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MICROTITER MICROMASS CULTURES OF LIMB-BUD MESENCHYMAL CELLS

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SUMMARY

A method is described for growing high-density micromass cultures of chick and mouse limb mesenchyme cells in 96-well microtiter plates (μTμM cultures). Rapid quantitative estimates of chondrogenic expression were obtained by automated spectrophotometric analysis of Alcian-blue-stained cartilage matrix extracts performed in the wells in which the cells had been grown. Quantitative estimates of myogenic expression were obtained similarly using anti-sarcomere myosin monoclonal antibody and modified ELISA techniques. This μTμM-ELISA method may be adapted for use with other antigens for which specific antibodies are available. These methods were used to compare cartilage and muscle differentiation in 1 to 4 d μTμM cultures grown in serum-containing (SCM) and defined (DM) media. The DM contains minimal additives (insulin, hydrocortisone, and in some cases, ascorbate or transferrin) and supports both chondrogenesis and myogenesis. The colorimetric analyses agree well with the morphologic appraisal of chondrogenesis and myogenesis. Similar numbers of cartilage nodules formed in all cultures, but in DM the nodules failed to enlarge; explaining the reduced matrix synthesis in DM as compared with SCM, and suggesting that nodule enlargement is a discrete, serum-dependent step. Studies of selected additives to DM show that transferrin enhances myogenesis, ascorbic acid enhances chondrogenesis, and retinoic acid inhibits chondrogenesis. Together, the μTμM system, in situ colorimetric assays of chondrogenesis and myogenesis, and DM will allow rapid prescreening of teratogens and screening of various bioactive compounds (e.g., hormones, growth factors, vitamins, adhesion factors) for effects on limb mesenchymal cell differentiation.

Key words: limb-bud mesenchyme; microtiter plates; chondrogenesis; myogenesis; serum-free medium; ELISA.

INTRODUCTION

Limb-bud mesenchymal cells, when grown in high-density cultures, can differentiate into a number of cell types, including cartilage and muscle, and have been used extensively for studies of in vitro chondrogenesis (26). Formerly, large numbers of these cells were used to achieve the high cell densities required for chondrogenesis (6,23,32). Development of the micromass culture technique (1) provided a way to obtain similar results with a fraction of the number of cells previously needed per culture. Largely due to its scaled-down size, the micromass culture system is now widely used in studies of in vitro limb chondrogenesis, and has been adapted as a means for prescreening potential teratogens (9,13,34) and for studies of the differentiation of other mesenchymal cell populations (e.g., 33).

The findings reported here extend the usefulness of the micromass culture model by adapting it to 96-well microtiter plates. This facilitates the handling of large numbers of cultures and makes more economical use of plasticware, media, and reagents. Further, the microtiter micromass (μTμM) system facilitates automation of the seeding, feeding, and biochemical analysis of the cultures.

Other enhancements described include the development of a basal serum-free medium that contains a minimum of additives and supports both chondrogenesis and myogenesis, and an adaptation of standard ELISA methods that can be used to quantitate a variety of differentiation-related antigens. Together, the methods described allow rapid quantitation of both chondrogenic and myogenic differentiation in the same vessel in which the cells are cultured.

MATERIALS AND METHODS

Media. The serum-containing medium (SCM) used in this study consisted of a stock of 60% Ham's F12 (GIBCO, Grand Island, NY) and 40% Dulbecco's modified Eagle's medium (high glucose, GIBCO), hereafter referred to as F12/DMEM, supplemented with 10% fetal bovine serum and antibiotics. The defined medium (DM) was modified from a formulation described previously (16,30) and was composed of F12/DMEM supplemented with 5 μg/ml insulin (Collaborative Research, Waltham, MA), 100 n*M* hydrocortisone (Sigma, St. Louis, MO), and antibiotics. For some cultures, the DM was further supplemented with 5 μg/ml chicken transferrin (Conalbumin; Sigma) (DM+T), or 50 μg/ml L-ascorbic acid (Sigma) (DM+C). The defined

medium for mouse embryo limb-bud mesenchymal cells (DM/M) was composed of CMRL 1066 medium (GIBCO) (21) supplemented with 5 μg/ml insulin and 100 n*M* hydrocortisone.

Isolation of limb mesenchymal cells. Cell suspensions were prepared from the wing buds of stage (12) 23-24 White Leghorn chick embryos or from the limbs of Day 10 1/2 mouse embryos. The limbs were excised from the embryos in a dish containing Puck's saline G (PSG) and dissociated in trypsin-collagenase (0.1% trypsin and 0.1% collagenase [Worthington, Freehold, NJ] in calcium- and magnesium-free PSG containing 10% chicken serum) by rotating at 70 rpm in a 37° C water bath. After 10 min, the limbs were pipetted up and down with a Pasteur pipette to obtain a cell suspension. An equal volume of SCM was added to inactivate the enzymes and the cells were centrifuged, washed, and resuspended in the appropriate medium before being passed through four layers of Nitex 20 nylon mesh (Tetko).

Inoculation of the cultures. Cell density was determined using a hemacytometer and adjusted to 2.33×10^6 cells/ml. One hundred fifty-microliter aliquots of this suspension (3.5×10^5 cells) were inoculated into each well of a 96-well, tissue-culture treated, flat-bottomed microtiter plate (Corning, Costar) to achieve a confluent monolayer on the culture surface. Earlier attempts at spotting 10-μl drops of the standard 2×10^7 cells/ml suspension (1) usually resulted in the droplet contacting the well wall, droplet collapse, and nonuniform cell distribution. Five-microliter drops were more successful, but sometimes gave the same result and were difficult to place consistently in the exact center of the well; a situation that would have complicated the spectrophotometric analyses described below. The 150-μl size of the aliquot was derived from repeated trials in which larger and smaller volumes resulted in uneven cell distribution. Comparison of DM cultures, in which the cells were inoculated directly in DM vs. those in which they were first inoculated in SCM, allowed to attach for an hour at 37° C, then washed and covered with DM, revealed no significant differences in either the total DNA/well at 8 or 24 h, or in the amount of Alcian-blue-stained matrix/well at 4 d. It was concluded that including serum in the inoculum was not required for cell attachment. Thus, the 150-μl inoculum eliminates a) concerns about dehydration during the attachment period, b) the need to return and flood the well with additional culture medium after the attachment period, and c) the need for careful spotting of droplets into the wells, decreasing the time required for inoculation and making possible automation of the inoculation procedure when large numbers of cultures are needed.

