# Alterations in Chondrocyte Cytoskeletal Architecture during Phenotypic Modulation by Retinoic Acid and Dihydrocytochalasin B–induced Reexpression

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*Abstract.* The differentiated phenotype of rabbit articular chondrocytes was modulated in primary culture by treatment with 1 μg/ml retinoic acid (RA) and reexpressed in secondary culture by treatment with the microfilament-disruptive drug dihydrocytochalasin B (DHCB) in the absence of RA. Because the effective dose of DHCB (3 μM) did not elicit detectable cell rounding or retraction, the nature and extent of microfilament modification responsible for induction of reexpression was evaluated. The network of microfilament stress fibers detected with rhodamine-labeled phalloidin in primary control chondrocytes was altered by RA to a "cobblestone" pattern of circularly oriented fibers at the cell periphery. Subsequent treatment with DHCB resulted in rapid changes in this pattern before overt reexpression. Stress fibers decreased in number and were reoriented. Parallel arrays of long fibers that traversed the cell were evident, in addition to fiber fragments and focal condensations of staining. Immunofluorescent staining of intermediate filaments revealed a marked decrease in complexity and intensity during RA treatment but no change during reexpression. An extended microtubular architecture was present throughout the study. These results clearly identify microfilaments as the principal affected cytoskeletal element and demonstrate that their modification, rather than complete disruption, is sufficient for reexpression. The specificity of DHCB and the reorientation of these filaments before reexpression of the differentiated phenotype suggests a causative role in the mechanism of reexpression.

CHONDROCYTES exhibit high levels of synthesis of cartilage-specific proteoglycan and type II collagen as a major part of their differentiated phenotype. The expression of this phenotype has been shown to be responsive to changes in cell shape, the maintenance (13) or resumption (5) of spherical conformation promoting the expression of the differentiated phenotype. In contrast modulating agents such as fibronectin (17, 26) and retinoic acid (RA)[1] (4), which appear to enhance both adhesion and spreading of cultured chondrocytes, also enhance the rate at which the differentiated phenotype is lost. These observations, together with the results of more recent studies on the effects of microfilament-modifying drugs on chondrogenesis (18, 27), suggest the involvement of some element of the cytoskeleton in modulation of the chondrocyte phenotype.

To study this involvement, the cytoskeletal architecture of rabbit articular chondrocytes has been examined by immunofluorescent techniques during both RA modulation of the differentiated phenotype and its subsequent reexpression as induced by the microfilament-modifying drug dihydrocytochalasin B (DHCB). Transitions in the synthesis of genetically distinct collagen types were used as markers for defined phenotypic change, and are reported separately (4, 6). Briefly, RA modulation of the phenotype was marked by a cessation of type II collagen synthesis and a transient stimulation of type III and type I trimer synthesis. DHCB-induced reexpression of the differentiated phenotype was marked by a loss of type III and type I trimer synthesis and a return of type II synthesis. Notably this induction of reexpression did not involve a resumption of spherical cell shape; rather the cells remained as a well-spread confluent monolayer.

The purpose of this study was to determine the rate and extent of microfilament modification responsible for the initiation of phenotypic reexpression in the absence of cell rounding, and to evaluate the extent to which other cytoskeletal structures might be involved. We report here that both RA modulation and DHCB-induced reexpression resulted in marked microfilament modification, and modification caused by DHCB preceded the resumption of type II collagen synthesis. The organization of intermediate filaments and microtubules appeared to be largely independent of these changes.

1. *Abbreviations used in this paper*: DHCB, dihydrocytochalasin B; RA, retinoic acid.

## Materials and Methods

### Cell Culture

Chondrocyte monolayer cultures were established from the articular cartilage of the humeral head of 8-wk-old New Zealand White rabbits and grown in medium containing 10% FBS as previously described (6). After 10 d of culture in the absence or presence of 1 μg/ml RA (Sigma Chemical Co., St. Louis, MO), control and RA-modulated cells were replated at $4.0 \times 10^4$ cells/cm$^2$ as secondary cultures grown in plastic chamber slides (Miles Laboratories Inc., Naperville, IL). Cells were allowed to attach for 4 d in the absence or presence of RA.

