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Collagen and Stretch Modulate Autocrine Secretion of Insulin-like Growth Factor-1 and Insulin-like Growth Factor Binding Proteins from Differentiated Skeletal Muscle Cells*

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Carmen E. Perrone‡, Daniela Fenwick-Smith, and Herman H. Vandenburgh

From the Department of Pathology, Brown University School of Medicine and The Miriam Hospital, Providence, Rhode Island 02906

Stretch-induced skeletal muscle growth may involve increased autocrine secretion of insulin-like growth factor-1 (IGF-1) since IGF-1 is a potent growth factor for skeletal muscle hypertrophy, and stretch elevates IGF-1 mRNA levels *in vivo*. In tissue cultures of differentiated avian pectoralis skeletal muscle cells, nanomolar concentrations of exogenous IGF-1 stimulated growth in mechanically stretched but not static cultures. These cultures released up to 100 pg of endogenously produced IGF-1/μg of protein/day, as well as three major IGF binding proteins of 31, 36, and 43 kilodaltons (kDa). IGF-1 was secreted from both myofibers and fibroblasts coexisting in the muscle cultures. Repetitive stretch/relaxation of the differentiated skeletal muscle cells stimulated the acute release of IGF-1 during the first 4 h after initiating mechanical activity, but caused no increase in the long-term secretion over 24–72 h of IGF-1, or its binding proteins. Varying the intensity and frequency of stretch had no effect on the long-term efflux of IGF-1. In contrast to stretch, embedding the differentiated muscle cells in a three-dimensional collagen (Type I) matrix resulted in a 2–5-fold increase in long-term IGF-1 efflux over 24–72 h. Collagen also caused a 2–5-fold increase in the release of the IGF binding proteins. Thus, both the extracellular matrix protein type I collagen and stretch stimulate the autocrine secretion of IGF-1, but with different time kinetics. This endogenously produced growth factor may be important for the growth response of skeletal myofibers to both types of external stimuli.

Insulin-like growth factors (IGFs)[1] are potent mitogens involved in stimulating skeletal muscle growth (1–4). They increase amino acid uptake and protein synthesis, decrease protein degradation, and stimulate the proliferation and differentiation of skeletal muscle cells (2, 5–10). IGF's have been shown to be secreted from several mammalian skeletal muscle cell lines (8, 11, 12). A number of studies have revealed that IGF-2 is released during myoblast proliferation while IGF-1 efflux is observed during skeletal muscle differentiation (1, 8). Increases in IGF-1 mRNA have been observed during muscle regeneration after injury (13–15), and during work-induced compensatory hypertrophy (16). It has been suggested that the increased secretion of IGF-1 during work-induced hypertrophy (16) may promote the accumulation of proteins in skeletal muscle cells by an autocrine mechanism but the level of IGF-1 release from skeletal muscle cells undergoing hypertrophy is not known.

The mitogenic effects of insulin-like growth factors are regulated by their binding proteins (reviewed in Refs. 17–20). IGF binding proteins are released from cells which also secrete insulin-like growth factors (1, 18, 21, 22). They have been well characterized in serum *in vivo* (23) and in conditioned medium from tissue-cultured fibroblasts, liver cells, smooth muscle, decidual cells, and mammalian skeletal myoblasts (reviewed in Refs. 1, 18, 21, and 22). The efflux of IGF binding proteins from these cultured cells correlates with changes in the secretion of IGF-1. Thus, during C2 skeletal muscle cell line differentiation, increased secretion of IGF-1 is accompanied by increased release of IGF binding proteins (1). There are no reports on IGF binding protein efflux during either skeletal muscle repair or skeletal muscle hypertrophy.

This study was conducted to first establish whether primary cultures of differentiated avian skeletal muscle cells secrete IGF-1 and IGF binding proteins in a manner similar to tissue-cultured mammalian skeletal muscle cell lines. Second, using blocking antibodies, we determined whether IGF-1 secreted by the muscle cells could act as a autocrine/paracrine growth stimulator. Third, we determined the effect of repetitive mechanical stimulation on the sensitivity of the cells to exogenous IGF-1. Finally, the effect of mechanical stimulation on the autocrine secretion of IGF-1 and IGF binding proteins from the cultured avian pectoralis muscle cells was examined. The results indicate that IGF-1 is an autocrine/paracrine growth factor in differentiated avian pectoralis skeletal muscle cultures. Repetitive mechanical stimulation of the muscle cells increased the sensitivity of the cells to exogenous IGF-1, and acutely stimulated IGF-1 release; but it had no long-term effect on either IGF-1 or IGF binding protein release. In contrast, the release of IGF-1 and IGF binding proteins from the muscle cells was dramatically stimulated by embedding the cells in a three-dimensional collagen type I matrix after myofiber formation. This stimulated release of IGF-1 by type I collagen may be responsible for its ability to stimulate skeletal myofiber growth *in vitro* (24).

EXPERIMENTAL PROCEDURES

Materials—Fertilized Leghorn chicken eggs were purchased from Beaver River Farm, Kingstown, RI. Silicone rubber elastic membranes were from Dow Corning Corp., Midland, MI. Rat tail type I collagen was obtained from Collaborative Biomedical Products, Bedford, MA. Eagle's basal medium, penicillin, glutamine, and trypsin were from Life Technologies, Inc., Grand Island, NY. C18 Sep-Pak cartridges were obtained from Waters, Division of Millipore, Bedford, MA. Protein assay kits were purchased from Pierce. ^{125}I-Insulin-like growth factor-1, donkey anti-rabbit antibody, and L-[U-^{14}C]phenylalanine were from

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‡ To whom correspondence should be addressed: Dept. Lab Medicine, The Miriam Hospital, 164 Summit Ave., Providence, RI 02906. Tel.: 401-331-8500 (ext. 4275); Fax: 401-751-2398.

[1] The abbreviations used are: IGF, insulin-like growth factor; MM medium, muscle maintenance medium; PGM, program.

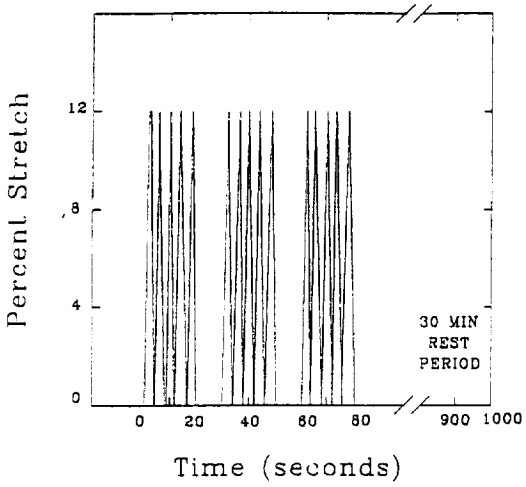

FIG. 1. **Cell stretch/relaxation activity (TRIAL39.PGM).** Differentiated skeletal muscle cells were mechanically stimulated with five 12% stretches and relaxations of the substratum over a 20-s period followed by a 10-s rest period. The pattern was repeated twice more, followed by a 30-min rest period.