After inoculation, the plates were maintained in a 37° C water-jacketed incubator in a humidified atmosphere of 5% CO_2:95% air. The first medium change was made at 5 to 9 h after inoculation at which time 250 μl of fresh medium was added to each well. Subsequent medium changes were made at 12-h intervals. Medium changes at longer (24 h) intervals reduced the amount of Alcian-blue-stained matrix produced by cells cultured in SCM, but not by cells in DM. It may be possible in the future to decrease the frequency of medium changes by developing a medium enriched for certain nutrients or growth factors.

Treatment with retinoic acid. A stock solution (0.5 mg/ml) of all-trans retinoic acid (RA, Sigma) in 100% ethanol was prepared and stored in the dark at −20° C. This stock was diluted with DM to prepare a series of secondary stocks at various RA concentrations from which 100-μl aliquots could be added to the appropriate wells to give the desired final concentrations (100, 75, 50, and 25 ng/ml). Control cultures received ethanol equal to the highest concentration (0.015%) in the experimental cultures. Fresh RA was added at every medium change. It is worth noting that RA stock solutions reach saturation at concentrations approaching 2 mg RA/ml 100% ethanol and 1 mg/ml 95% ethanol.

Histologic methods. After the appropriate culture periods, the cultures were fixed with fixatives previously determined to provide optimal results for the subsequent analytic methods. Cultures destined for morphologic analysis were fixed in Kahle's fixative for 15 min, then washed with distilled water, stained overnight for cartilage with 0.5% Alcian blue in 0.1 *N* HCl (17), washed with 0.1 *N* HCl, and covered with 70% ethanol. When the plates were carefully sealed with Parafilm and stored at 4° C to reduce evaporation, storage for up to 7 d had no effect on subsequent staining for myoblasts. Staining of the myogenic cells in these cultures was accomplished as described previously (29). Briefly, the cultures were rehydrated and washed several times with 0.02 *M* phosphate buffered saline (PBS), covered with 0.3% H_2O_2 in methanol to inactivate endogenous peroxidase, and washed again (and after each subsequent step) with PBS. Each well was filled with 5% normal rabbit serum (NRS) in PBS for 10 min to reduce nonspecific binding of the secondary antibody, and the plates were incubated with hybridoma culture supernatant containing the antisarcomere myosin primary antibody, MF-20 (2) for 45 min, rabbit-anti-mouse IgG (secondary antibody) for 45 min, 40 μg/ml mouse peroxidase-antiperoxidase in PBS containing 1% NRS for 45 min. Diaminobenzidine (DAB) substrate (5 mg DAB + 2 μl 30% H_2O_2 in 10 ml PBS) was then added, and color development was monitored microscopically for 4 min before stopping the reaction by rinsing with distilled water and covering with glycerine. Glycerine-mounted cultures may be kept in the cold indefinitely for later analysis or photography. Photographs were taken through a Wild M-5 stereomicroscope after diluting the glycerine with several washes of distilled water and removal of the last wash to near dryness.

Quantitation of chondrogenesis. Cultures destined for Alcian blue extraction were washed twice with PSG and fixed in acidified alcohol (2% glacial acetic acid in ethanol), as this fixative was determined to result in consistently, although not dramatically, higher spectrophotometric readings and lower background values, suggesting more complete dye extraction, especially for cultures grown in SCM. After fixation, the cultures were rehydrated through 95 and 70% ethanol in preparation for staining. The degree of chondrogenic expression was

quantitated by a modification of the method described by Hassel and Horigan (13). Fixed cultures were stained overnight with 0.5% Alcian blue in 0.1 *N* HCl and rinsed twice with 0.1 *N* HCl to remove any unbound dye. The final rinse with distilled water was aspirated from each well to near dryness. One hundred microliters of 4 *M* guanidinium HCl was then added to each well and the plate was sealed with Parafilm and allowed to stand overnight at 4° C. The plate was then allowed to warm to room temperature, and absorbance values for triplicate cultures were read on a Titertek microtiter plate reader using a 600-nm filter (the maximum absorbance of the Alcian blue used had been previously determined to occur at 595 nm). After obtaining the gross readings, the fluid was removed, the wells washed twice with distilled water, and 100 μl 4 *M* guanidinium HCl was delivered to each well. The plate was read again to obtain blank values, which were then subtracted from the gross values to give the net absorbance of the extracted Alcian blue stain. Nodule counts were obtained microscopically with the aid of a reticle grid whose demarcations were measured using a stage micrometer.

Quantitation of myogenesis (μTμM-ELISA). Cultures destined for μTμM-ELISA analysis were rinsed with PSG, fixed for 30 min in acidified alcohol, covered with 70% ethanol, stored overnight at 4° C, and postfixed for 3 h in 10% buffered formalin to inactivate endogenous phosphatase. Kahle's, acidified alcohol or formalin fixatives used alone did not inactivate the endogenous phosphateses and gave excessively high background readings. Fixed cultures were washed four times with 0.02 *M* PBS (pH 7.4) and rinsed once with 0.01 *M* PBS (pH 9.0). Each well was then filled with 5% NRS in 0.01 *M* PBS (pH 9.0) for 45 min to coat the walls of the wells and prevent nonspecific binding of the primary and secondary antibodies. Each well was then rinsed quickly with 0.25% NRS in 0.02 *M* PBS containing 0.02% Tween 20 (PBS-Tween). The experimental wells then received 100 μl of 50% MF-20 hybridoma supernatant in PBS-Tween, whereas control wells received 100 μl of 0.25% NRS in PBS-Tween. Experimental and control wells were incubated at room temperature for 45 min and then rinsed four times with PBS-Tween. Each well then received 100 μl of alkaline phosphatase-conjugated rabbit anti-mouse IgG (Zymed, South San Francisco, CA) diluted 1:2000 from the supplier's stock in 0.25% NRS in PBS-Tween. The plate was carefully sealed with Parafilm and incubated overnight at 4° C. Shorter incubations with higher concentrations of conjugated secondary antibody yielded higher blank readings. The wells were then washed four times with PBS-Tween. The final wash was replaced with 100 μl of PNPP substrate solution (1 part *p*-nitrophenyl-phosphate substrate stock [Zymed] to 100 parts 0.75 *M* amino-methyl-propanediol, pH 10.3). The reaction was allowed to proceed for 40 min at room temperature and the plate was then read on the microtiter plate reader using the 405-nm filter. The values obtained for the blank wells (receiving no primary antibody) were subtracted from those obtained for the corresponding experimental wells to give a net value.

