Induction of Chondrogenesis in Limb Mesenchymal Cultures by Disruption of the Actin Cytoskeleton

NINA C. ZANETTI and MICHAEL SOLURSH
Department of Zoology, University of Iowa, Iowa City, Iowa 52242

ABSTRACT Cell shape is known to influence the chondrogenic differentiation of cultured limb bud mesenchyme cells (Solursh, M., T. F. Linsenmayer, and K. L. Jensen, 1982, *Dev. Biol.*, 94: 259–264). To test whether specific cytoskeletal components mediate this influence of cell shape, we examined different cytoskeleton disrupting agents for their ability to affect chondrogenesis. Limb bud cells cultured at subconfluent densities on plastic substrata normally become flattened, contain numerous cytoplasmic microtubules and actin bundles, and do not undergo spontaneous chondrogenesis. If such cultures are treated with 2 μg/ml cytochalasin D during the initial 3–24 h in culture, the cells round up, lose their actin cables, and undergo chondrogenesis, as indicated by the production of immunologically detectable type II collagen and a pericellular Alcian blue staining matrix. Cytochalasin D also induces cartilage formation by high-density cultures of proximal limb bud cells, which normally become blocked in a protodifferentiated state. In addition, cytochalasin D was found to reverse the normal inhibition by fibronectin of chondrogenesis by proximal limb bud cells cultured in hydrated collagen gels. Agents that disrupt microtubules have no apparent effect on the shape or chondrogenic differentiation of limb bud mesenchymal cells. These results suggest an involvement of the actin cytoskeleton in controlling cell shape and chondrogenic differentiation of limb bud mesenchyme. Interactions of the actin cytoskeleton and extracellular matrix components may provide a regulatory mechanism for mesenchyme cell differentiation into cartilage or fibrous connective tissue in the developing limb.

An apparent correlation between cell shape and differentiation in a number of different experimental systems has supported the hypothesis that cell shape and the cytoskeleton may affect the expression of certain genes (8, 14, 17). In the cases examined so far, the actin cytoskeleton undergoes reorganization during cell shape changes that are coupled to altered gene expression. For example, cultured corneal epithelial cells respond to certain matrix molecules by reorganizing their basal surface and doubling their production of collagen (34, 35). Reorganization of the basal cytoplasm involves the formation of an orderly meshwork of actin bundles. Spiegelman and his associates (31, 32, 33) have found that when certain sublines of 3T3 cells undergo adipogenesis, the acquisition of a spherical cell shape and loss of actin filaments are prerequisites to initiation of biosynthesis of lipogenic enzymes. Other systems in which altered gene expression appears linked to reorganization of the actin cytoskeleton include the production of procollagenase by cultured fibroblasts (1) and cAMP-dependent hormone-induced shape changes in cultured fibroblasts, bone cells, and thyroid cells (4, 5, 39, 42).

Several studies have pointed to an influence of cell shape on chondrogenic gene expression. Continued expression of the cartilage phenotype by chondrocytes in vitro is enhanced when cells are maintained in a round configuration by culturing them in agarose (6) or on poly(2-hydroxyethylmethacrylate) (12), whereas chondrocytes grown on lens capsule flatten and dedifferentiate (40). Initiation of chondrogenesis by mesenchymal cells is also influenced by cell shape. Differentiation of dissociated, prechondrogenic limb mesenchyme into cartilage normally requires that the cells be plated at densities higher than confluence (7), presumably so that essential cell–cell interactions can take place (30). However, limb mesenchymal cells can undergo chondrogenesis at low cell densities if they are cultured in suspension (19, 29). Single, isolated limb mesenchymal cells in collagen gels (28) are also capable of chondrogenic differentiation as demonstrated by the presence of immunologically detectable type II collagen.

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In both these culture conditions, cells are maintained in a round configuration.

In this study we have investigated whether specific cytoskeletal elements are involved in the cell shape changes correlated with enhanced chondrogenesis by limb mesenchymal cells. Embryonic limb bud cells were cultured under conditions not normally permissive for chondrogenesis, and specific microtubule- and microfilament-disrupting agents were examined for their ability to alter cell shape and induce chondrogenesis.

MATERIALS AND METHODS

Cell Cultures: Cell suspensions were prepared from stage 22–24 (13) White Leghorn chick embryos (Welp Hatchery, Bancroft, IA) as described previously (2). Wing buds were removed, rinsed in calcium- and magnesium-free saline G, dissociated in 0.1% trypsin–collagenase for 10 min at 37°C, and pipetted up and down. 2 ml of medium was added, after which the cells were collected by centrifugation and resuspended in 5 ml of medium. The cells were filtered through two layers of No. 20 Nitex (Tetko, Inc., Elmsford, N.Y.) and counted on a hemacytometer (American Optical Corp., Buffalo, N.Y.). For some experiments, wing buds were dissected into proximal and distal portions (36) before dissociating and plating.

For spot cultures, 2×10^5 (high-density "micromass" cultures) of 5×10^4 (subconfluent cultures) cells were inoculated as a 10-μl dot in the center of a 35-mm tissue culture dish. After 1 h in a 37°C incubator for cell attachment, the dishes were flooded with 2 ml of culture medium (Ham's F_{12} nutrient mixture containing 10% fetal calf serum and antibiotics). Medium was changed daily. In some experiments, cells were plated at clonal density (5×10^4 cells/60-mm culture dish). These cultures were fed by addition of medium on alternate days. In some experiments, limb bud cells were cultured in collagen gels as described previously (28). Rat tail collagen was prepared by a modification of the procedure of Elsdale and Bard (11). To prepare 1 ml of collagen culture medium, the collagen was diluted ⅓ with sterile distilled water to a concentration of ~1.0 mg/ml, then 7.5 ml of the diluted collagen was added to a mixture of 0.1 ml of 10× Ham's F_{12} medium, 0.1 of ml fetal calf serum, and 0.05 ml of a 1 mg/ml solution of human plasma fibronectin (Bethesda Research Laboratories, Gaithersburg, MD) in 0.1 M sodium bicarbonate, pH 11. Limb bud cells, collected by centrifugation, were resuspended in the collagen medium at a final concentration of 5×10^6 cells/ml. 10-μl dots containing 0.5×10^5 cells in collagen were quickly inoculated onto the center of a 35-mm tissue culture dish. After 5 min the gels were flooded with 2 ml of F_{12} medium, containing 50 μg/ml fibronectin and, in some cases, 2 μg/ml cytochalasin D. Cultures were fed once during the culture period by addition of medium. Cultures were fixed and stained with Alcian blue after 7 d.

