and correspond to the previously described data from mouse calvaria (Fessler & Fessler, 1978) and other cells (Sage & Bornstein, 1982).

corresponding to fibronectin was seen in media from both cell types; however, this band was more intense in calvarial osteoblasts than in myoblasts.

Discussion

In the present paper we have identified the major collagenase-sensitive [^3H]proline-labelled proteins synthesized by G8-1 clonal mouse skeletal-muscle cells as $\alpha 1$ chains of type I collagen. Type III procollagen was detected in the media, but not in the cells, in immunoprecipitation experiments (Fig. 4a). A band corresponding to type III procollagen was also observed in $(\text{NH}_4)_2\text{SO}_4$ precipitates of media (Fig. 2 and Fig. 5). However, disulphide reduction did not significantly increase the intensity of the $\alpha 1$ band, and only peptides corresponding to type I collagen were seen in CNBr digests of $\alpha 1$ chains. The most reasonable interpretation of these results is that: (1) type III procollagen is synthesized, rapidly secreted and thus does not accumulate in the cells; (2) much greater amounts of $\alpha 1$ type I collagen are produced,

secreted and processed than type III collagen; and (3) more of the unprocessed pro- $\alpha 1$ (I) than pro- $\alpha 1$ (III) accumulates in the cells. Despite the conclusive evidence for $\alpha 1$ (I) collagen as the major secreted collagen, no $\alpha 2$ (I) collagen or its precursors were observed. We conclude that the major secreted collagen of these cells is $\alpha 1$ (I) collagen, possibly in the form of $\alpha 1$ (I) trimer. In addition, we demonstrated that these cells also secrete both type IV (Fig. 4b) and amounts of type V collagen (Fig. 4a).

The ability of cultured skeletal-muscle cell lines to synthesize collagenous proteins has been studied by using several different systems. Rat L6 myogenic cells were shown to secrete several unidentified collagenase-sensitive, high- M_r proteins with high levels of proline and low levels of methionine (Schubert *et al.*, 1973; Garrels, 1979). A non-fusing continuous human embryonal rhabdomyosarcoma cell line apparently synthesized primarily type III collagen as well as minor proportions of types I and V (Kreig *et al.*, 1979). In primary 'clonal' quail skeletal-muscle cell cultures, more than 85% of the hydroxyproline incorporated into proteins was found to be present as soluble media protein (Lipton, 1977).

Immunofluorescence studies (Durance *et al.*, 1977; Bailey *et al.*, 1979a,b), and extraction of chick muscle tissues (Ketley *et al.*, 1976) have shown that collagen types I, III and V are present in muscle and enriched in the epimysium, perimysium and endomysium respectively. These collagen types have also been extracted from muscle tissue (Durance *et al.*, 1977; Ketley *et al.*, 1976; Fujii & Murota, 1982). In addition, biosynthetic and immunofluorescence studies have shown that these collagen types are synthesized in primary cultures, and suggest that myogenic cells can synthesize these collagen types as well as type IV collagen, laminin and fibronectin (Sasse *et al.*, 1981; Durance *et al.*, 1979a,b; Kundu *et al.*, 1981a,b; von der Mark *et al.*, 1981; Köhl *et al.*, 1982; Duksin *et al.*, 1983). In these studies, type I collagen was the predominant species that was synthesized, but both $\alpha 1$ and $\alpha 2$ chains were observed. We also find type I collagen, but only the $\alpha 1$ chain, as the predominant collagen in clonal mouse myoblast cultures.

Several other cell types have been found to secrete $\alpha 1$ (I) trimer in culture (Crouch & Bornstein, 1978; Munksgaard *et al.*, 1978; Mayne *et al.*, 1975). Type I trimer has also been demonstrated in normal tissues, including embryonic-chick tendons and calvaria (Jimenez *et al.*, 1977) and human skin (Uitto, 1979). Thus the presence of type I trimer in G8-1 skeletal-muscle cells is not without precedent. However, the fact that this component represents a large portion of the secreted pepsin-

resistant proline-labelled protein secreted into the medium is unusual. Other cells have been reported to synthesize smaller proportions of type I trimer (Crouch & Bornstein, 1978; Mayne *et al.*, 1975), but the high proportion of type I trimer produced by G8-1 cells recommends them as a potential system for studying the genetic regulation of synthesis of $\alpha 1$ chains, and for analysis of the processing of $\alpha 1$ pre-procollagen chains.