DNA assay. Values for total DNA in cultures grown in DM vs. SCM at the various times tested were obtained using a modification of the assay described by Brunk et al. (5). Briefly, cells for time 0 DNA values were obtained by delivering identical aliquots of the same cell suspension used to inoculate the cultures into 1.5-ml microcentrifuge tubes. Cells for other time points were obtained by washing the cultures twice with PSG, delivering 200 μl of trypsin-collagenase to each well, incubating the plates at 37° C for 2 to 15 min while monitoring cell detachment microscopically and transferring the detached cells to separate 1.5-ml microcentrifuge tubes. Routinely, some scraping with a small policeman fashioned from Tygon tubing was necessary to improve recovery from 3- and 4-d cultures. The recovered cells were centrifuged at top speed for 2 min in a Beckman microcentrifuge, the pellet washed twice with 0.02 *M* PBS and once with the DNA assay buffer (5), then frozen at −20° C in 200 μl DNA assay buffer. On the day of the assay, the pellets were thawed, disrupted by sonication, and assayed for DNA content on a Turner fluorometer using chicken liver DNA as standard and Hoechst 33258 (Polysciences) as the fluor (5).

Protein assay. Values for total tissue protein/culture were obtained using a modification of the method of Lowry et al. (18). Briefly, unfixed cultures were washed several times with PSG, scraped from the wells with a small policeman and transferred with two 100-μl washes

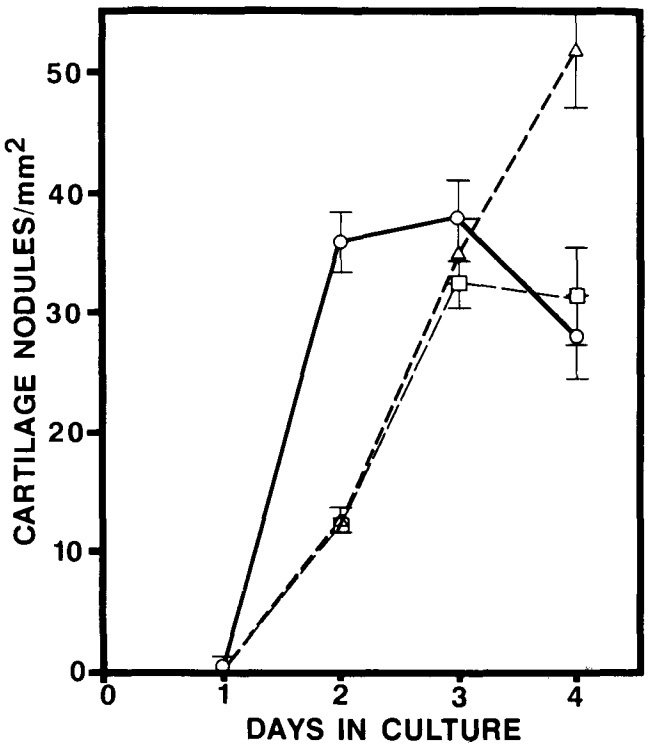

FIG. 1. Number of cartilage nodules vs. days in culture for chick limb mesenchymal cells grown in SCM (○), DM (△), and DM+T (□). Compare with Fig. 2. By 3 d, number of nodules/culture is similar for all media and begins to level off. Increase between Days 3 and 4 in cultures grown in DM is due to increased staining intensity of some internodular connections (also counted as nodules) (*see* Figs. 2 *G*, *J* and 3). Decrease between Days 3 and 4 in cultures grown in SCM is due to fusion of adjacent nodules accompanying nodule enlargement (*see* Fig. 2 *I*, *L*).