All cultures were maintained in a 37°C incubator with a humidified atmosphere of 95% air/5% CO_2.

Fixation and Histology: Spot cultures and clonal density cultures were rinsed in saline G and fixed in Kahle's fixative for 10 min. To visualize sulfated glycosaminoglycans in cartilage matrix, we stained cultures with Alcian blue at pH 1 overnight (18). After examination of Alcian blue staining cells or nodules, some cultures were restained with Carazzi's hematoxylin. Collagen cultures were stained by a similar procedure except that cultures were fixed in 10% buffered formalin, and the staining time in Alcian blue was reduced to 30 min.

Immunofluorescence: Cultures to be stained for type II collagen were rinsed twice in saline G and once in phosphate-buffered saline (PBS) at room temperature. They then were fixed in 70% ethanol. Before being stained, cultures were incubated for 5 min in a 1:1 mixture of 95% ethanol:ether to permeabilize cell membranes. Cultures were stained as described previously (27). All steps were carried out at room temperature. Cultures were rehydrated for 10 min in PBS, then incubated for 30 min in a 1/800 dilution of mouse ascites fluid containing monoclonal antibody to type II collagen (20). The cultures were washed four times with PBS, then were incubated for 20 min with the fluorescein-conjugated IgG fraction of rabbit anti-mouse IgG (heavy and light chains; Cappel Laboratories Inc., Cochranville, PA) diluted 1/300 in PBS. To intensify fluorescence, we then rinsed cultures four times in PBS and incubated for 20 min in the fluorescein-conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories). After four more PBS rinses, cover slips were placed over the cultures, with paraphenylene diamine as mounting medium (16).

Cultures to be stained with antibodies directed against cytoskeletal elements were fixed in ice-cold methanol for 5 min (22), then rehydrated in PBS at room temperature for 10 min. Cultures were then stained with rabbit antibody to actin or tubulin (Miles Scientific, Naperville, IL) diluted 1/10 in PBS, for 30 min. The cultures were rinsed four times in PBS and incubated for 20 min in the fluorescein-conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories). After four more rinses in PBS, the cultures were mounted in paraphenylene diamine.

All cultures stained for immunofluorescence were observed and photographed with a Leitz Planopak illuminator with an L_2 filter cube (E. Leitz, Inc., Rockleigh, NJ).

Scanning Electron Microscopy: Cultures were rinsed in saline G, then fixed in ½ strength Karnovsky's fixative in 0.1 M cacodylate buffer, pH 7.2, for 30 min at room temperature. The fixed samples were rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO_4 for 45 min at 2°C, and dehydrated through a series of ethanols. Cultures were critical-point–dried with liquid CO_2 as transition fluid, and sputter coated with palladium-gold. Specimens were examined with a Jeol JSM-35C scanning electron microscope at 13 KV (JEOL USA, Electron Optics Div., Peabody, MA).

Chemicals: Cytochalasin D, obtained from Sigma Chemical Co. (St. Louis, MO), was dissolved in dimethylsulfoxide at a concentration of 1 mg/ml, then was diluted with distilled water to make a stock solution of 100 μg/ml. The stock solution was routinely diluted 1/50 in culture medium. In control experiments, dimethylsulfoxide at the final concentration used (0.2%) had no apparent effect on cell survival or differentiation.

Nocodazole was purchased from Sigma Chemical Co., dissolved in dimethylsulfoxide at a concentration of 4 mg/ml, and then diluted with distilled water to make a stock solution of 40 μg/ml.

Colcemid was obtained from CIBA Pharmaceutical Co. (Summit, NJ) and dissolved in distilled water to make a stock solution of 5 μg/ml.

RESULTS

Effect of Cytochalasin D on Subconfluent Limb Bud Cell Cultures

To test whether the actin cytoskeleton affects cell shape and chondrogenic expression of limb bud mesenchyme, cells were plated as subconfluent spot cultures and treated at various times with cytochalasin D. At cell densities less than confluence chondrogenesis normally does not occur (2, 7). In subconfluent cultures, the cells initially appear round, but within 24 h, become flattened and fibroblastic (Fig. 1 *a*). In contrast, cells cultured in the presence of 2 μg/ml cytochalasin D remain round (Fig. 1 *b*). This effect on cell shape is reversible, to the extent that in cultures treated with cytochalasin D for 24 h, the cells once again flatten within 6 h after removal of the drug (Fig. 1 *e*).

Cytochalasin D is believed to affect cell shape by disrupting the actin cytoskeleton (24, 41). To detect such an effect, we examined subconfluent cultures by immunofluorescence microscopy with antibodies directed against actin. After 24 h in culture, cells fed with control medium exhibit an extensive network of actin cables (Fig. 2 *a*). Thick cables are especially prominent at the lower cell surface which is attached to the culture dish. In parallel cultures treated with cytochalasin D, actin cables are not seen, but rather most cells exhibit diffuse staining and one or two bright patches of intracellular fluorescence (Fig. 2 *b*). If cytochalasin D is removed from the medium, actin cables reappear in the cells within 24 h (Fig. 2 *c*).