Amersham. Anti-IGF-1 rabbit antibody was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program. IGF-1 standards were from Intergen Co., Purchase, NY. Polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad. All other chemicals were from Sigma.

Cell Cultures—Embryonic avian skeletal muscle cells were enzymatically isolated from 12-day *in ovo* pectoralis muscle using standard dissection techniques (25). The cells were plated on collagen-coated wells of plastic culture dishes or the elastic substratum wells of a mechanical cell stimulator (Cell Kinetics Inc., Providence, RI) at a final density of 7,950 cell/mm^2 as described previously (25). The cultures were maintained at 37 °C in a humidified 5% CO_2 incubator in Eagle's basal medium containing 10% horse serum, 5% chicken embryo extract, 50 units/ml penicillin, 2 mM glutamine (85/10/5). At the high plating density used in these studies myofiber formation was initiated within 36 h of plating and well formed myofibers were evident by 72 h. Some cultured cells were embedded in a three-dimensional collagen gel matrix (400 μg of collagen/well) 72 h postplating as described previously (25). To prepare cultures depleted of fibroblasts and enriched for myofibers, the cultures were treated with 10 μM cytosine arabinoside for 24 h at day 3 postplating. After cytosine arabinoside treatment, the cells were rinsed once, and incubated in 85/10/5 medium, or embedded in the collagen gel matrix. Fibroblast enriched cultures were obtained by plating newly isolated avian muscle cells in culture flasks for 50 min at 37 °C. The attached cells, which are mainly fibroblasts, were rinsed twice in 85/10/5 medium, and incubated in this medium for 72 h. The cells were resuspended with 0.025% trypsin for 15 min, collected by centrifugation, resuspended in 85/10/5 medium, filtered through 20–30-μm pore-size Nitex filters to remove residual myofibers, and plated in a culture flask. After reaching confluency, the cells were subcultured a second time by the same protocol, plated on collagen coated 4-well plates at a final density of 530 cells/mm^2, and used for the experiments when confluent. In some experiments, the confluent fibroblast cultures were also embedded in a collagen gel 72 h postplating (24).

Mechanical Stimulation—On day 6 postplating, collagen-embedded and noncollagen-embedded muscle cells were rinsed for 2 h (four 30-min rinses) in basal Eagle's medium containing 50 units/ml penicillin, and 2 mM glutamine. The rinsed cells were incubated in defined serum-free medium consisting of basal medium Eagle's, 50 units/ml penicillin, 2 mM glutamine, 0.835 mg/liter ferrous sulfate, 0.05 mg/liter sodium selenate, and 125 mg/100 ml of bovine serum albumin (muscle maintenance medium: MM medium) as described previously (25). Half of the 36 culture wells were maintained as static controls in the mechanical cell stimulator while the other 18 wells were mechanically stimulated by a pattern of activity which induces skeletal muscle hypertrophy (25) (five 12% substratum stretches and relaxations over a 20-s period followed by a 10-s rest period). This pattern was repeated twice more, followed by a 30-min rest period after the third mechanical stimulus (TRIAL39.PGM, Fig. 1). The cells were mechanically stimulated by stretching the substratum with 2-mm diameter vertically moving prongs centered on the bottom of each well. Cell stretch equals substratum stretch in this model system, as determined by morphometric measurements (25). In experiments involving changes in stretch intensity, cells were mechanically stimulated by TRIAL39.PGM but the percent stretch was varied from 6.7 to 21% by varying prong height in the different wells. In experiments where the frequency of stretch was increased, the skeletal muscle cells were stretched and relaxed 12% by TRIAL39.PGM but with a rest period of 5 min rather than 30 min. All cells grown in plastic culture plates or in the mechanical cell stimulator were kept on a rotary shaker (40 rpm) at 37 °C when mechanically stimulated to eliminate medium stirring differences between control and stretch groups.

Extraction of Insulin-like Growth Factor-1 from Conditioned Medium—Conditioned medium was collected at various times and stored at −80 °C. Insulin-like growth factor-1 was extracted from the medium following the procedure of Brier *et al.* (26). Briefly, the medium was thawed, and incubated for 1 h at 21 °C with an equal volume of 0.5 N HCl to free IGF-1 from its binding proteins. The acidic medium was passed through C18 Sep-Pak columns (prewashed with isopropyl alcohol, methanol, and 4% (v/v) acetic acid), and recycled once. IGF binding proteins were washed through the columns with 4% acetic acid, and IGF-1 was eluted from the columns with absolute methanol. Recovery of IGF-1 with this method was approximately 70% based on the extraction and collection of IGF-1 standards. The IGF-1 containing eluates were dried under nitrogen for approximately 40 min, and stored at −80 °C. Control culture medium incubated at 37 °C for an equal time period but in the absence of cells contained no measurable IGF-1 by this assay technique.

IGF-1 Determination—IGF-1 was determined using a modification of the radioimmunoassay technique of Furlanetto *et al.* (27). Dried samples were reconstituted in RIA buffer (200 mg/liter protamine sulfate, 30 mmol/liter $NaH_2PO_4 \cdot H_2O$, 0.05% (v/v) Tween 20, 0.02% (w/v) sodium azide, and 0.01 M EDTA, pH 7.4). Sample aliquots were incubated 48 h at 4 °C with anti-rabbit IGF-1 primary antibody (1:10,000 dilution). The mixture was incubated overnight at 4 °C with approximately 20,000 cpm of ^{125}I-IGF-1 tracer. IGF-1-primary antibody complexes were precipitated with donkey anti-rabbit antibody for 15 min at room temperature, and collected by centrifugation at 2,000 rpm for 15 min at 4 °C. The supernatant was decanted, and the radioactivity in the pellet measured with a Berthold Multi-Crystal Counter LB2104. This method could reproducibly detect 12 to 1,000 pg of IGF-1 standards.

IGF-1 Neutralizing Antibody Assay—Differentiated myofiber cultures were rinsed and incubated from day 5 to day 7 postplating in MM medium with either 25 or 250 μg/ml anti-IGF-1 antibody. During the last 4–6 h of incubation, protein synthesis rates were measured as outlined below.