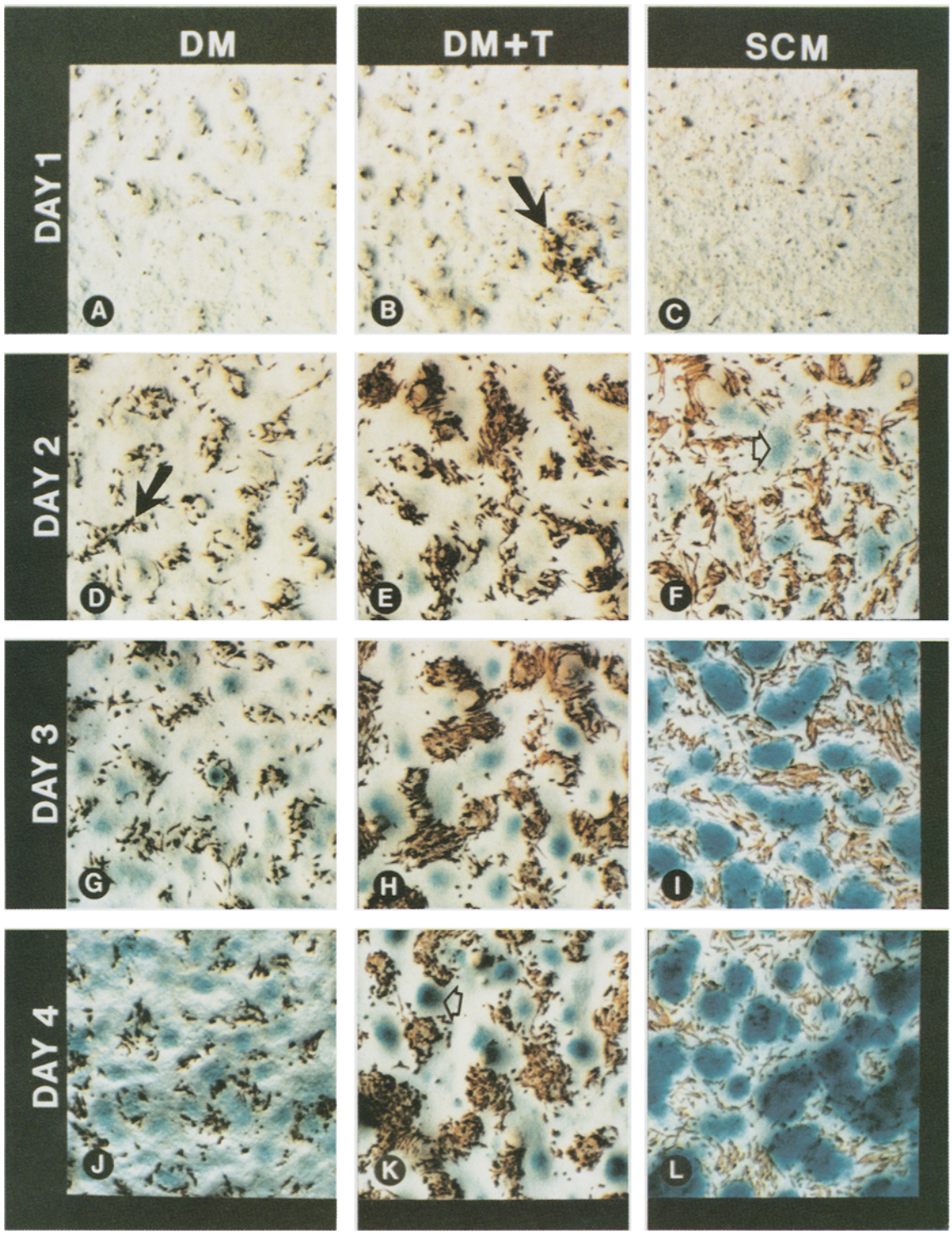

FIG. 2. Light photomicrographs of μTμM cultures of stage 23-24 chick limb mesenchymal cells grown in SCM (*C,F,I,L*) DM (*A,D,G,J*), and DM+T (*B,E,H,K*) for the culture periods indicated. Cartilage nodules (*open arrows*) are demonstrated by Alcian blue stain. Differentiated myoblasts (*solid arrows*) are demonstrated by MF-20 immunoperoxidase technique. *Note* the later appearance of nodules in DM and DM+T than in SCM. In DM and DM+T, cartilage nodules fail to enlarge significantly once they have formed. MF-20-positive myoblasts are most abundant and more likely to form clusters in cultures grown in DM+T. MF-20-positive myoblasts gradually increase in number between Days 2 and 4 in DM and DM+T and decrease in SCM during this same period. ×368.

of 0.1 *N* NaOH to separate 1.5-ml microcentrifuge tubes. Assays were performed on duplicate 50-μl (Days 3 and 4) or 100-μl (Days 0, 1, and 2) aliquots of the NaOH extracts and compared with similar aliquots of bovine serum albumin (Pentex fraction 5; Miles, Elkhart, IN) in 0.1 *N* NaOH as standards.

RESULTS

Morphological Analysis

Patterns of chondrogenesis in 4-d μTμM cultures. Regardless of the medium, similar numbers of randomly distributed Alcian-blue-positive cartilage nodules formed in all cultures by Day 4 (Fig. 1). In contrast, the size, degree of segregation from internodular cells, and staining intensity of the individual nodules all showed medium-dependent differences. As expected, cells cultured in SCM formed large, well-defined nodules that stained intensely with Alcian blue (Fig. 2 *L*). Cells grown in both serum-free media (DM and DM+T) were similar in that they formed smaller nodules than in SCM, but differed from one another in that the individual nodules in DM-containing cultures (Fig. 2 *J*) were less well segregated from internodular tissue and stained less intensely than those that formed in DM+T (Fig. 2 *K*). Mouse limb cells grown in DM/M for comparison produced a similar random pattern of small, intensely stained nodules, but differed from chick cultures with respect to the presence of narrow, Alcian-blue-positive strands bridging the internodular tissue to connect the nodules (Fig. 3).

Medium-Dependent Difference in Nodule Development

Day 1. Within 24 h after seeding the cultures, obvious cellular aggregates appear in the DM and DM+T cultures (Fig. 2 *A,B*), whereas cell aggregation is less apparent in the SCM cultures (Fig. 2 *C*). It is not clear whether all of these aggregates subsequently give rise to cartilage nodules, because many contain MF-20-positive myoblasts that appear in clusters at later stages in DM and DM+T cultures.

Day 2. Definitive chondrogenesis has begun in SCM cultures as indicated by the presence of numerous, small Alcian-blue-staining nodules (Fig. 2 *F*). By Day 2, the number of nodules/unit area of the cultures has already reached a maximum in SCM (Fig. 1), whereas in the serum-free media, few definitive nodules have formed (Figs. 1 and 2 *D,E*), although many pale-staining, precartilage aggregates are discernible.

Day 3. Definitive cartilage nodules are seen in all cultures. Those in SCM cultures have enlarged (Fig. 2 *I*) but have not increased in number (Fig. 1). In contrast, the nodules in the serum-free cultures (Fig. 2 *G,H*) have increased in number to a level similar to that seen for cells cultured in SCM (Fig. 1), but are smaller in size and more closely resemble the nodules in the 2-d SCM cultures than those in the 3-d SCM cultures.

Day 4. Cartilage nodule enlargement continues through Day 4 in SCM cultures and is accompanied by the fusion of adjacent nodules (Fig. 2 *L*), whereas nodules in serum-free media, although exhibiting an increase in staining intensity, do not enlarge significantly compared with their size in 3-d cultures and remain comparable in size to those in 2-d SCM cultures (Fig. 2 *J,K*). The number of nodules is similar regardless of the medium used, although a slight increase is seen in the DM-containing cultures (Fig. 1).

Medium-Dependent Differences in In Vitro Myogenesis

Myogenesis was monitored immunohistochemically using MF-20, a monoclonal antibody directed against sarcomere myosin (2). MF-20-positive cells are not present initially, but differentiate in culture from mesenchymal precursors in the inoculum (29).

Day 1. There are slightly more MF-20-positive myoblasts present in the serum-free-medium-containing cultures on Day 1 (Fig. 2 *A,B*) than in SCM (Fig. 2 *C*). Cultures grown in DM+T (Fig. 2 *B*) are already beginning to form clusters of differentiated myoblasts.