To investigate whether disruption of the actin cytoskeleton might affect later chondrogenic expression by limb bud mesenchyme cells, subconfluent cultures treated at varying times with cytochalasin D were fixed on day 4 of culture and stained with Alcian blue to detect cartilage matrix (Table I and Fig. 3). No Alcian blue staining nodules are found in cultures in control medium (Fig. 3 *a*). In contrast, cultures exposed to cytochalasin D for as little as 3 h form small nodules (Table I). In some cases Alcian blue staining matrix surrounds single, isolated cells. Nodule formation increases with prolonged

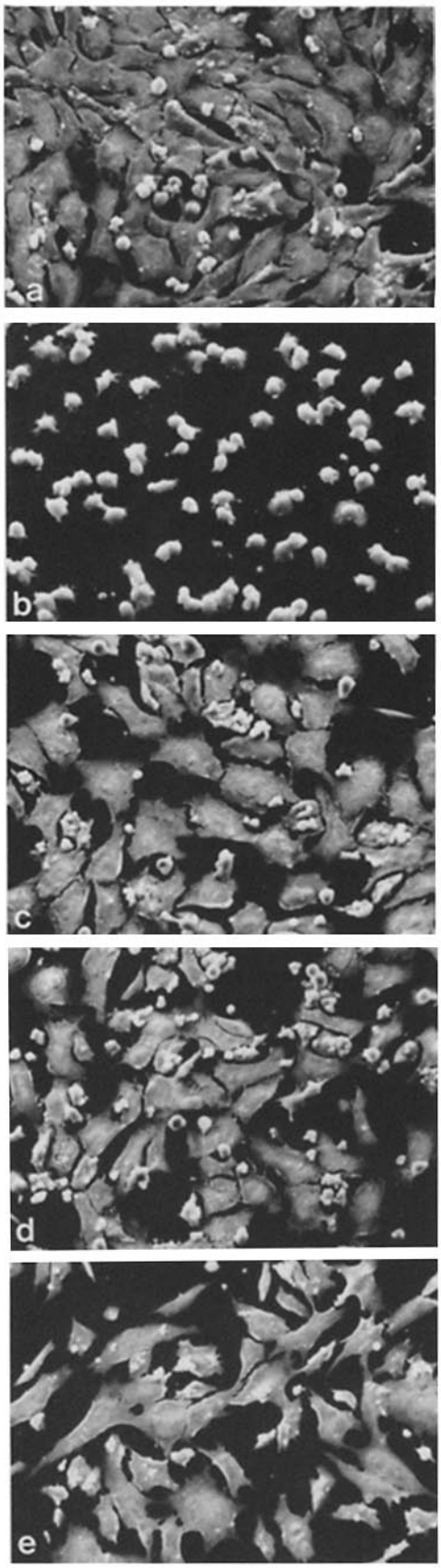

FIGURE 1 Scanning electron micrographs of limb bud mesenchymal cells plated as subconfluent spot cultures. Cultures were fixed after the first 24 h in culture in the presence of control medium (*a*) or medium containing 2 μg/ml cytochalasin D (*b*), 0.05 μg/ml colcemid (*c*), or 0.4 μg/ml nocodazole (*d*). Many of the control and nocodazole-treated cells have flattened and spread on the culture dishes. Cytochalasin-D-treated cells appear spherical. Cells in *e* were fixed 6 h after removal of cytochalasin D from the medium after a 24-h treatment. These cells have returned to a flattened configuration. Some cell loss due to detachment has occurred in all the treated cultures and accounts for the apparent reduction in cell density seen in these preparations as compared with control cultures. × 250.

exposure to cytochalasin D, to the extent that cells treated with cytochalasin D for the first 24 h in culture form numerous small nodules that merge together, making nodules difficult to count (Fig. 3*b*). However, hematoxylin staining reveals that not all cells become chondrogenic in response to cytochalasin D (Fig. 3*d*). The time and the duration of cytochalasin D treatment appear to be important in its effect on chondrogenesis. As shown in Table I, the drug is most effective during the first day in culture. If cells are plated in control medium and cytochalasin D is added after 24 h, scanning electron microscopy indicates that many of the cells do not become spherical, but only partially retract on the tissue culture dish. Therefore, the retention of a flat cell shape is correlated with the formation of relatively few cartilage nodules.

The ability of cytochalasin D to affect cell shape and chondrogenic differentiation of subconfluent cultures of limb bud mesenchyme has been tested over a range of concentrations (Table II). In cultures exposed to 1–2 μg/ml cytochalasin D for 24 h, 80–95% of the cells become round, and cartilage nodules appear by day 4. At lower concentrations of cytochalasin D (0.01–0.1 μg/ml), the percentage of rounded cells is not greatly enhanced over control cultures, and the cultures fail to produce Alcian blue staining nodules. Immunologically detectable actin cables are present in cells treated for 24 h with 0–0.1 μg/ml cytochalasin D but are largely disrupted by the higher concentrations that also stimulate nodule formation.

It was important to consider whether cytochalasin D alters the differentiation of prechondrogenic mesenchymal cells or only enhances cartilage matrix production by a few early chondrocytes that might be present in the stage 23–24 limb buds routinely used for preparing cell suspensions. We, therefore, tested cytochalasin D on subconfluent cultures of limb bud mesenchyme from younger embryos. Even cells from limb buds of stage 22 embryos form Alcian blue staining nodules in response to a 24-h treatment with 2 μg/ml cytochalasin D. Because cartilage specific products are not detected in intact limb buds earlier than stage 24 (10), these results strongly suggest that cytochalasin D influences the differentiation of mesenchymal cells that have not yet begun chondrogenesis.

To confirm that cells with pericellular Alcian blue staining matrix in cytochalasin D-treated cultures are indeed chondrocytes, we examined some cultures by immunofluorescence microscopy, using a monoclonal antibody directed against type II collagen (20). When cultures are fixed and stained on day 4, cultures receiving cytochalasin D on day 0 contain many clusters of cells that stain intensely for type II collagen (Fig. 4*d*). Staining is confined to intracellular vesicles, a pattern typical of limb chondrocytes cultured in ascorbate-deficient medium (27). Staining is not observed in untreated,