IGF-1 mRNA Determination—Total RNA was extracted using the RNAzol B method (CINNA/Biotecx, Houston, TX), and yielded 1–2 μg/10^6 cells, as determined spectrophotometrically. The integrity of the RNA was checked by agarose gel electrophoresis by standard techniques (28). Northern blots for IGF-1 mRNA were performed by separating 10–20 μg of total RNA on 1% agarose gels, transferring the RNA by capillary action to Biotrans membranes (ICN, Costa Mesa, CA), baking for 2 h at 68 °C, prehybridizing at 42 °C for 1 h (5 × Denhardt's, 5 × SSC, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 250 μg/ml salmon sperm DNA, 50% formamide), and hybridizing overnight at 42 °C in prehybidization solution containing 10^6 cpm of ^{32}P-labeled IGF-1 antisense probe. Posthybridization washes were performed according to the ICN Biotrans protocol. The membranes were exposed to Hyperfilm x-ray film (Amersham) for 24 h at −80 °C using 1 intensifying screen. Ribonuclease protection assays for IGF-1 mRNA determination were also performed (28) on 20–40 μg of total RNA, using a commercially available kit (RBA-II, Ambion, Austin, TX). A pPCR2 BlueScript plasmid (gift of P. Rotwein) containing the cDNA sequence for chicken IGF-1 was used to prepare the IGF-1 mRNA probe. The plasmid was linearized at the *Bam*HI site, and ^{32}P-labeled antisense IGF-1 probe prepared with [^{32}P]CTP (Amersham) MAXIscript T3 transcription kit (Ambion). The probe was purified on 5% polyacrylamide, 8 M urea gels. Linearized pTRIPLEscript plasmid (Ambion) containing 1 250-base pair mouse actin gene fragment was utilized as a control template. In all experiments, Torula yeast RNA served as a negative control, while adult mouse liver total RNA, chicken 12-day embryo skeletal muscle, and eye total RNA served as positive controls.

Biochemical Assays—Cells were collected, rinsed twice in phenol red-free Earle's balanced saline solution, and stored at −80 °C. Protein assays were performed on cell sonicate aliquots using the bicinchonic acid protein assay as described previously (25). Protein synthesis was determined using L-[U-^{14}C]phenylalanine incorporation into trichloro-

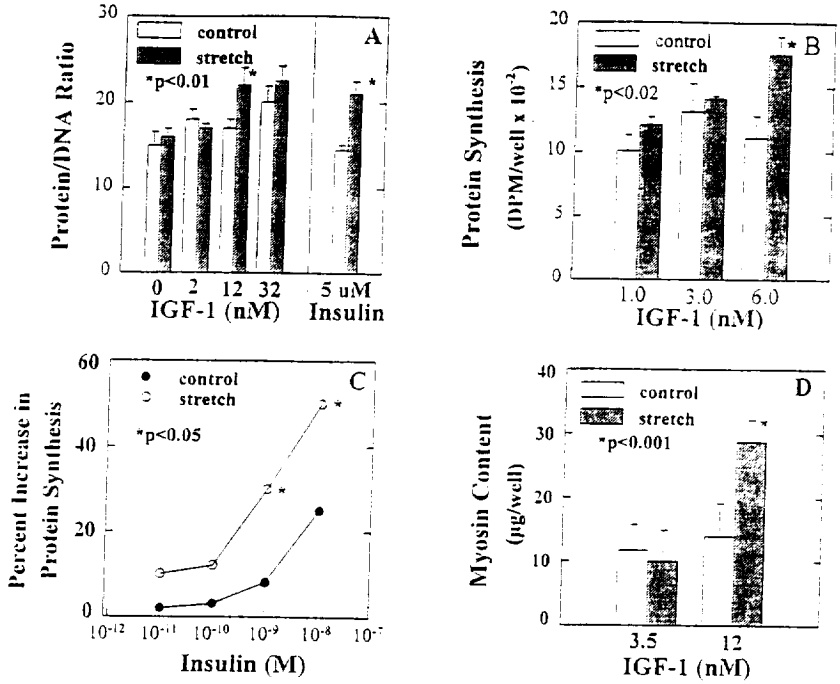

FIG. 2. **Effect of IGF-1 and insulin on protein/DNA ratio (*A*), protein synthesis (*B* and *C*), and myosin content (*D*) in control and mechanically-stimulated skeletal muscle cells.** Cultures in *A* and *D* were mechanically stimulated for 48 h, while those in *B* and *C* were for 2 h, in serum-free MM medium containing the level of exogenous IGF-1 or insulin indicated on the *x* axis. Values are expressed as the mean ± S.E. of 4–6 samples and compared by unpaired *t* test.

acetic acid-insoluble material during a 4–6-h incubation period as described previously by Vandenburgh *et al.* (29). Incorporation is linear during this time period and excess nonradioactive phenylalanine was included in the medium (0.5 mM) to allow rapid equilibration of the intracellular and extracellular amino acid pools (30). DNA was measured fluorometrically by the modified method of Labarca and Paigen (31).

Gel Electrophoresis and Ligand Blotting—IGF binding proteins in the conditioned medium were examined using gel electrophoresis and ligand blotting (23, 32, 33). Four parts of conditioned medium were mixed with one part nonreducing sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 12.5% (w/v) sodium dodecyl sulfate, and 0.05% (w/v) bromphenol blue), boiled for 5 min, and cooled to 21 °C. The proteins in the conditioned medium were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis prepared according to Laemmli (32). The proteins were transferred to nitrocellulose paper by Western blotting (33), and identified by the ^{125}I-IGF-1 ligand blotting technique (23). Briefly, the nitrocellulose membrane was air-dried at room temperature, soaked for 30 min (4 °C) in saline (0.15 M NaCl, 0.5 mg/ml sodium azide, 0.01 M Tris-HCl, pH 7.4) with 3% (v/v) Nonidet P-40, 2 h (4 °C) in saline with 1% bovine serum albumin, and 10 min (4 °C) in saline with 0.1% (v/v) Tween 20. The washed membranes were incubated overnight (4 °C) in saline, 1% (w/v) powdered milk, 0.1% Tween 20, and 500,000 cpm of ^{125}I-IGF-1. After extensive rinsing in saline, the membrane was air-dried, and exposed to x-ray film for varying times at −80 °C with two intensifying screens. The amounts of labeled binding proteins were determined quantitatively by densitometric scanning of preflashed x-ray autoradiographs using a computerized image analysis system (JAVA and PEAK FIT, Jandel Scientific, Corte Madera, CA).

Statistical analyses of the data were performed by *t* tests for unpaired values using a statistical software program (SIGMASTAT, Jandel Scientific).