Day 2. Regardless of the medium, MF-20-positive myoblasts increase dramatically in number between 1 and 2 d of culture and become concentrated between the developing cartilage nodules (Fig. 2 *D,E,F*). The level of increase and degree of clustering are medium dependent. DM cultures have noticeably fewer MF-20-positive myoblasts than either DM+T or SCM. Myoblasts in DM+T cultures segregate into tight clusters; those in DM form looser clusters; and those in SCM appear more randomly distributed in the internodular areas.

Day 3. The number of MF-20-positive myoblasts present has increased in the serum-free cultures (Fig. 2 *G,H*), but seems to remain the same or decrease slightly in SCM (Fig. 2 *I*). In addition, myoblasts in SCM cultures have spread and become more stellate and the intensity of their immunoperoxidase staining has declined. Occasional multinucleated myotubes are seen in all cultures. The

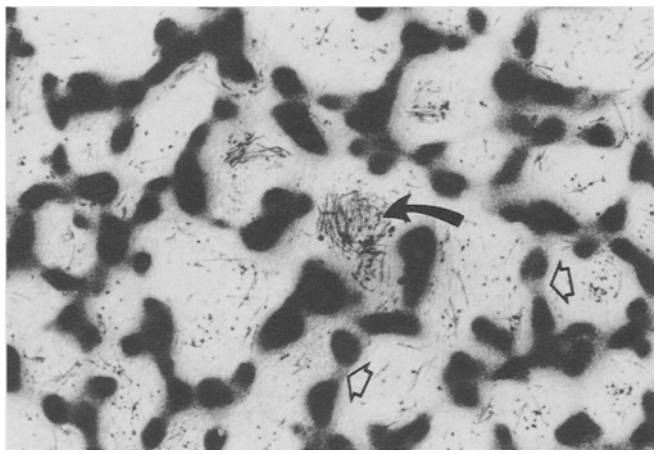

FIG. 3. Light photomicrograph of a μTμM culture of 10.5-d mouse embryo limb mesenchymal cells cultured for 4 d in DM/M. Cartilage nodules demonstrated by Alcian blue stain. Differentiated myoblasts (*solid arrow*) demonstrated by MF-20 immunoperoxidase technique. *Note* the narrow, pale Alcian-blue-positive connections (*open arrows*) between some nodules. ×490.

myoblasts in serum-free media remain predominantly in tight clusters whereas those in SCM are still randomly arranged within the internodular tissue.

Day 4. MF-20-positive myoblast numbers continue to increase in the serum-free cultures (Fig. 2 *J,K*) and decrease in SCM (Fig. 2 *L*). In all media, the number of small, rounded MF-20-positive cells increases.

As described previously for standard micromass cultures (29), in both 3- and 4-day SCM cultures, several MF-20-positive myoblasts are seen embedded in the cartilage