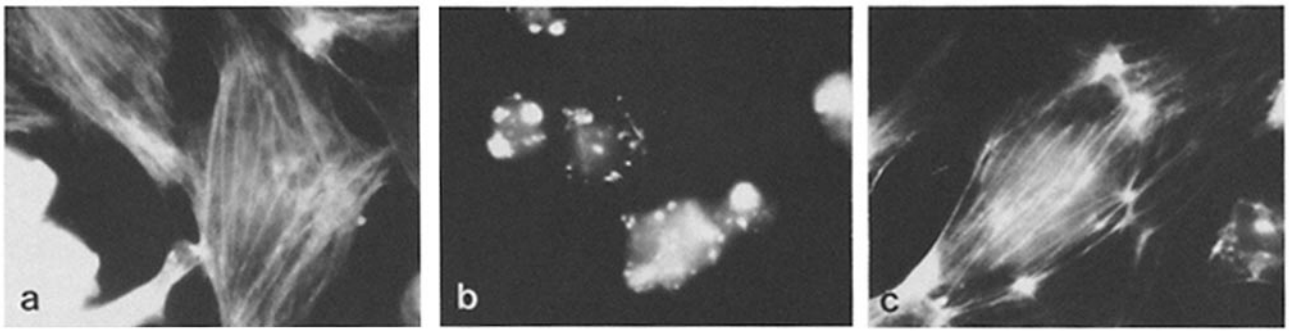

FIGURE 2 Immunofluorescence microscopy of subconfluent spot cultures stained with antiactin antibody. Cells were fixed after 24 h in control medium (a), 24 h in medium containing 2 μg/ml cytochalasin D (*b*), or 24 h in cytochalasin-D–containing medium followed by 20 h in control medium (c). Large actin cables are present in cells of control cultures (a). In spherical cytochalasin-D–treated cells (*b*), actin cables are absent. The cells stain diffusely, with a few bright patches of intracellular fluorescence. Actin cables reappear in cells of treated cultures after cytochalasin D is removed from the medium (*c*). × 640.

TABLE I

*Effect of Cytoskeleton-disrupting Agents on Chondrogenesis by Subconfluent Limb Bud Mesenchymal Cells**

Drug added	Time of treatment	Nodules/culture‡
		n
(Control)	—	0 (±0)
Cytochalasin D (2 μg/ml)	Initial 3 h	9.0 (±6.7)
Cytochalasin D	Initial 6 h	19.5 (±27.1)
Cytochalasin D	Initial 12 h	56.8 (±38.8)
Cytochalasin D	Initial 24 h	>100§
Cytochalasin D	Second 24 h	7.0 (±2.5)
Nocodazole (0.4 μg/ml)	Initial 24 h	0 (±0)
Colcemid (0.05 μg/ml)	Initial 24 h	0 (±0)

* Limb bud mesenchymal cells at stages 23–24 were plated as subconfluent spot cultures and fed F_{12} medium (control) or F_{12} medium that contained drugs at the times indicated. After treatment with the drugs, the cells were switched to and fed daily with control medium. Cultures were fixed on day 4 and stained with Alcian blue.

‡ Results are means and standard deviations of four replicate cultures and are representative of three experiments. Numerous floating cells are observed in cytochalasin D-treated cultures, hence counts of Alcian blue staining cells represent a low estimate of the number of cells undergoing chondrogenesis. Cell detachment may also account for the wide variance among replicate treated cultures.

§ Nodules are continuous and cannot be counted.

control cultures (Fig. 4*e*). Some cultures were fixed and stained at various times to determine the earliest time of appearance of type II collagen in response to cytochalasin D. In cultures treated with the drug from the start of culture, faint staining for type II collagen can be detected at 24 h after plating (Fig. 4*b*) and increases progressively with time (Fig. 4*c* and *d*). Staining is not detected at 12 h (Fig. 4*a*).

Because cell–cell interaction is known to be important in chondrogenesis of limb mesenchymal cells in vitro (30), it was important to verify that cytochalasin D was not stimulating cartilage formation in subconfluent cultures merely by enhancing cell aggregation. In experiments where cells were plated at clonal densities (5×10^4 cells/60 mm-dish) and fixed after 10 d, cultures receiving cytochalasin D for 24 h after plating contained 10–300 single cells with Alcian blue staining matrix (Fig. 5). Alcian blue staining cells do not appear in cultures fed with control medium.

In both clonal and subconfluent cultures treated with cytochalasin D, numerous floating cells are observed at the time of fixation. Counts of Alcian blue staining cells therefore represent a minimal estimate of the percentage of cells undergoing chondrogenesis. Cell detachment may also account for the wide range in the number of chondrocytes observed in treated cultures.

An additional result of treating clonal density and subconfluent cultures with cytochalasin D is the appearance of many binucleated cells. Unlike effects of cytochalasin D on cell shape and actin cables, the effect on binucleation is irreversible during the culture period used in these experiments. To test whether binucleation rather than cell shape might constitute the major driving force for cytochalasin D-induced chondrogenesis, we scored single chondrocytes appearing in cytochalasin D-treated clonal density cultures for bi- or mononuclearity. 36% of single cells producing Alcian blue staining matrix are mononucleated; hence binucleation is not obligatory for chondrogenic differentiation in these cultures.

Effect of Microtuble-disrupting Agents on Chondrogenesis by Limb Bud Cells

To investigate whether the state of cytoplasmic microtubules, as well as actin filaments, might influence chondrogenic differentiation, we examined the effect of microtubule-disrupting agents on subconfluent limb bud mesenchymal cell cultures. Unlike cytochalasin D, Colcemid (Fig. 1*c*) and nocodazole (Fig. 1*d*) do not induce rounding of the cells. When cultures are stained with antibody to tubulin, the cells in control cultures display a fine network of microtubules, often radiating from the center of the cell (Fig. 6*a*). In cultures treated with 0.4 μg/ml nocodazole, microtubules are not observed (Fig. 6*b*); however, when these cultures are stained with anti-actin antibodies, the cells appear to retain a normal distribution of actin cables (Fig. 6*c*). Nocodazole was tested on subconfluent cultures at concentrations ranging from 0.0004 to 0.4 μg/ml and for exposures of 6–24 h. The lowest of these concentrations caused no observable alteration of immunologically detectable microtubules. Neither Colcemid nor any of the tested concentrations of nocodazole stimulate cartilage nodule formation by subconfluent limb bud cells.