The absence of $\alpha 2(I)$ chains or their precursors in the present study might be explained by at least three possibilities. First, it is possible that G8-1 cells do not synthesize the mRNA for pro- $\alpha 2(I)$ collagen, having repressed or lost the gene for $\alpha 2(I)$ chains. Secondly, these cells might synthesize the message, but the mRNA is not translated because it is either unstable or in other ways unsuitable for translation. The third possibility is that the mRNA for procollagen $\alpha 2(I)$ is translated but the polypeptide product is unusually unstable. Reports of low levels of translatable and/or hybridizable collagen type I in fibroblastic cell lines have appeared (Adams *et al.*, 1977; Rowe *et al.*, 1978; Sobel *et al.*, 1981; Sandmeyer *et al.*, 1981; Avvedimento *et al.*, 1981). Recently, Marsilio *et al.* (1984) demonstrated a lack of translatable pro- $\alpha 2(I)$ collagen mRNA in the W8 cell line. Deak *et al.* (1983) found that cultured skin fibroblasts from a patient with osteogenesis imperfecta produced an altered pro- $\alpha 2(I)$ collagen that was not detectable in the medium but was present, in reduced quantities, in the cells. Further experiments are needed to determine why G8-1 myoblast cultures, employed in the current experiments, have no detectable levels of secreted $\alpha 2(I)$ collagen. We have found that G8-1 cells differentiate phenotypically as skeletal muscle (Beach *et al.*, 1982a,b; Romstedt *et al.*, 1983). Others have found that G8-1 cells differentiate extensively in culture, including formation of synapses with neuronal cells (Moore *et al.*, 1981; Walsh & Phillips, 1981; Christian *et al.*, 1977; Noble *et al.*, 1978; Romstedt *et al.*, 1983; Sugiyama, 1977) when grown on exogenous collagen. Since we have cultured G8-1 cells directly on plastic for these experiments, it appears that the collagens produced are able to support the differentiation of G8-1 muscle cells *in vitro*, perhaps to the same extent as is seen with primary muscle cell culture. In separate experiments we have observed basal-lamina and collagen-fibril formation in a reticular layer in 10-day myotube cultures by using electron microscopy (R. L. Beach, J. S. Rao, J. W. Hamilton & B. W. Festoff, unpublished work). The present and other results (Beach *et al.*, 1982a,b) demonstrate that cultured clonal muscle cells are able to produce many of their own extracellular matrix components, making them suitable '*in vitro*' models for

the study of extracellular-matrix metabolism in muscle.

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References

- Adams, S. L., Sobel, M. E., Howard, B. H., Olden, K., Yamada, K., de Crombrughe, B. & Pastan, I. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3399–3403
- Avvedimento, V. E., Yamada, Y., Lovelace, E., de Crombrughe, B. & Pastan, I. (1981) *Nucleic Acids Res.* **9**, 1123–1131
- Bailey, A. J., Shellswell, G. B. & Duance, V. C. (1979a) *Nature (London)* **278**, 67–69
- Bailey, A. J., Duance, V. C., Sims, T. J. & Beard, H. K. (1979b) *Front. Matrix Biol.* **7**, 49–59
- Beach, R. L., Kelly, P. T., Babitch, J. A. & Cotman, C. W. (1981) *Brain Res.* **225**, 75–93
- Beach, R. L., Burton, W. V., Hendricks, W. J. & Festoff, B. W. (1982a) *J. Biol. Chem.* **257**, 11437–11442
- Beach, R. L., Burton, W. B., Hamilton, J. & Festoff, B. W. (1982b) *Neurosci. Abstr.* **8**, 417
- Beach, R. L., Popiela, H. & Festoff, B. W. (1983) *FEBS Lett.* **156**, 151–156
- Bornstein, P. & Traub, W. (1979) in *The Proteins* (Neurath, H. & Hill, R. L., eds.), vol. 4, pp. 411–632, Academic Press, New York
- Chiquet, M., Eppenberger, H. M. & Turner, D. C. (1981) *Dev. Biol.* **88**, 220–235
- Christian, C. N., Nelson, P. G., Peacock, J. & Nirenberg, M. (1977) *Science* **196**, 995–998
- Crouch, E. & Bornstein, P. (1978) *Biochemistry* **17**, 5499–5509
- De La Haba, G., Kamal, H. M. & Tiede, D. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2729–2732
- Deak, S. B., Nicholls, A., Pope, F. M. & Prockop, D. J. (1983) *J. Biol. Chem.* **258**, 15192–15197
- Duance, V. C., Restall, D. J., Beard, H., Bourne, F. J. & Bailey, A. J. (1977) *FEBS Lett.* **79**, 248–252
- Duance, V. C., Black, C. M., Dubowitz, V., Hughes, G. R. V. & Bailey, A. J. (1979a) *Muscle Nerve* **3**, 487–490
- Duance, V. C., Stephens, H. R., Dunn, M., Bailey, A. J. & Dubowitz, V. (1979b) *Nature (London)* **284**, 470–472
- Duksin, D., Kalchheim, C. & Vogel, Z. (1983) *J. Biol. Chem.* **258**, 14585–14591
- Ehrisman, R., Chiquet, M. & Turner, D. C. (1981) *J. Biol. Chem.* **256**, 4056–4062