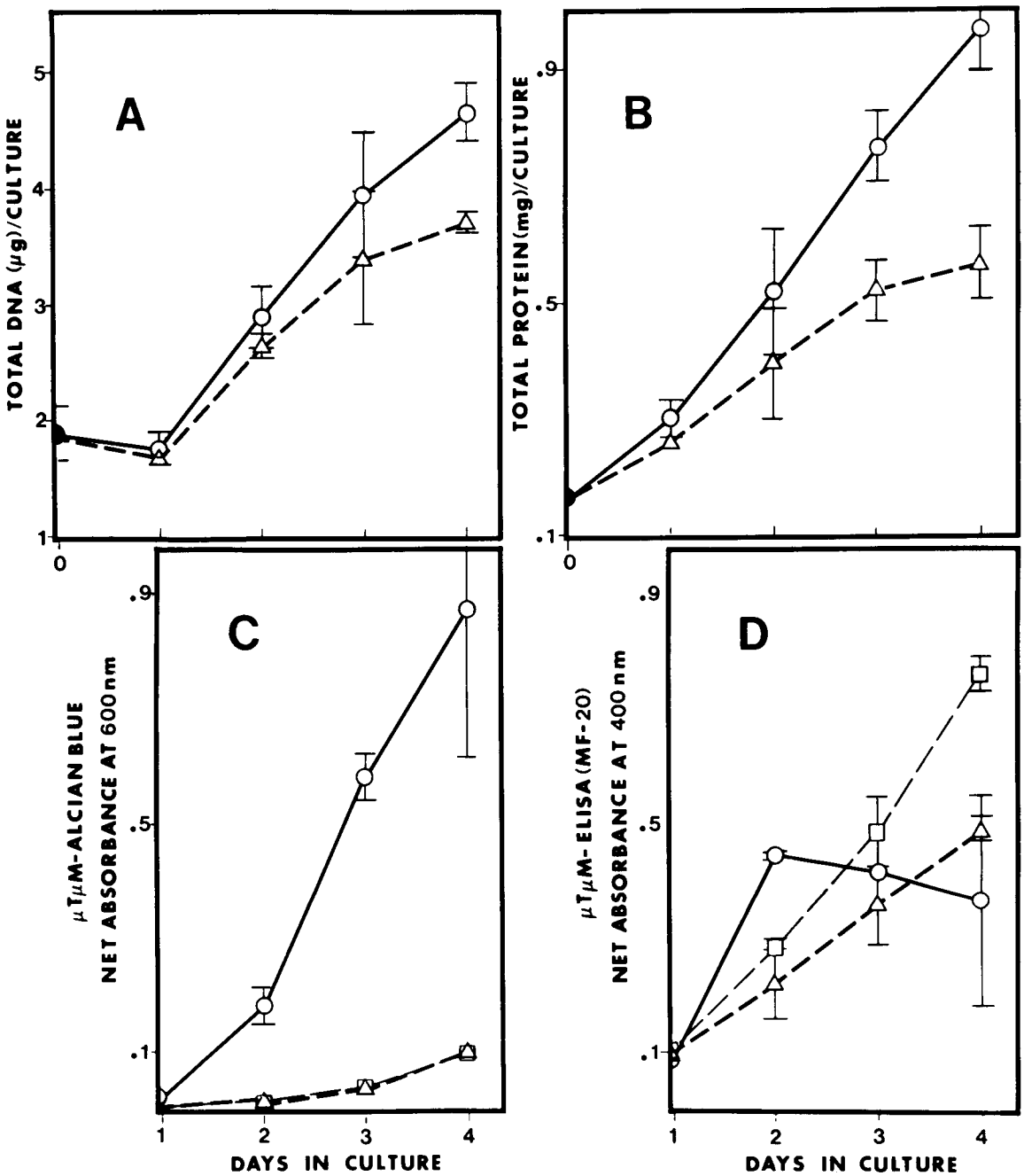

FIG. 4. Biochemical analyses performed on μTμM cultures of stage 23–24 chick limb mesenchymal cells grown in SCM (○), DM (△), or DM+T (□). *A*, total DNA per culture as a function of days in culture. There is a slightly greater rate of DNA accumulation in SCM than in DM. *B*, total protein per culture as a function of days in culture. There is a greater rate of protein accumulation in SCM than in DM. *C*, colorimetric estimation of cartilage differentiation as a function of days in culture using the absorbance of extractable Alcian-blue-stained cartilage matrix as an indicator. Chondrogenesis is dramatically greater in SCM than in DM or DM+T. Compare with morphology in Fig. 2. *D*, μTμM-ELISA: colorimetric estimation of muscle differentiation as a function of days in culture using the antisarcomere myosin monoclonal antibody, MF-20, as the primary antibody, alkaline phosphatase-conjugated rabbit antimouse IgG as the secondary antibody and *p*-nitrophenylphosphate as the substrate. Myogenic expression peaks at Day 2 in SCM before declining, whereas that in defined medium shows a gradual increase. Addition of transferrin to DM (DM+T) enhances myogenesis. Compare with morphology in Fig. 2.

nodules (Fig. 2 *I,L*). The majority of these cells are small and rounded and few if any express the bipolar, spindle-shaped morphology characteristic of the myoblasts in the internodular regions. Intranodular MF-20-positive cells appeared much less frequently in the smaller nodules of 3- and 4-d serum-free cultures (Fig. 2 *G,H,J,K*) and 2-d SCM cultures (Fig. 2 *F*).

Biochemical Analysis

DNA and protein. Values obtained for total DNA/culture (Fig. 4 *A*) and total tissue protein/culture (Fig. 4 *B*) suggest a comparable but not unexpectedly slower growth rate for cells maintained in DM relative to those grown in SCM.

Quantitation of chondrogenesis. The degree of chondrogenic expression, as measured by the absorbance of Alcian blue extracted per culture in a fixed volume of eluant (100 μl GuHCl), is significantly reduced in cultures grown in DM (Fig. 4 *C*), although measurable chondrogenesis does occur by Day 3 in this medium. There was no significant difference in the amount of extractable Alcian-blue-stained matrix material at any time between DM and DM+T.

Quantitation of myogenesis. μTμM-ELISA analysis showed marked medium-dependent differences in chronologic trends of myogenic expression (Fig. 4 *D*). Myogenesis in SCM reaches a peak by Day 2 and then shows a slow decline. In contrast, the data indicate that myogenesis in the serum-free media increases more gradually and continues to Day 4. Inclusion of transferrin in the defined medium (DM+T) enhances myogenesis over that in DM alone (Figs. 2 and 4 *D*).

Effects of retinoic acid. To determine whether μTμM cultures of limb mesenchyme and the defined medium described could be used in prescreening potential teratogens for dose-dependent effects on chondrogenesis, the effects of a known skeletal teratogen, all-trans RA were studied (Fig. 5). The data show that this method is capable of detecting dose-dependent effects of RA on the production of extractable Alcian-blue-positive cartilage matrix materials in 4-d cultures. Under these conditions, a maximal inhibition of chondrogenesis is achieved at 75 ng RA/ml (Fig. 5 *A*). DNA assays of companion cultures suggest a slight enhancement of cell proliferation by RA at a concentration of 25 ng/ml and a slight reduction at a concentration of 150 ng/ml, but that intermediate concentrations do not significantly alter the total amount of DNA/culture from that of cultures treated with the vehicle alone (Fig. 5 *B*). Together, these data indicate a dose-dependent decrease in the amount of extractable Alcian-blue-stained matrix material per unit DNA that reaches a maximum at 75 ng RA/ml (Fig. 5 *C*). The slight stimulatory effect of 25 ng/ml RA on the DNA content of the cultures may reflect a nutritional requirement for retinoids that is lacking in the defined medium but provided in more appropriate amounts in culture media supplemented with serum. This suggestion is supported by preliminary data that show a two- to threefold enhancement of matrix accumulation by 4-d μTμM cultures grown in DM supplemented with 5 to 10 ng/ml of RA compared to cultures grown in DM alone (Paulsen and Solursh (35).