Effect of Cytochalasin D on Proximal Limb Bud Cell Cultures

Because cytochalasin D appears to overcome the need for high cell densities for in vitro limb chondrogenesis, it was of interest to investigate whether disruption of the actin cytoskeleton by this drug stimulates cartilage formation under other conditions in which limb bud cells are blocked in chondrogenic differentiation. Swalla et al. (36) reported that

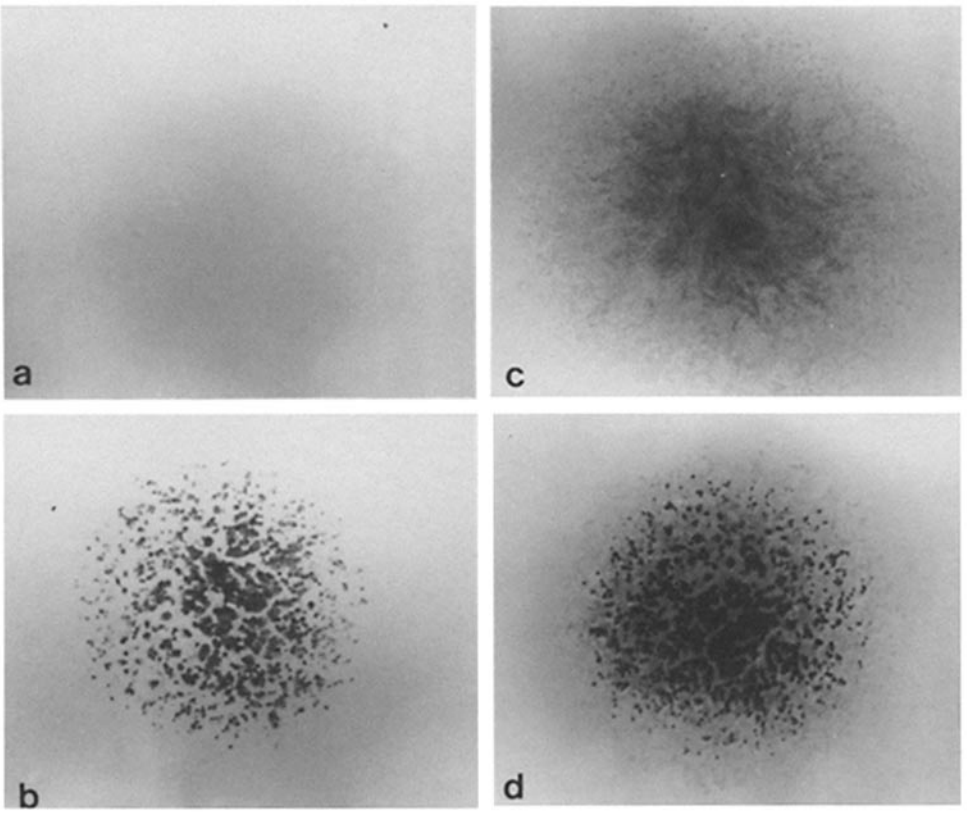

FIGURE 3 Effect of cytochalasin D on chondrogenesis of limb bud mesenchymal cells. Subconfluent spot cultures fixed on day 4 of culture were stained with Alcian blue (*a* and *b*), then counterstained with hematoxylin (*c* and *d*). Control cultures (*a* and *c*) contain no Alcian blue staining nodules. Cultures fed medium containing cytochalasin D for the first 24 h in culture (*b* and *d*) contain numerous nodules. × 9.6.

TABLE II

*Effect of Various Concentrations of Cytochalasin D on Cell Shape and Chondrogenic Differentiation by Subconfluent Limb Bud Mesenchymal Cells**

Concentration of cytochalasin D	Rounded cells‡	Alcian blue-staining§ nodules
μg/ml	%	
0	32.6	0
0.01	43.7	0
0.1	51.2	0
1.0	78.7	+
2.0	96.5	+

* Limb bud mesenchymal cells at stages 23–24 were plated as subconfluent spot cultures and fed F_{12} medium containing cytochalasin D at the indicated concentrations. After 24 h cells were switched to F_{12} medium, and thereafter fed daily with medium lacking cytochalasin D.

‡ Cultures were fixed at 24 h after plating and were examined by scanning electron microscopy. Representative areas of the cultures were photographed, and all cells scored as flattened, round, or bipolar. At least 300 cells were counted for each treatment.

§ Cultures were fixed on day 4 and stained with Alcian blue. Three cultures were examined for each treatment. Samples designated as positive for cartilage formation (+) contained at least 10 Alcian blue staining nodules per dish.

when cells from the proximal half of the stage 23 or 24 chick limb bud are cultured as high-density micromass cultures, they remain in a protodifferentiated state characterized by production of low levels of type II collagen but without accumulation of cartilage matrix. Cells in these proximal cultures appear flattened during the first day of culture and form few if any nodules after 4 d of culture in control medium (Fig. 7*a*). In contrast, proximal micromass cultures in medium containing 2 μg/ml cytochalasin D for the first 24 h after plating form numerous Alcian blue staining nodules by day 4 (Fig. 7*c*). Just as for subconfluent cultures, cytochalasin D stimulates nodule formation by proximal cells after as little as a 3-h exposure (Fig. 7*b*), and is most effective when present during the first 24 h of culture (compare Figs. 7*c* and *d*).

A useful method for studying cell–matrix interactions in development has been to culture limb mesenchymal cells in three-dimensional hydrated collagen gels (28). Using this culture system, Swalla et al. (37) have demonstrated that chondrogenesis by proximal limb bud cells is inhibited by exogenous fibronectin. Presumably, fibronectin promotes interaction of cells with the collagen gel to cause cell flattening. To investigate whether this sensitivity of proximal cells to fibronectin might involve the actin cytoskeleton, we fed proximal cells cultured in collagen gels medium containing both fibronectin and cytochalasin D. The presence of cytochalasin D during the first day of culture appears to block the inhibitory effect of fibronectin on the chondrogenic differentiation of these cells, such that after 7 d the treated cultures contain single, large, spherical cells surrounded by Alcian blue staining matrix (Fig. 8).