RA was then removed from treated cultures to begin the reexpression period. These cells were maintained in 2 or 10% FBS in the absence or presence of DHCB ($3 \times 10^{-6}$ M) (Sigma Chemical Co.) from day 4 to day 18 of secondary culture. Cells were examined by immunofluorescence 1, 2, 7, and 14 d after the removal of RA.

### Immunofluorescence Microscopy

Microfilament-containing stress fibers were stained with rhodamine-labeled phalloidin (Molecular Probes Inc., Junction City, OR). The cells were rinsed briefly in calcium and magnesium-free Dulbecco's PBS (Gibco, Grand Island, NY) containing 5 mM EGTA, and then fixed in 4% (wt/vol) paraformaldehyde in PBS-EGTA for 20 min at 37°C. The cells were then immersed in 47.5% ethanol, containing 5 mM EGTA, for 15 min at room temperature and finally held in PBS-EGTA for 5 min before staining (14). The cells were stained with a 1:20 dilution of rhodamine phalloidin in PBS-EGTA for 30 min at 37°C. The cells were then rinsed in several changes of PBS-EGTA and mounted in a 1:1 mix of glycerol and PBS-EGTA.

Microtubules were stained using a mouse mAb raised against native chick brain microtubules (Amersham Corp., Arlington Heights, IL). All antibody and labeled streptavidin dilutions were made in PBS-EGTA containing 1 mg/ml BSA and 0.1% (wt/vol) sodium azide. Cells were rinsed briefly with 60 mM Pipes buffer, pH 6.9, containing 25 mM Hepes, 10 mM EGTA, and 2 mM magnesium chloride, and then immersed in the same buffer containing 0.15% (vol/vol) Triton X-100 for 2 min at room temperature (19). Treatment with Triton X-100 removed many soluble proteins without altering the microtubule cytoskeletal network and resulted in increased clarity of the fluorescent image. After rinsing briefly in the initial buffer the cells were fixed in methanol containing 5 mM EGTA for 6 min at −20°C (16). The cells were held in PBS-EGTA for 5 min before staining with a 1:500 dilution of the mAb for 1 h at 37°C and then rinsed three times in PBS-EGTA. The cells were then incubated for 1 h at 37°C with a 1:50 dilution of biotinylated second antibody, raised against mouse Ig (Amersham Corp.), and rinsed three times in PBS-EGTA. Detection was carried out by incubation for 30 min at 37°C with a 1:50 dilution of Texas Red–labeled streptavidin (Amersham Corp.), the cells rinsed and mounted as described.

Intermediate filaments were stained using a mouse mAb raised against porcine eye lens vimentin (Amersham Corp.). Cells were fixed as described for microfilament staining with the exception that the paraformaldehyde solution contained 0.5 mg/ml Saponin (Sigma Chemical Co.). Incubation with first and second antibodies and detection with Texas Red–labeled streptavidin was as described for microtubule staining, however a 1:5 dilution of the monoclonal first antibody was used.

The stained cells were viewed using a photomicroscope III (Carl Zeiss Inc., Thornwood, NY), equipped with epifluorescent illumination and a filter suitable for rhodamine. Photomicrographs were taken using Tri-X-Pan ASA 400 film (Eastman Kodak Co., Rochester, NY).

## Results

Preliminary studies with a variety of fixation procedures revealed that no single procedure gave optimum results with all three filament systems. The different procedures used with each type of filament were selected or modified from existing procedures based on the criteria of integrity of structure, continuity of filaments, and brightness and clarity of image. Microfilament-containing stress fibers stained in cells that had been fixed with methanol appeared faint and fragmented or punctate. However the same fibers stained in cells fixed with paraformaldehyde, the procedure used routinely, appeared bright and continuous. In contrast smoothly curving microtubules were only seen in cells fixed in methanol. At no time were the cells allowed to dry, because this distorted both the shape of the cell and the orientation of the filaments.