- Fessler, J. H. & Fessler, L. I. (1978) *Annu. Rev. Biochem.* **47**, 129–162
- Fessler, L. I., Robinson, W. J. & Fessler, J. H. (1981) *J. Biol. Chem.* **256**, 9646–9651
- Fujii, L. & Murota, K. (1982) *Anal. Biochem.* **172**, 449–452
- Garrels, J. I. (1979) *Dev. Biol.* **73**, 134–152
- Hauschka, S. D. (1972) in *Growth, Nutrition and Metabolism of Cells in Culture* (Rothblat, G. H. & Cristofalo, V. J., eds.), vol. 2, pp. 67–130, Academic Press, New York
- Hauschka, S. D. & Konigsberg, I. R. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **55**, 119–126
- Hauschka, S. D. & White, N. K. (1972) in *Research in Muscle Development and the Muscle Spindle* (Banker, B. B. & Przybylski, R. J., eds.), pp. 53–71, Excerpta Medica, Amsterdam
- Jiminez, S. A., Bashey, R. I., Benditt, M. & Yankowski, R. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1354–1361
- Ketley, J. N., Orkin, R. W. & Martin, G. R. (1976) *Exp. Cell Res.* **99**, 261–268
- Konigsberg, I. R. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 3, pp. 1779–1810, Academic Press, New York
- Krieg, T., Timpl, R., Alitalo, K., Kurkinen, M. & Vaheri, A. (1979) *FEBS Lett.* **104**, 405–409
- Kühl, U., Timpl, R. & von der Mark, K. (1982) *Dev. Biol.* **93**, 344–354
- Kundu, K. K., Thompson, E. J. & Yasin, R. (1981a) *Biochem. Soc. Trans.* **9**, 76–77
- Kundu, K. K., Thompson, E. J. & Yasin, R. (1981b) *Biochem. Soc. Trans.* **9**, 551–552
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lipton, B. H. (1977) *Dev. Biol.* **61**, 153–165
- Marsilio, E., Sobel, M. E. & Smith, B. D. (1984) *J. Biol. Chem.* **259**, 1401–1404
- Mayne, R., Vail, M. S. & Miller, E. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4511–4515
- Moore, S. E., Phillips, E. & Walsh, F. S. (1981) *Neurochem. Int.* **3**, 27–36
- Munksgaard, E. C., Rhodes, M., Mayne, R. & Butler, W. T. (1978) *Eur. J. Biochem.* **82**, 609–617
- Noble, M. D., Brown, T. H. & Peacock, J. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3488–3492
- Popiela, H., Ellis, S. & Festoff, B. W. (1982) *J. Neurosci. Res.* **8**, 547–567
- Puri, E. C., Caravati, M., Perriard, J. C., Turner, D. C. & Eppenberger, H. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5297–5301
- Romstedt, K., Beach, R. L. & Festoff, B. W. (1983) *Muscle Nerve* **6**, 283–290
- Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. H., Bornstein, P. & Palmiter, R. D. (1978) *Biochemistry* **17**, 1581–1590
- Sage, H. & Bornstein, P. (1982) *Methods Enzymol.* **82**, 96–126
- Sandmeyer, S., Gallis, B. & Bornstein, P. (1981) *J. Biol. Chem.* **256**, 5022–5028
- Sasse, J., von der Mark, H., Kühl, U., Dessau, W. & Von Der Mark, K. (1981) *Dev. Biol.* **83**, 79–89
- Schubert, D., Tarikas, H., Humphreys, S., Heineman, S., & Patrick, J. (1973) *Dev. Biol.* **33**, 18–37
- Sobel, M. E., Yamamoto, T., de Crombrughe, B. & Pastan, I. (1981) *Biochemistry* **20**, 2678–2684
- Sugiyama, H. (1977) *FEBS Lett.* **84**, 257–260
- Sugiyama, H., Yamashita, Y. & Murakami, F. (1982) *J. Neurochem.* **39**, 1038–1046
- Uitto, J. (1979) *Arch. Biochem. Biophys.* **192**, 371–379
- von der Mark, K., Sasse, J., Dessau, W., von der Mark, H., Kühl, U. & Herrman, H. (1981) in *International Cell Biology 1980–1981* (Schweiger, H. G., ed.), pp. 542–551, Springer-Verlag, New York
- Walsh, F. S. & Phillips, E. (1981) *Dev. Biol.* **81**, 229–237
- Wong, G. L. & Cohn, D. V. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3167–3171
- Yaoita, H., Foidart, J. M. & Katz, S. I. (1978) *J. Invest. Dermatol.* **70**, 191–193