DISCUSSION

Effect of Cytoskeleton-disrupting Agents on Limb Bud Cell Chondrogenesis

We have examined the effect of cytoskeleton-disrupting agents on chondrogenic differentiation by cultured limb bud mesenchyme. Three different culture systems were tested: whole limb mesenchyme at subconfluent densities, proximal limb bud cells in high density micromass cultures, and proximal limb bud cells in hydrated collagen gels containing fibronectin. In these three culture systems, the cells normally remain flattened and nonchondrogenic. In contrast, when the actin cytoskeleton is disrupted by addition of cytochalasin D, the cells round up and undergo chondrogenesis, as indicated by production of type II collagen and an Alcian blue staining

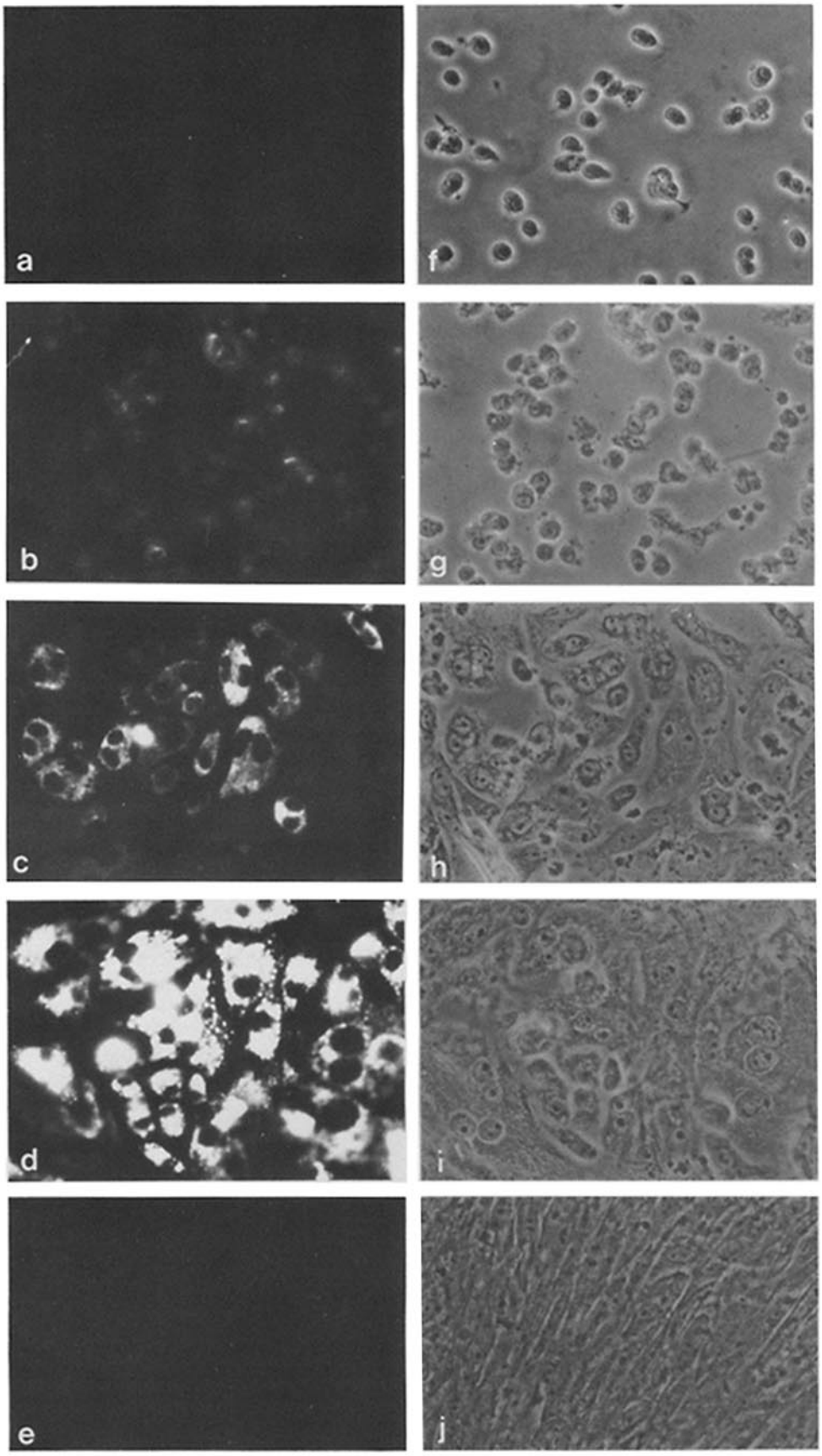

FIGURE 4 Phase-contrast microscopy (*f–j*) and immunofluorescence microscopy (*a–e*) of subconfluent spot cultures stained with antibody directed against type II collagen. Cultures in *a–d* were fed F_{12} medium containing 2 μg/ml cytochalasin D during the first 24 h of culture, and were fixed at various times after plating. Cells receiving cytochalasin D contain no immunologically detectable type II collagen at 12 h (*a*). Faint fluorescence appears in cultures fixed after 24 h (*b*). A progressive increase in intracellular staining is seen in treated cultures fixed at 48 h (*c*) and on day 4 (*d*). Cells in the presence of cytochalasin D appear round (*f* and *g*). In *h* and *i*, cytochalasin D was removed at 24 h, and the cells have become flattened. Cells fed control medium and fixed on day 4 (*e* and *j*) are flattened and do not stain for type II collagen. × 256.

extracellular matrix. These results extend our previous observation that maintaining cells in a round configuration stimulates cartilage formation by single limb bud cells (28, 29). In the present investigation, a transient exposure of limb bud mesenchymal cells to cytochalasin D (3–24 h) permanently alters the subsequent differentiation of these cells. This result is consistent with an earlier observation (29) that if prechondrogenic cells are maintained in suspension for 12–24 h, they

acquire the ability to form cartilage colonies, even when plated at low density.

The cytoskeleton of limb bud cells plated at subconfluent densities on plastic substrata normally contains both actin bundles and microtubules. Treatment of such cultures with cytochalasin D causes rounding of the cells and loss of actin cables. In contrast, nocodazole disrupts the network of microtubules, but leaves immunologically detectable actin bundles. Furthermore, the cells remain flattened after this treatment. These observations suggest that actin filaments rather than cytoplasmic microtubules control the shape of cultured limb bud cells. Cell shape changes in other types of cultured cells have also been reported to involve selective alteration of the actin cytoskeleton (4, 5, 42). Although disruption of actin cables stimulates chondrogenesis by limb bud mesenchymal cells, our results do not distinguish between a direct effect of the cytoskeleton on gene expression or an indirect effect mediated by the resulting change in cell shape.