### Alterations in Microfilament Organization

Treatment of primary rabbit chondrocytes with 1 μg/ml RA resulted in loss of the differentiated chondrocyte phenotype and replacement by a modulated phenotype. This was accompanied by the loss of the polygonal morphology and colony forming capacity that are characteristic of control primary chondrocytes (4). The RA-modulated chondrocytes formed a uniform and continuous monolayer and showed no colony formation even after 14 d of primary culture. This change in morphology was accompanied by a profound alteration in microfilament/stress fiber architecture. The unoriented stress fiber pattern of control chondrocytes (Fig. 1 *a*) was replaced by a more ordered array of fibers concentrated at, and aligned with, the cell periphery. Cell boundaries, often obscured in control cells, were also very distinct in RA-modulated chondrocytes (Fig. 1 *b*), a reflection of the marked contact inhibition of growth observed in these cultures.

Reexpression of the differentiated phenotype was induced in secondary culture, following the removal of RA, by culture in the presence of $3 \times 10^{-6}$ DHCB (6). After 7 d the pattern of stress fiber organization induced by RA had been lost (Fig. 1 *c*). The total number of fibers was significantly reduced and those that remained were aligned with the longitudinal axis of the cell in a near parallel array. Focal condensations of actin were also present in many of the cells. RA-modulated chondrocytes cultured for this period of time in the absence of RA and DHCB, maintained much of the RA-induced pattern of stress fiber organization. Cell boundaries remained distinct and the fibers were still concentrated at the cell periphery (Fig. 1 *d*).

By 14 d of culture in the presence of DHCB a further decrease in the number of fibers was noticed, however some long fibers remained in alignment with the longitudinal axis and some overgrowth of cells was evident (Fig. 1 *e*); indeed phase contrast micrographs of these cells revealed limited colony formation (6). Cells cultured in the absence of DHCB for 14 d had lost much of the RA-induced pattern of stress fiber organization, and contact inhibition of growth was less marked (Fig. 1 *f*). However these cells still retained an extensive, though more uniform, meshwork of stress fibers, and cell boundaries were still detectable.

### Alterations in Microtubule Organization

In contrast to the microfilament/stress fiber network, the organization of microtubules was largely unaffected by either RA or DHCB additions to the cultures. Both control (Fig. 2 *a*) and RA-modulated chondrocytes (Fig. 2 *b*) were shown to contain extensive networks of these filaments. Again RA-modulated cells showed clearer cell–cell separation than control cells. After 14 d of secondary culture in the presence of DHCB the cells still displayed a well-ordered and apparently intact microtubule cytoskeleton (Fig. 2 *c*), closely resembling that seen in cells cultured in the absence of DHCB (Fig. 2 *d*). This shows the integrity of the microtubule net-