RESULTS

Stretch Responses of Skeletal Muscle Cells to IGF-1 and Insulin—The relationship between mechanical stimulation, cell growth, and IGF-1 was first examined by performing protein synthesis-IGF-1 dose-response studies on collagen-embedded static control and mechanically stimulated skeletal muscle cultures. As previously reported for control muscle cell cultures (4), nanomolar concentrations of IGF-1 did not stimulate protein synthesis or cell growth (Fig. 2*A*). In contrast, at concentrations which were ineffective in static cultures, IGF-1 stimulated cell growth (Fig. 2*A*) and protein synthesis (Fig. 2*B*) in mechanically-stimulated cells. In addition, myosin heavy chain content was also increased in mechanically-stimulated cells by doses of IGF-1 (12 nM) that were ineffective in control static cultures (3.5 nM *versus* 12 nM, Fig. 2*D*). Insulin, at a concentration of 5 μM, was inactive in stimulating cell growth in static cultures, but the same concentration caused a significant increase in protein/DNA ratios in mechanically-stimulated cells (Fig. 2*A*). The effect of insulin on protein synthesis in the muscle cell cultures was also enhanced significantly by stretch (Fig. 2*C*). Insulin was active only at pharmacological doses in stimulating muscle cell growth since most of its growth-stimulatory effects are via the IGF-1 receptor, for which it has a low affinity (4). Similar results were obtained in three separate experiments. These data indicate that mechanical stimulation increases the sensitivity of skeletal muscle cells to exogenously added IGF-1 and insulin.

Effect of Collagen on the Autocrine Secretion of IGF-1 from Differentiated Skeletal Muscle Cells—One mechanism by which stretch could increase the cell's growth response to exogenously added IGF-1 would be by supplementing this with endogenously produced IGF-1. Insulin-like growth factors have been reported in conditioned medium from mammalian skeletal muscle cell lines but not primary avian muscle. Therefore, the endogenous secretion of IGF-1 from differentiated avian skeletal muscle cells was examined. The influence of embedding the muscle cells in a three-dimensional collagen gel matrix on IGF-1 efflux was measured first since the muscle cells withstand long-term repetitive stretch better when supported by an extracellular matrix (24). Collagen-embedded day 6 muscle cultures grown in plastic culture dishes were found to release 5.1 ± 0.9 pg of IGF-1/μg of protein from 0 to 24 h and 3.4 ± 0.6 pg of IGF-1/μg of protein from 24 to 48 h, which was 3–11 times greater than IGF-1 efflux from noncollagen-embedded cells (Fig. 3). The level of IGF-1 release varied significantly between different cell preparations, from 3 to 34 pg/μg of protein/24 h. The reason for this wide fluctuation in IGF-1 release from primary cell cultures is not known but it has been also found for other growth factors released from these cells (30). Each experiment was therefore repeated with at least two different cell preparations.

Within the same cell preparation, IGF-1 release was always greater when the muscle cells were grown on the elastic membranes of the mechanical cell stimulator compared to plastic

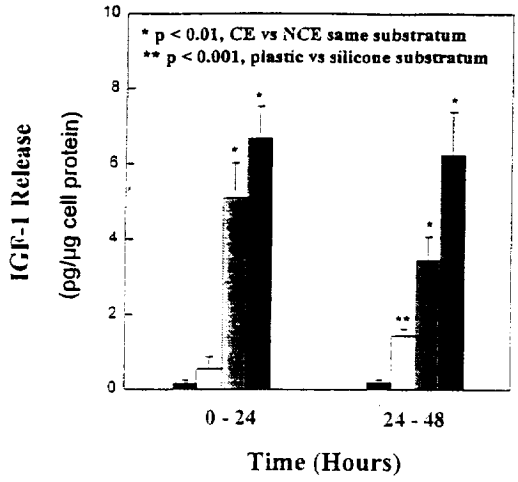

FIG. 3. **Effect of collagen on IGF-1 release from skeletal muscle cells grown on plastic tissue culture dishes, or on silicone rubber membranes.** On day 3 postplating, cultured muscle cells were fed either fresh 85/10/5 medium or embedded in a collagen gel matrix. On day 6 the cells were rinsed and incubated in defined-serum free medium. Conditioned media were collected for the 0–24 and 24–48 h time periods, and analyzed for IGF-1. The values represent the mean ± S.E. of five to eight samples, and are compared using the unpaired *t* test. *NCE*, noncollagen embedded; *CE*, collagen embedded. ■, NCE, plastic; □, NCE, silicone; ▨, CE, plastic; ■, CE, silicone.

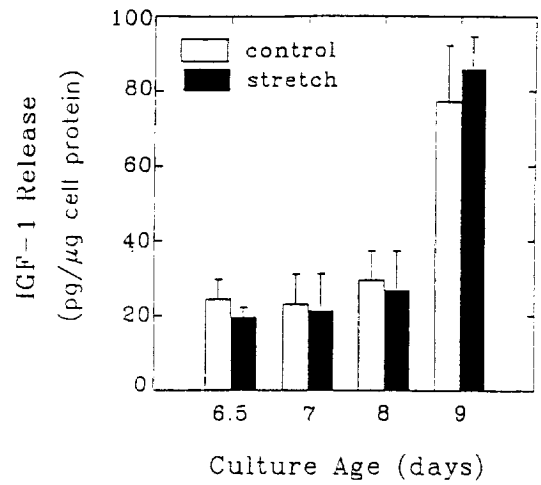

FIG. 4. **Effect of stretch on IGF-1 release from skeletal muscle cells.** Six-day-old collagen-embedded cells were incubated in defined MM medium, and stimulated mechanically every 30 min as outlined under "Experimental Procedures." Conditioned medium was collected at 0.5, 1, 2, and 3 days after initiating stretch, and IGF-1 released into the medium was measured by radioimmunoassay. Results are expressed as the mean ± S.E. of three to six values per group and compared by *t* test for unpaired values ($p > 0.05$ for all control *versus* stretch groups).

culture dishes. Thus, noncollagen-embedded skeletal muscle cells grown on elastic membranes released 2.6-fold more IGF-1 after 24 h, and 7.8-fold more after 48 h, compared to cells on rigid plastic dishes (Fig. 3). When embedded in a collagen matrix, the muscle cells growing on the elastic membranes released 1.3- and 1.8-fold more IGF-1/μg of protein after 24 and 48 h of incubation in defined medium, respectively, compared to those grown on plastic culture dishes (Fig. 3). In subsequent experiments, controls were therefore always run with the same cell preparation growing on identical substrata.