The observation that cytochalasin D induces chondrogenesis in both low-density limb bud cell cultures and high-density proximal cell cultures indicates that disruption of the actin cytoskeleton can overcome two different types of blocks in mesenchymal cell chondrogenesis. In low-density cultures, cells are stimulated to synthesize type II collagen under conditions where normally no immunologically detectable type II collagen is produced. Cytochalasin D also promotes cartilage matrix formation by proximal limb cells, which are otherwise blocked in a protodifferentiated state in which they produce type II collagen but not a pericellular cartilage matrix. In both of these situations, the level of control on chondrogenic gene expression by the cytoskeleton or cytochalasin D is presently unknown.

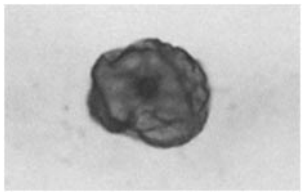

FIGURE 5 Single cell in culture of limb bud mesenchymal cells plated at clonal density (5×10^4 cells/60-mm dish), fixed on day 10, and stained with Alcian blue and hematoxylin. The cell is surrounded by Alcian blue staining matrix. × 320.

Evidence for Interactions of Fibronectin and the Actin Cytoskeleton in the Control of Chondrogenesis

Several investigations have suggested that the actin cytoskeleton may control gene expression through association with cell surface fibronectin. During adipogenesis of 3T3 preadipocytes, cell rounding and loss of actin bundles consistently precede the initiation of synthesis of lipogenic enzymes (31, 33). If preadipocytes are treated with fibronectin, actin cables remain intact, and the cells do not differentiate (32). This block of adipogenesis by fibronectin can be alleviated by treating the cells with cytochalasin D to cause actin disassembly (33). Courtois et al. (9) have observed a similar correlation between loss of cell surface fibronectin and disruption of actin cables during the changes in cell shape that accompany the response of lens epithelial cells to retinal extract. Structural relationships between actin filaments and fibronectin have been demonstrated by immunocytochemical techniques (15, 25) and by the observation that treatment of cultured fibroblasts with cytochalasin D results in loss of cell surface fibronectin (3, 21).

Experiments with proximal limb bud cells in hydrated collagen gels have provided evidence that a functional relationship between the actin cytoskeleton and fibronectin may be important in limb chondrogenesis. Proximal cells normally undergo chondrogenesis in collagen gels but are inhibited from doing so in gels containing fibronectin (37). This block in differentiation is overcome when cell spreading is prevented by treating cells with cytochalasin D. It is noteworthy that proximal cells also become chondrogenic when they are cultured in multilayers (5×10^5 cells/10-μl dot) on plastic (unpublished observation). In both these situations, chondrogenesis of proximal cells occurs when cell-substratum attachments are reduced. Proximal cells may be especially sensitive to this type of control mechanism because they possess greater cell surface adhesiveness than cells from other regions of the limb bud (23).

Possible Role of Cell Shape in the Developing Limb

We have demonstrated that in vitro chondrogenic differentiation of early limb bud mesenchyme is influenced by cell shape, possibly through interactions between the cytoskeleton

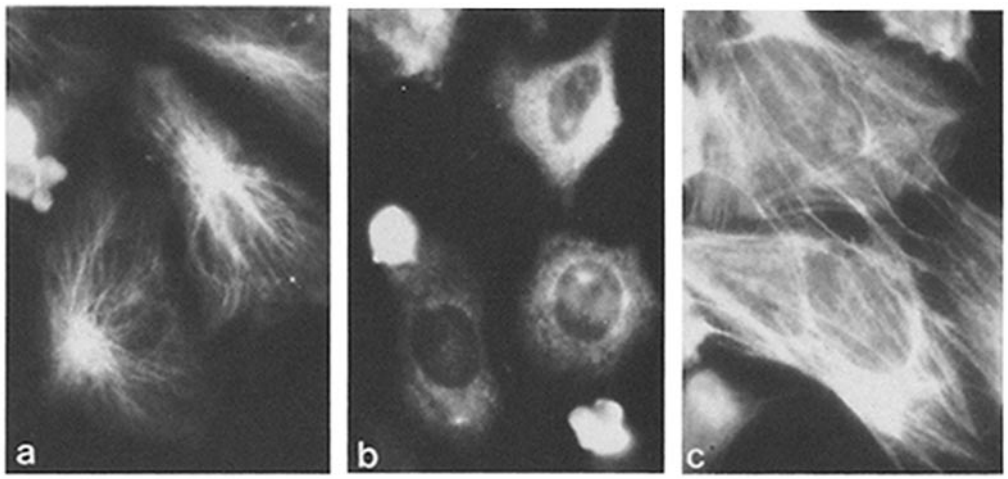

FIGURE 6 Immunofluorescence microscopy of cytoskeletal elements in cells of subconfluent spot cultures. Cultures were fixed after 24 h in control medium (*a*) or medium containing 0.4 μg/ml nocodazole (*b* and *c*), and were stained with antibodies directed against tubulin (*a* and *b*) or actin (*c*). The extensive network of microtubules present in untreated cells (*a*) is absent in nocodazole-treated cultures (*b*). Actin cables are present in nocodazole-treated cells (*c*). × 640.