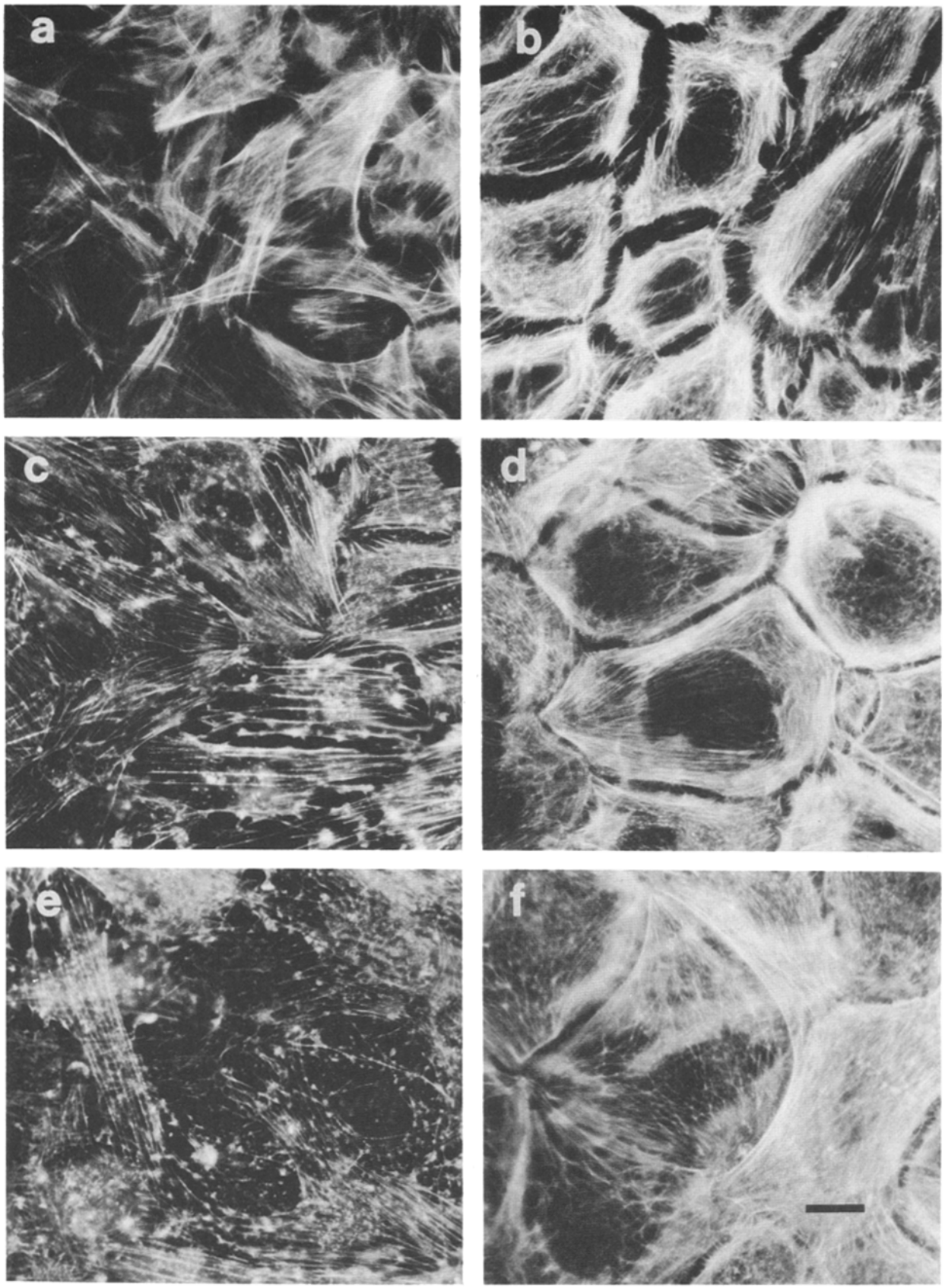


*Figure 1.* Fluorescent staining of actin-containing microfilament bundles with rhodamine-labeled phalloidin in rabbit chondrocytes. Cells were grown in primary and secondary culture in the absence (*a*) and presence (*b*) of RA. The RA-modulated cells were then cultured in the absence of RA and in the absence (*d* and *f*) and presence (*c* and *e*) of DHCB for 7 (*c* and *d*) and 14 d (*e* and *f*). Bar, 20 μm.