To ascertain whether the increased IGF-1 found in conditioned medium from collagen-embedded cells was trapped within the collagen gels from prior incubation with serum and chicken embryo extract containing medium, collagen gels were prepared in 4-well plates with 85/10/5 medium, but without cells, and treated the same way as the muscle cell cultures. After rinsing the gels by the normal protocol, they were incubated in serum-free medium for a 24-h period, and conditioned medium collected for IGF-1 analysis. The collagen gels without cells released an average of 381 ± 42 pg of IGF-1/well/24 h, compared to 1,790 ± 270 pg of IGF-1/well/24 h observed in conditioned medium from collagen-embedded cells grown in plastic culture plates. To further examine this question, the amount of IGF-1 trapped from 85/10/5 medium in collagen gels in the presence of skeletal muscle cells was determined by preparing the collagen gels with medium containing tracer levels of ^{125}I-IGF-1. Fresh medium containing tracer levels of ^{125}I-IGF-1 was added to the cultures every 24 h. The 6–7-day-old cultures were then rinsed by the normal protocol, and the release of radioactivity measured over a 24-h period. The rinsed muscle cells embedded in the collagen matrix released 6.88% of the total initial medium radioactivity over a 24-h period. This equaled 42 pg of IGF-1/well trapped by the collagen gels, 10–15-fold less than the IGF-1 released from collagen-embedded cells into the medium during this time period. The ^{125}I-IGF-1 measured in homogenates of the collagen-embedded cells from these experiments was 1.9% of the total radioactivity in the original 85/10/5 medium. These results indicate that only a small percent of the IGF-1 released into the conditioned medium resulted from IGF-1 trapped from serum and embryo extract containing medium.

Effect of Mechanical Stimulation on IGF-1 Release from Cultured Skeletal Muscle Cells—To assess the effect of stretch on IGF-1 release, 6-day-old collagen-embedded cultures of differentiated skeletal muscle cells grown on silicone rubber membranes were mechanically stimulated 12% every 30 min from day 6 to day 9 postplating as outlined under "Experimental Procedures." The efflux of IGF-1 into the medium over a 12–24 h period was approximately 20–80 pg/μg of protein, and there was no significant difference between control and stretched cultures (Fig. 4). No responses to mechanical stimulation were observed when muscle cells were stretched for up to days 10 and 11 postplating (data not shown).

The effect of different patterns of mechanical stimulation on IGF-1 efflux from the collagen-embedded muscle cells was examined next. The cells were mechanically stimulated 6.7–21% every 30 min for 24 h with the same frequency as in TRIAL39.PGM. No significant differences in IGF-1 efflux were observed among the different stretch intensity groups (Fig. 5*A*). Similarly, a 6-fold increase in the frequency of mechanical stimulation (5-min rest periods, TRIAL52.PGM) showed no effect on the release of IGF-1 from the muscle cells (Fig. 5*B*).

To examine the time course of IGF-1 efflux with stretch, day 6 noncollagen-embedded cells were mechanically stimulated using the TRIAL39.PGM activity pattern, and conditioned medium was collected at 1, 2, 4, 8, 12, and 24 h of stretch, with fresh medium added to the cultures at each time point. Noncollagen-embedded cultures were used in these kinetic studies to eliminate the collagen as a potential diffusion barrier. While total accumulated release of IGF-1 over the 24-h incubation period (*i.e.* addition of released IGF-1 at all the time points) was not significantly different in these noncollagen-embedded cultures (control static cultures: 18.7 pg of IGF-1/μg of protein/24 h; stretched cultures: 16.4 pg of IGF-1/μg of protein/24 h), as found for the collagen-embedded culture experiments described above, the kinetics of IGF-1 release was significantly different between control and stretched cells. IGF-1 release from static control cells increased rapidly during the first 4 h and then increased at a slower rate over the remaining 20-h period (Fig. 6). IGF-1 release from stretched cells was signifi-

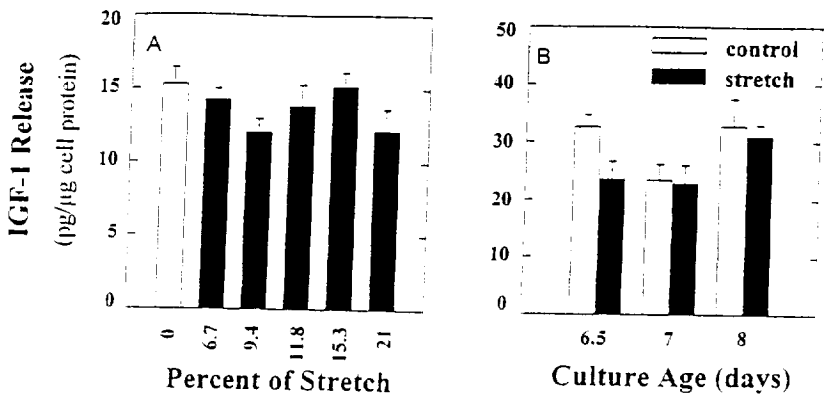

FIG. 5. **Effect of stretch intensity and frequency on IGF-1 efflux.** Collagen-embedded skeletal muscle cells were switched to defined MM medium from day 6 to day 8 postplating. Cultures in *A* were mechanically stimulated for 24 h by the same frequency pattern as outlined in Fig. 1, but with varied percent intensities of stretch. This experiment was performed with the same cell preparation by varying prong heights between wells as described under "Experimental Procedures." Cultures in *B* were mechanically stimulated every 5 min instead of every 30 min by the same pattern of activity as outlined in Fig. 1. Results are expressed as the mean ± S.E. of six values per group and compared by *t* test for unpaired values.

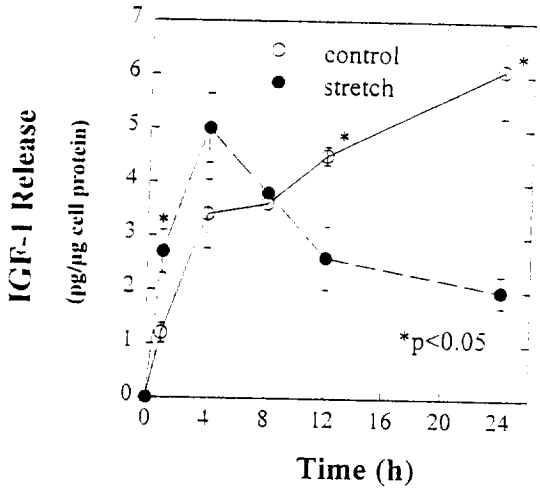

FIG. 6. **Time course of IGF-1 efflux from noncollagen-embedded skeletal muscle cells.** The cells were mechanically stimulated by TRIAL39.PGM. The media was removed at each time point, and fresh defined MM media added. IGF-1 content was assayed in each sample as outlined under "Experimental Procedures." Results are expressed as the mean ± S.E. of 2–3 values and compared by *t* test for unpaired values.

cantly increased during the first hour of stretch compared to static controls, reaching a maximum at 4 h of mechanical stimulation (Fig. 6). IGF-1 efflux then declined in these cultures even though mechanical stimulation continued. This pattern of IGF-1 release was observed in three different experiments involving noncollagen-embedded muscle cells, and in four different experiments using collagen-embedded cells (data not shown).