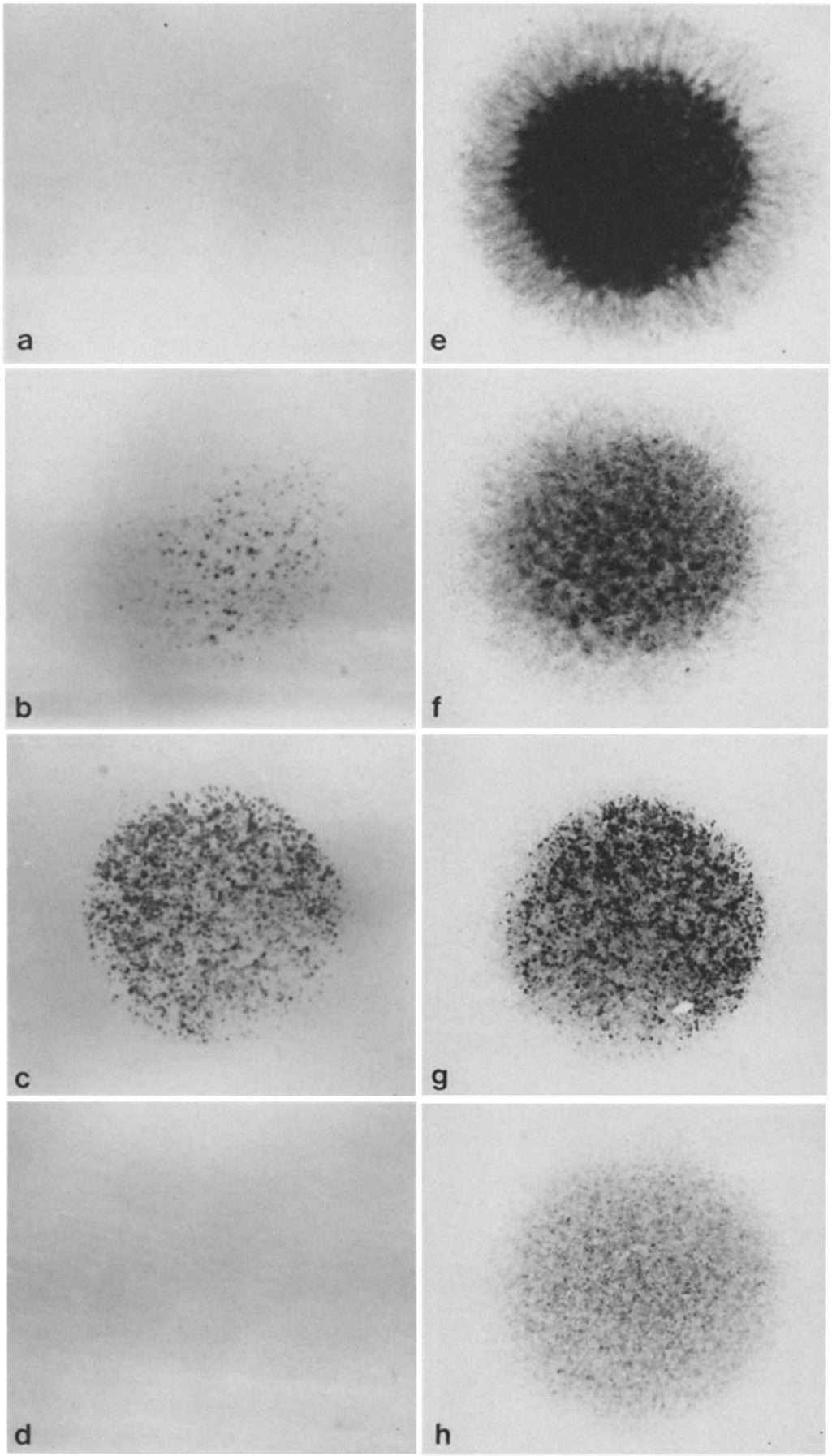

FIGURE 7 Effect of cytochalasin D on high-density spot cultures of proximal limb bud mesenchymal cells. Cultures were fixed on day 4 and were stained with Alcian blue (*a–d*) followed by hematoxylin (*e–h*). Cultures fed control medium (*a*) contain no Alcian blue staining nodules. Nodules appear in cultures treated with 2 μg/ml cytochalasin D during the initial 3 h (*b*). An increased number of nodules appears in cultures treated with cytochalasin D during the initial 24 h (*c*). Nodules do not appear when cells are fed cytochalasin D only during the second 24 h of culture (*d*). × 9.

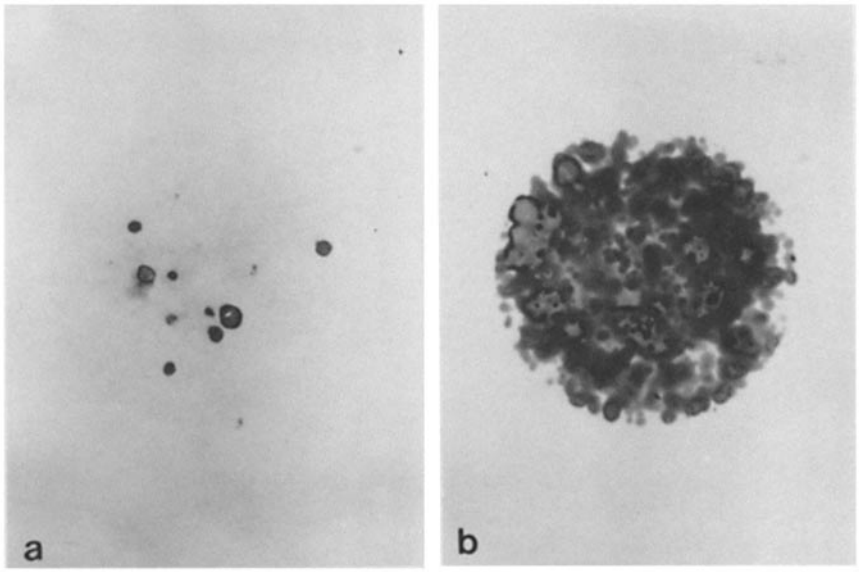

FIGURE 8 Proximal limb bud mesenchymal cells cultured in hydrated collagen gels containing human plasma fibronectin. Cultures were fixed after 7 d and stained with Alcian blue. The culture in (*a*) was fed F_{12} medium containing 50 μg/ml human plasma fibronectin; few Alcian blue staining nodules are apparent. The culture in *b* was fed as in *a* but with the addition of 2 μg/ml cytochalasin D during the first 24 h in culture. The gel contains numerous large spherical cells with pericellular Alcian blue staining matrix. × 9.

and extracellular matrix. The question remains whether cell shape and the cytoskeleton are involved in controlling limb chondrogenesis in vivo. This possibility would be consistent with the observation that mesenchymal cells in prechondrogenic condensations often appear round (26, 38). Differential distribution of specific matrix components, such as fibronectin or type I collagen, in the early limb bud could affect the shape and, therefore, the differentiation of potentially chondrogenic cells in different regions of the limb. Alternatively, early limb bud cells may possess temporal and positional variations in their ability to respond to certain matrix molecules or altered cell shape. The results presented here are consistent with a role of cell shape in the choice of mesenchyme cells to differentiate into fibroblasts or chondrocytes. Perhaps a similar mechanism operates in determining the differentiation of other precursor cell types.

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