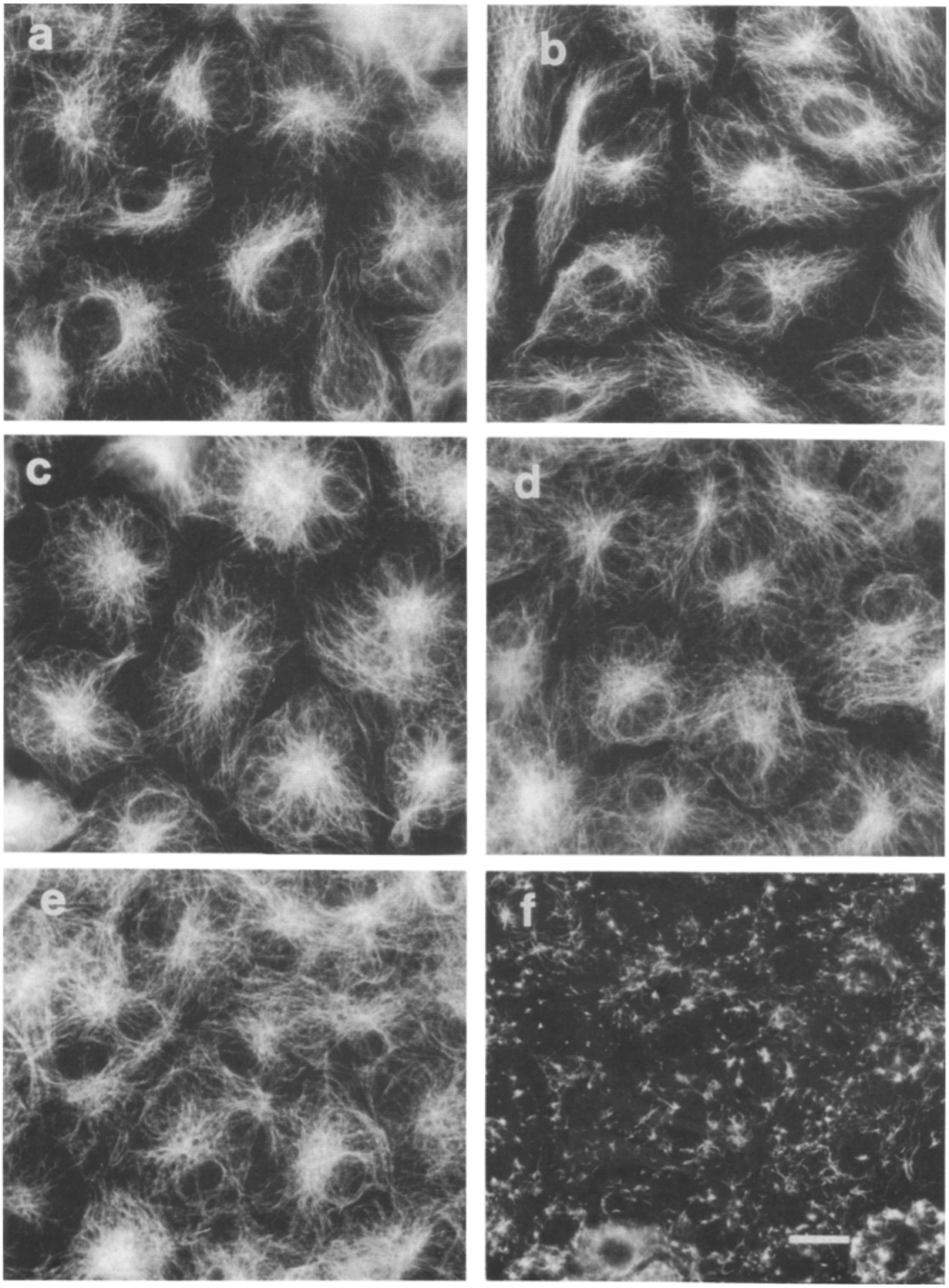


*Figure 2.* Immunofluorescent staining of microtubules in rabbit chondrocytes. Cells were grown in primary and secondary culture in the absence (*a*) and presence (*b*) of RA. The RA-modulated cells were then cultured in the absence of RA and in the absence (*d*) and presence (*c*) of DHCB for 14 d. RA-modulated cells were also cultured for 7 d in the absence of RA and in the absence (*e*) and presence (*f*) of the microtubule disrupting agent, colchicine. Bar, 20 μm.