Effect of Mechanical Stimulation on IGF-1 mRNA Levels in Cultured Skeletal Muscle Cells—Work induced hypertrophy has been reported to increase IGF-1 mRNA levels in skeletal muscle; therefore, we attempted to determine if mechanical stretch has the same effect on cultured skeletal muscle cells. Total RNA was isolated from control and mechanically-stimulated cells, and analyzed for IGF-1 mRNA by both Northern blotting and nuclease protection assays using an antisense probe for avian IGF-1 mRNA. Using either technique, no IGF-1 mRNA could be detected in total RNA isolated from either control or stretched muscle cultures (data not shown). The same results were obtained from noncollagen-embedded and collagen-embedded cells in four separate experiments (data not shown). Thus, IGF-1 mRNA levels were too low to be measured in the cultured muscle cells by this assay technique. *In vivo* avian skeletal muscle also contains extremely low levels of IGF-1 mRNA.[2]

Comparison of IGF-1 Secretion from Myofibers and Fibroblasts—Because avian skeletal muscle cultures contain both myofibers and fibroblasts, we determined which cell type contributes to the IGF-1 released into the medium, and which cell type was stimulated to release IGF-1 when collagen-embedded. Noncollagen and collagen-embedded mixed cultures containing both cell types, myofiber-enriched cultures, and fibroblast only cultures were prepared as outlined under "Experimental Procedures." At day 6 postplating the cells were rinsed, and incubated for 24–48 h in defined serum-free medium. Both myofiber-enriched cultures and confluent fibroblast cultures released IGF-1 under both noncollagen- and collagen-embedded conditions (Fig. 7). Noncollagen-embedded cells released lower amounts of IGF-1 than collagen-embedded cells in both cell types (Fig. 7, *A versus B*). On a per unit of microgram of cellular protein basis, collagen-embedded fibroblast cultures produced 1.7–2.4 times more IGF-1 than collagen-embedded myofibers at 24 and 48 h of incubation in defined medium. Interestingly, on a per unit protein basis, mixed cultures effluxed less IGF-1 than either of the two cell types alone.

Autocrine/Paracrine Effect of IGF-1 Released from Differentiated Skeletal Muscle Cells—Insulin-like growth factors can modulate anabolic processes in a number of cells including those from which they originate (12). Therefore we examined the effect of locally released IGF-1 on the differentiated skeletal muscle cells. Noncollagen-embedded and collagen-embedded skeletal muscle cells were preincubated in serum-free medium in the presence or absence of anti-IGF-1 antibody for 48 h, and L-[U-^{14}C]phenylalanine incorporation into cellular proteins followed over a 4-h incubation period. Compared to control cells, protein synthesis was decreased 52 and 29% in the antibody-treated noncollagen-embedded and collagen-embedded cells, respectively (Fig. 8).

IGF Binding Protein Secretion from Cultured Skeletal Muscle Cells—The physiological responses of insulin-like growth factors are modulated by IGF binding proteins, and their secretion might be altered by collagen embedding or mechanical stimulation. The release of IGF binding proteins from the differentiated avian skeletal muscle cultures was therefore examined. Gel electrophoresis and ligand blotting of conditioned medium from the skeletal muscle cultures revealed the presence of three IGF binding proteins of molecular masses 31, 36, and 43 kilodaltons (kDa) (Fig. 9). The 36-kDa band was the predominant secreted binding protein from the avian cells. The effects of collagen and stretch on the efflux of these binding proteins was studied over a 24-h period. Compared to noncollagen-embedded static muscle cells, cells embedded in a colla-

[2] P. Rotwein, personal communication.

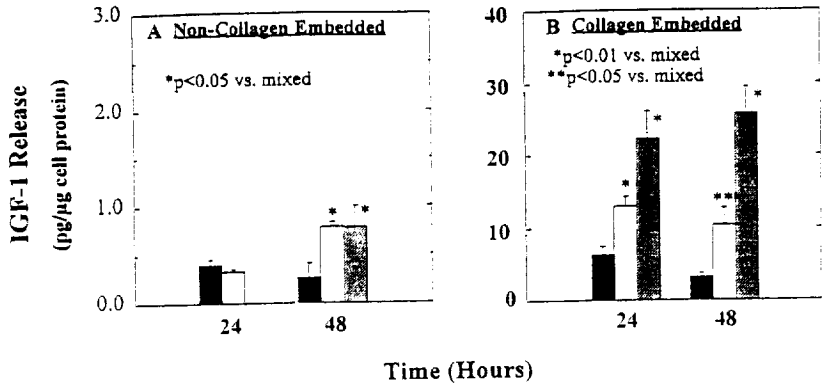

FIG. 7. **Collagen-induced efflux of IGF-1 from skeletal muscle mixed cultures, skeletal myofiber-enriched, and fibroblast-enriched cultures.** Myofiber-enriched cultures and fibroblast only cultures were prepared as described under "Experimental Procedures." Six-day-old cultures were incubated in defined MM medium for 24–48 h and IGF-1 efflux measured from noncollagen-embedded (*A*) and collagen-embedded (*B*) cells. Results are expressed as the mean ± S.E. of four values and compared by unpaired *t* test. ■, mixed cultures; □, myofiber-enriched cultures; ▨, fibroblast-enriched cultures.