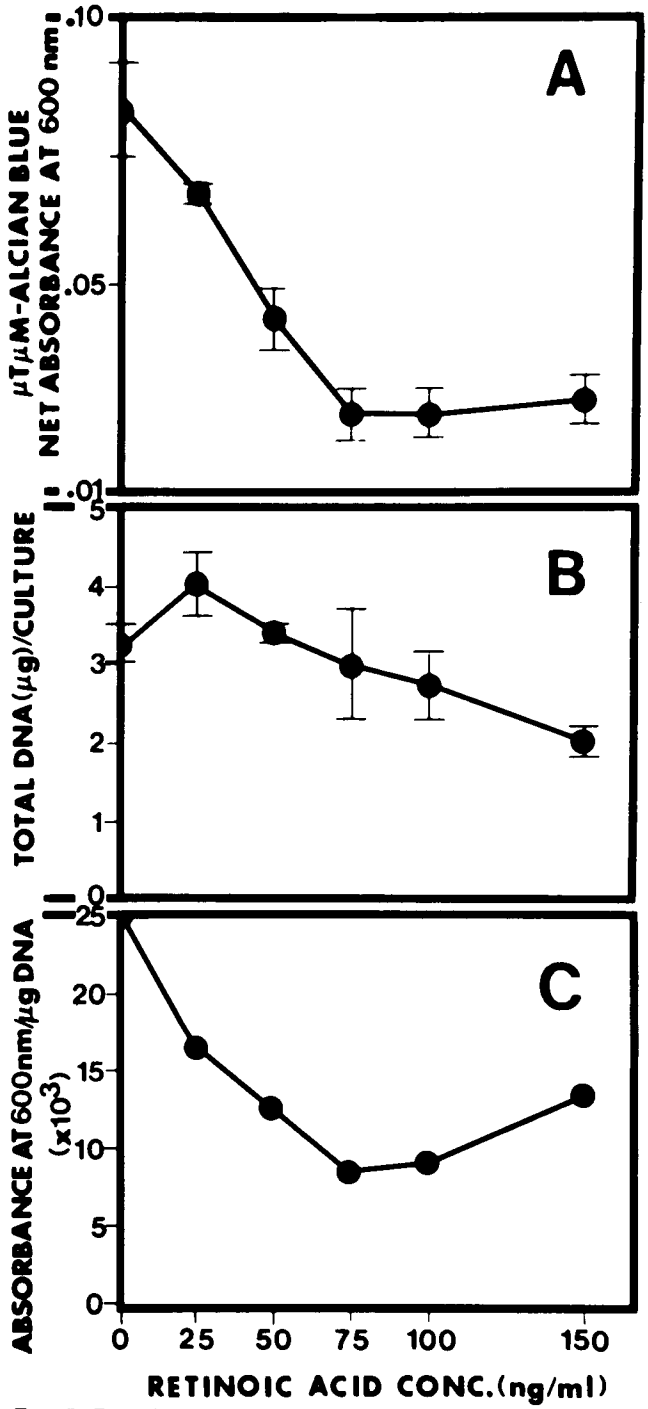

FIG. 5. Dose-dependent effects of all-trans RA in 4-d cultures of chick limb mesenchymal cells grown in defined medium. *A*, dose-dependent inhibition of chondrogenesis. Complete inhibition occurs at 75 ng RA/ml. Colorimetric estimation of levels of chondrogenesis were obtained using absorbance of extractable Alcian-blue-stained matrix as an indicator. *B*, effects on total DNA/culture. Slight enhancement of DNA accumulation over 4 d in culture by 25 ng RA/ml ($P = 0.0282$). Slight inhibition of DNA accumulation over 4 d in culture by 150 ng RA/ml ($P = 0.0017$). Intermediate values do not differ significantly from control ($P > 0.08$). *C*, dose-dependent effect of RA expressed as a function of extractable Alcian-blue-stained cartilage matrix per unit DNA in 4-d companion cultures.

Effects of vitamin C. This same method was used to determine the effect on chondrogenic expression of supplementing the defined medium with a standard concentration (50 μg/ml) of vitamin C (L-ascorbic acid). Previous studies (11,28) led us to suspect that this modification was likely to enhance matrix production by these cells. Indeed, the addition of ascorbic acid (DM+C) effectively doubled the amount of extractable Alcian-blue-stained matrix material by Day 4 of culture ($P = 0.0004$) without dramatically affecting nodule size. However, cells grown in DM+C still produced about fourfold less matrix per unit DNA than cells grown in SCM. Addition of ascorbic acid to the DM increased the total amount of DNA per culture by Day 4 less significantly ($P = 0.0224$) (Fig. 6), but to a level (4.44 ± 0.64 μg/culture) approximating that for cultures grown in SCM (4.65 ± 0.23).

DISCUSSION

Microtiter micromass cultures. This report describes modifications of the micromass culture system (1) whereby limb-bud mesenchymal cells are grown at high densities in 96-well microtiter plates in either SCM or DM. In combination with a histochemical technique devised by Hassell and Horigan (13) involving automated spectrophotometric analysis of guanidinium HCl-extracts of Alcian-blue-stained cartilage matrix material, the μTμM system allows rapid quantitation of chondrogenesis using an ELISA plate reader on the same plate in which the cells are grown. Similarly, quantitative estimates of myogenesis can be obtained by using a monoclonal anti-sarcomere-myosin antibody (MF-20) (2) and exploiting colorimetric antibody quantitation techniques devised for ELISA. The latter technique (μTμM-ELISA) may be adapted for the quantitation of any antigen for which a specific antibody is available and will be useful in quantitating the effects of a variety of treatments, including potential teratogens, hormones, vitamins, and growth factors on the production of many differentiation-related macromolecules by the cultured cells.

The quantitative estimates of chondrogenesis and myogenesis obtained with the Alcian-blue extraction and μTμM-ELISA methods (Fig. 4) agree well with the morphologic features of these cultures (Fig. 2). Although the fine points in morphology (e.g., shape and staining intensity of individual myoblasts, degree of segregation of cartilage nodules from internodular tissue) are not accessible through these methods, they do provide a more rapid and quantitative approach to analyzing the level of chondrogenic and myogenic expression in these cultures.

Advantages of the defined medium. Although studies of chondrogenesis in serum-containing cultures have provided a great deal of useful information (26), in vitro analysis of the precise requirements for the maintenance and differentiation of cultured cells is possible only through the use of chemically defined, serum-free media (3,4,24). Difficulties encountered in previous attempts to devise defined media for limb mesenchyme cells mainly involved problems in initially achieving and then maintaining cell-substrate attachment (15,24,31,36), requiring cells to be grown as pellets in suspension (15) or in media supplemented with fibronectin and either bovine serum albumin (BSA) or fetuin (24,36). In the absence of such additives, the loss of both chondrogenic and myogenic cells from the initial inoculum, as estimated from recovery of incorporated [^{3}H]thymidine and total DNA, is much more dramatic in serum-free cultures than in serum-containing cultures (31). As indicated by the continued increase of total DNA over 4 d in serum-free culture and the close correspondence between the DNA values for serum-containing and defined medium (Fig. 4 *A*), the cell attachment problem was not as great under the conditions described here. Although the serum-containing cultures produced significantly greater levels of extractable Alcian-blue-stained matrix material than did the serum-free cultures, there was greater culture-to-culture variation in the serum-containing cultures for most data points (SD indicated by *error bars* in figures) for all parameters studied. Variability may be even more pronounced when attempts are made to compare results obtained from different serum lots (unpublished observation) or different types of heterologous sera used in combination (8) or alone (22). Thus, in cases where long-term consistency in control