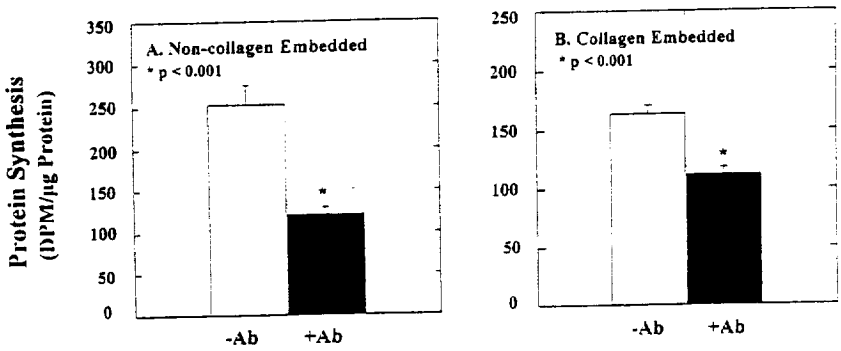

FIG. 8. **Effect of anti-IGF-1 antibody on protein synthesis in noncollagen-embedded and collagen-embedded skeletal muscle cells.** Five-day-old noncollagen-embedded and collagen-embeded skeletal muscle cells were rinsed and preincubated for 48 h in MM medium containing 25 and 250 μg of anti-IGF-1 rabbit antibody, respectively. Control cells were preincubated in MM medium without the antibody for 48 h. Protein synthesis was assayed over a 4–6-h time period, with or without the antibody. Values are expressed as the mean ± S.E. of 8 values and compared by unpaired *t* test.

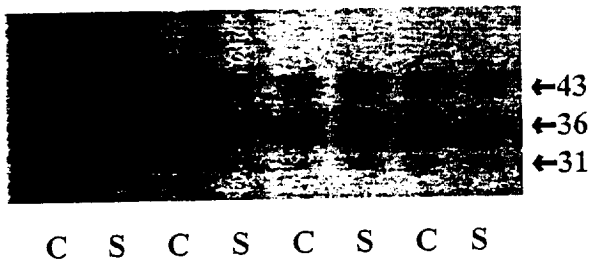

FIG. 9. **Detection of IGF binding proteins released from skeletal muscle cell cultures.** Conditioned medium was analyzed for IGF binding proteins using ligand blots as described under "Experimental Procedures." The autoradiography shows three binding proteins of molecular masses 31, 36, and 43 kDa. No significant differences in IGF binding protein levels were detected between control (*C*) and stretched (*S*) cultures.

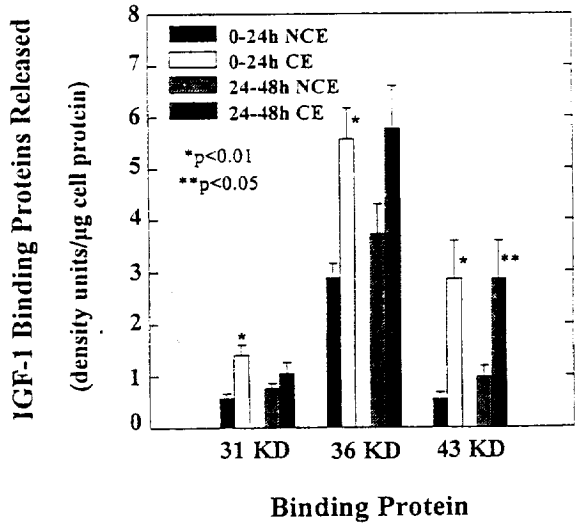

FIG. 10. **IGF binding proteins released from noncollagen-embedded and collagen-embedded skeletal muscle cells.** Differentiated muscle cells were grown either with or without collagen embedding as described under "Experimental Procedures." On day 6 postplating, the cells were rinsed for 2 h and incubated in defined medium for 0–24, and 24–48 h. Conditioned medium was analyzed for binding protein levels by ligand blotting and quantitative densitometric analysis as outlined under "Experimental Procedures." Values (arbitrary density units/μg of cell protein) are expressed as the mean ± S.E. of six samples per group and compared by unpaired *t* test. *NCE*, noncollagen embedded; *CE*, collagen embedded. All statistical analyses were done comparing NCE *versus* CE in the different groups.

gen gel matrix showed a 2.4-, 1.9-, and 5.2-fold increase in the release of the 31-, 36-, and 43-kDa binding proteins, respectively (Fig. 10). An increased release of IGF binding proteins was also observed after 48 h of incubation (Fig. 10). While the efflux of IGF binding proteins was modulated by collagen, mechanical stimulation of collagen-embedded muscle cells by TRIAL39.PGM for either 24 or 48 h had no effect on the release of these proteins (data not shown). To further examine the time course release of IGF binding proteins, skeletal muscle cells were stretched by TRIAL39.PGM, and conditioned medium was collected at 30 min, 1, 4, 8, and 12 h. The release of all three IGF binding proteins increased with time, but no stretch effect on the kinetics of binding protein release was observed (Fig. 11).

DISCUSSION

This is the first report assessing the efflux of IGF-1 from differentiated primary avian skeletal muscle cells in tissue culture. This study revealed that primary cultures of well-differentiated skeletal myofibers release IGF-1 in significant amounts. Autocrine secretion of IGF-1 has been hypothesized to be involved in work induced skeletal muscle growth *in vivo* (16), and we tested this hypothesis with an *in vitro* model of stretch-induced skeletal muscle growth. Mechanical stretch influenced the sensitivity of skeletal muscle cells to exogenously added IGF-1, and increased the acute but not long-term release of IGF-1 from these cells. On a nanomolar basis, the acute release of IGF-1 with stretch was found to be 20–40-fold less than the amount of recombinant IGF-1 required to stimulate muscle growth in mechanically stimulated cultures

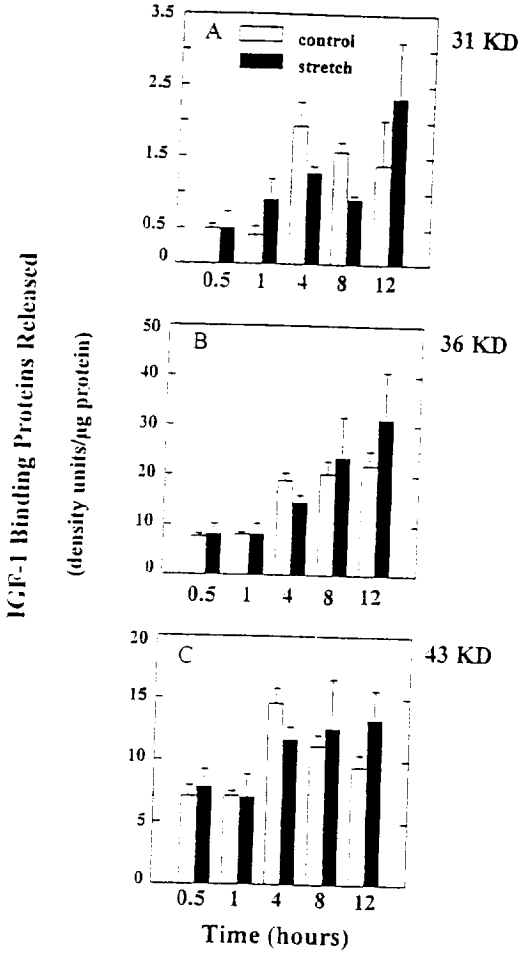

FIG. 11. **Time course of stretch-induced release of IGF binding proteins.** Differentiated skeletal muscle cells were embedded in a collagen gel on day 3 postplating, and mechanically stimulated by TRIAL39.PGM starting on day 6. Conditioned medium was collected at 0.5, 1, 4, 8, and 12 h of stretch, and analyzed for binding proteins by ligand blotting as described under "Experimental Procedures." Values are the mean ± S.E. of 4 samples per group and compared by unpaired *t* test.

in vitro. If the stretch-induced autocrine production of IGF-1 is involved in stretch-induced muscle growth, it must be either more biologically active or more accessible to the IGF-1 receptor than exogenously added recombinant human IGF-1.

The acute secretion of IGF-1 from cultured skeletal muscle cells in response to mechanical stimulation is very similar to the acute, but not long-term, stretch-induced release of atrial natriuretic peptide from cardiac cells (34). It may result from the release of already synthesized IGF-1, rather than newly synthesized IGF-1. Immunocytochemical studies demonstrate that the cytoplasm of myoblasts and newly formed myotubes contains increased IGF-1 levels during muscle regeneration *in vivo* (13, 14, 35). In the present study, skeletal muscle cells were utilized 3 or 4 days after myofiber formation *in vitro*, and it is possible that these cells also contain intracellular IGF-1 stores. During the first hour of mechanical stimulation *in vitro*, differentiated skeletal muscle cells appear to be partially damaged, based on temporary creatine kinase release and protease activation in the stretched skeletal muscle cells (25). The partial damage to the muscle cells by stretch could result in the release of the intracellular IGF-1, as part of a repair process.

Differentiated avian pectoralis muscle cells were found to secrete not only IGF-1 but also IGF binding proteins of molecular masses 31, 36, and 43 kDa. This is the first report on the secretion of IGF binding proteins from differentiated avian skeletal muscle cells. A number of studies have shown the presence of IGF binding proteins in human and chicken serum (18, 33), and human amniotic fluid (21), as well as in conditioned medium of tissue cultured liver cells (18), and mammalian muscle cell lines (1, 22). The C_2 muscle cell line secretes a single IGF binding protein of 29 kDa (22), while the C_2C_{12} cell line releases three binding proteins of molecular masses 24, 30, and 32 kDa (1). The three IGF binding proteins released from the primary avian skeletal muscle cells are similar in molecular mass to the binding proteins found in avian serum *in vivo* (28, 33, and 41 kDa) (33). Mechanical stimulation of the skeletal muscle cells had no significant effect on the efflux rate of IGF binding proteins at any of the time periods studied.

A second significant finding in this study was the increased release of IGF-1 and IGF binding proteins from skeletal muscle cells after embedding them in a three-dimensional type I collagen matrix. Collagen-embedded cells released 3–11 times more IGF-1 than noncollagen-embedded cells. There is evidence that IGF-1 stimulates collagen synthesis (36) but there appear to be no studies on the effect of collagen on IGF-1 release. Embedding the myofibers in a collagen gel matrix stimulates their hypertrophy (24, 37), possibly by activating IGF-1 synthesis and secretion as a paracrine/autocrine growth factor. The mechanism by which collagen enhances IGF-1 release from avian pectoralis muscle cells is not known. In differentiating hepatocytes, collagen promotes the activity of transcription factors resulting in the increased transcription of serum protein genes, such as albumin (38, 39). Collagen may interact with cell surface receptors resulting in increased transcription of the IGF-1 gene. Because collagen type I recognizes and binds to integrins (40–42), the effects of collagen on IGF-1 expression may be modulated via these receptors.

In addition to the differences in IGF-1 efflux from noncollagen-embedded and collagen-embedded cells, skeletal muscle cells grown on a silicone rubber substratum consistently released greater amounts of IGF-1 into the conditioned medium than when grown on plastic culture plates. These results indicate the importance of running proper controls of cells growing on identical substratum. The elastic substratum may have greater permeability than polystyrene plastic to gases such as oxygen and carbon dioxide, resulting in increased cellular activities and leading to elevated levels of IGF-1 production. Skeletal muscle hypoxia not only reduces muscle mass but also reduces oxidative metabolism in the muscle tissue (43).

The tissue cultures utilized in these experiments consisted of two main cell types, myofibers and fibroblasts. Lowe *et al.* (44) reported that fibroblasts are capable of synthesizing IGF-1 *in vivo*. Our experiments using enriched myofiber or confluent fibroblast cultures showed that both cell types are capable of releasing IGF-1. Whereas in mouse primary skeletal muscle cultures the muscle cells produce greater amounts of IGF-1 than fibroblasts (11), the avian fibroblasts released greater amounts of IGF-1 than the enriched myofiber cultures on a microgram cellular protein basis. But, since 80–90% of the cellular protein in the mixed avian muscle cultures utilized in this study arises from skeletal myofibers (30), the production of IGF-1 by the myofibers in these cultures on a microgram cell protein basis constitutes the major part of total IGF-1 release. It is difficult, however, to determine the exact contribution of each cell type in the mixed cultures since the two cell types appear to interact in regulating total IGF-1 efflux in a complex manner when co-cultured (Fig. 7), as found previously for the regulation of total protein degradation in the two cell types (45). IGF-1 secretion in mixed cultures was less than in either cell type alone, indicating some form of feedback inhibition

when the two cell types are cultured together.

IGF-1 secreted from cultured skeletal muscle cells can be considered an important autocrine factor. Our experiments showed that protein synthesis rates are significantly reduced in the muscle cells when incubated in the presence of anti-IGF-1 antibody. Similarly, [^{3}H]thymidine uptake in fetal rat myoblasts was blocked when these cells were incubated with a monoclonal antibody against human somatomedin (12). Locally produced IGF-1 therefore plays an important role in the maintenance of tissue-cultured skeletal muscle cells due to its effects on anabolic processes.

In summary, this paper shows that IGF-1 and IGF binding proteins are released from differentiated avian pectoralis muscle cell cultures, and that the long-term *in vitro* release of these proteins from the muscle cells is not significantly stimulated by stretch. Stretch-induced myofiber hypertrophy in cultured skeletal muscle cells may involve the short-term increase in IGF-1 secretion, changes in IGF-1 receptors, or a non-IGF-1-related mechanism. In addition, significant collagen-induced IGF-1 and IGF binding protein release from the differentiated muscle cells occurs *in vitro*. Further studies are needed to examine the mechanisms leading to collagen-induced IGF-1 and IGF binding protein synthesis and/or release from skeletal muscle cells.

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