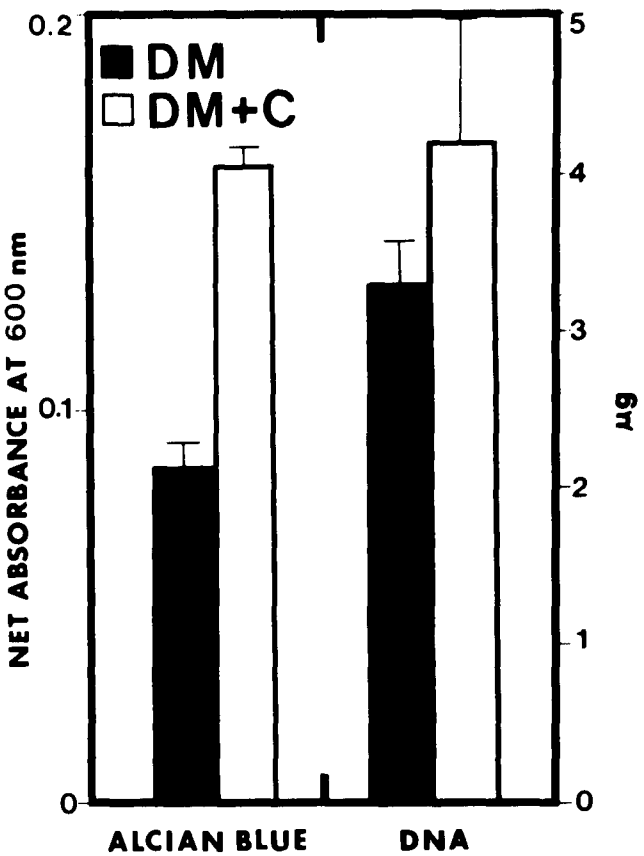

FIG. 6. Effects of 50 μg/ml L-ascorbic acid (vitamin C) on 4-d cultures of chick limb mesenchymal cells grown in defined medium. Approximately twofold enhancement of chondrogenesis by ascorbic acid over control value ($P = 0.0004$). Lesser enhancement of value for total DNA/culture over control ($P = 0.0224$).

values and comparability of the effects of various treatments are desired, the use of chemically defined media is preferable.

Effects of additives. The composition of the defined medium used in these studies was decided after a series of preliminary trials in which components of a serum-free medium described earlier by Kujawa and Caplan (16) and Syftestad et al. (30) were individually deleted to determine their relative importance. As has been demonstrated for a variety of other cell lines (3,4), insulin and hydrocortisone were required for maintaining chondrogenic and myogenic expression over the 4-d culture period. In the absence of either of these components, chondrogenesis may still occur (31) but falls to levels below the ability of the Alcian blue-extraction/ELISA-plate-reader technique to distinguish them from background. Higher insulin concentrations may enhance in vitro chondrogenesis, but further study will be required to distinguish between insulin-specific effects at physiologic concentrations and those observed at higher, pharmacologic doses, where insulin may be substituting for members of the insulin-like growth factor-somatomedin family (19). Nonessential components that had specific effects include transferrin, which dramatically enhances myogenesis, and ascorbic acid, which enhances chondrogenesis. The myotrophic effect of iron-complexed transferrin has been described previously (14) and there is recent evidence that iron alone may be substituted (10). The enhancement of cartilage matrix production by ascorbic acid is well documented (11,28). Additives tested, but which had no significant effect on chondrogenesis or myogenesis, include BSA and selenium.

Steps in in vitro chondrogenesis. Together, the morphologic and biochemical analyses indicate dramatic changes between Days 2 and 3 of culture in SCM that do not occur in DM or DM+T. The transition occurring in SCM, but not in the serum-free media, includes a significant increase in the rate of matrix accumulation as indicated by increased nodule size and the amount of extractable Alcian-blue-stained matrix present and an apparent decrease in internodular area. These observations suggest that chondrogenesis in SCM-containing cultures occurs in the following stepwise progression: a) formation of precartilage aggregates of chondrogenic cells, b) initiation of chondrogenesis within the aggregates resulting in the formation of small nodules by Day 2, c) nodule enlargement on Days 2 to 4 through proliferation of cells within the nodules (interstitial growth) or recruitment of additional chondrogenic cells from the internodular tissue at the nodule periphery (appositional growth) or both. Although steps a and b have been described previously (27), the existence of step c as a distinct step is discernable only by comparing chondrogenesis in serum-containing and serum-free cultures.

The results show that cells grown in serum-free medium reach step b more slowly (Day 3 instead of Day 2 for SCM) and do not reach step c by Day 4. This assertion is deduced from the following observaions: Similar numbers of nodules are ultimately formed in SCM and serum-free media, formation of the maximum number of nodules is delayed in serum-free cultures, the nodules in Day 3 and 4 serum-free cultures have a morphologic appearance similar to those in Day 2 SCM cultures, and finally, the nodules in serum-free cultures fail to enlarge significantly beyond the size of the initial aggregates by Day 4. The possibility that some serum-borne factor is required for or accelerates the transition between step b and c deserves further investigation, as does the mechanism underlying nodule enlargement. Significantly reduced secretion of extractable Alcian-blue-stained cartilage matrix material and failure of the nodules present to enlarge over 4 d of culture in the defined medium suggest that the serum-free media developed for these studies may be useful in future analyses of exogenous factors required for certain steps in in vitro chondrogenesis.

It should be noted that in standard micromass cultures (1) grown for 4 d in 35-mm dishes, similar numbers of nodules form in both media and the difference in nodule size is somewhat less dramatic, but nodules formed in such cultures in DM are still smaller than in SCM.

Lack of interdependence of chondrogenesis and myogenesis. Between Days 2 and 3 in SCM, myogenesis levels off and may even decrease as chondrogenesis increases dramatically. In addition, in comparing Day 4 DM+T cultures with Day 4 SCM cultures, DM+T enhances muscle expression and decreases cartilage expression, whereas SCM enhances cartilage expression and decreases muscle expression. From these isolated observations it is tempting to speculate, as others have done for similar reasons (7,20,25), that one cell type inhibits the differentiation of the other and that some sort of reciprocal relationship exists between chondrogenesis and myogenesis in cultures of limb mesenchyme (i.e. that chondrogenesis inhibits myogenesis or myogenesis inhibits chondrogenesis or both). However, Day-4 DM cultures express cartilage at levels more comparable to DM+T (low) and muscle at levels more comparable to SCM (low) arguing against a reciprocal relationship and for some degree of independence in the regulation of chondrogenesis and myogenesis in vitro (29).

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