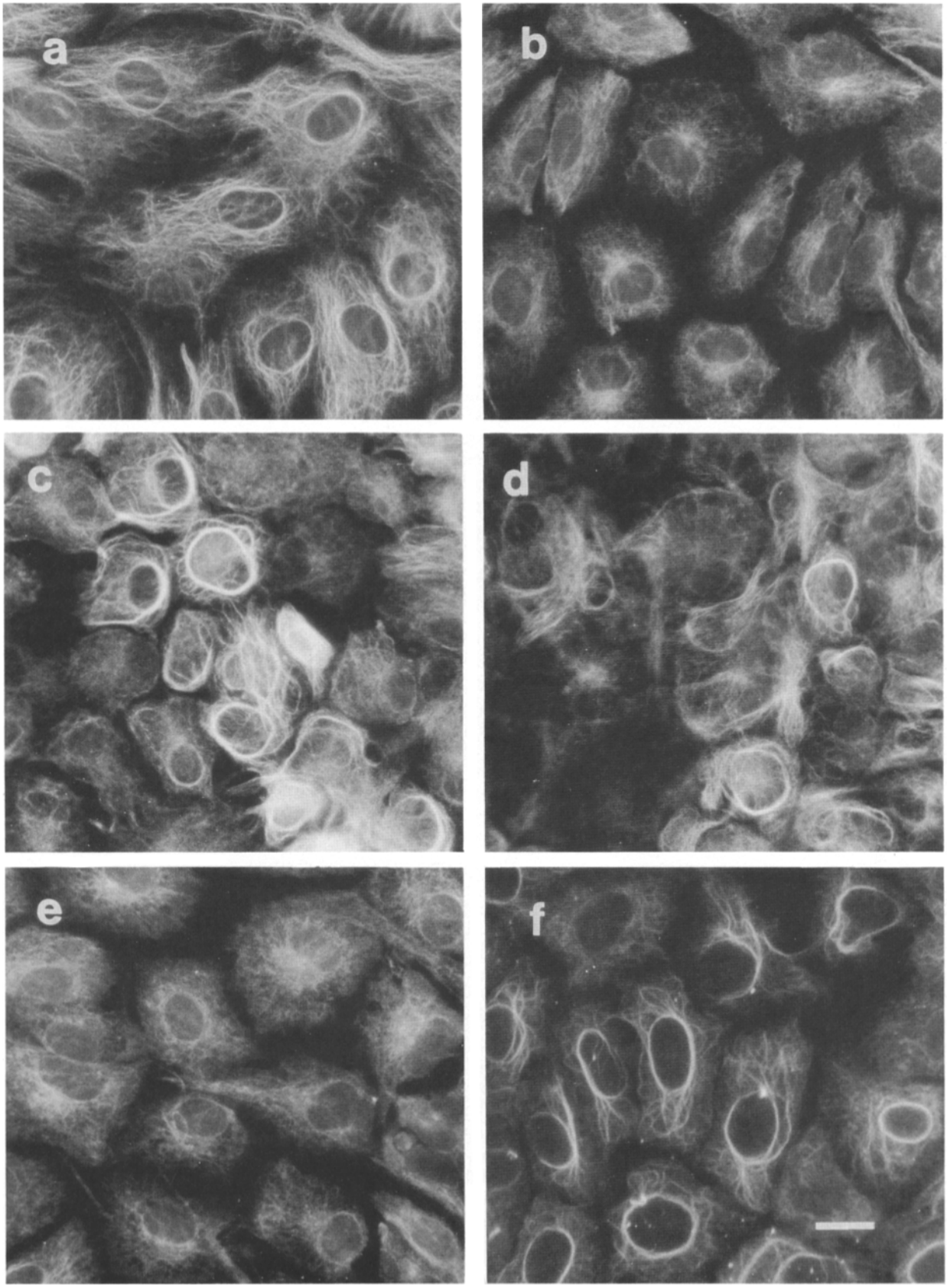


*Figure 3.* Immunofluorescent staining of intermediate filaments in rabbit chondrocytes. Cells were grown in primary and secondary culture in the absence (*a*) and presence (*b*) of RA. The RA-modulated cells were then cultured in the absence of RA and in the absence (*d*) and presence (*c*) of DHCB for 7 d. RA-modulated cells were also cultured for 2 d in the absence of RA and in the absence (*e*) and presence (*f*) of colchicine. Bar, 20 μm.

work in these cells to be independent from that of the microfilament/stress fiber organization. This independence has been observed in a variety of other cell types including mouse 3T3 cells and rat mammary fibroblasts (24). The specificity of the antibody staining of microtubules was demonstrated by culture of the cells in the presence of the microtubule disrupting agent, colchicine. RA-modulated chondrocytes were cultured for 7 d in the absence of RA, and in the absence (Fig. 2 *e*) and presence (Fig. 2 *f*) of $1 \times 10^{-6}$ M colchicine.

### *Alterations in Intermediate Filament Organization*

The formation and organization of the intermediate filament cytoskeleton, although modulated by RA, was not significantly affected by the addition of DHCB to the cultures. The formation of the well-developed and extensive intermediate filament network seen in control primary chondrocytes (Fig. 3 *a*) was clearly suppressed in RA-modulated cells (Fig. 3 *b*). The latter showed only faintly staining structures located near the nucleus. This is consistent with other studies, where vimentin synthesis and filament formation appeared to be responsive to changes in cell density and cell growth (3).

Continued culture of RA-modulated chondrocytes for 7 d in the absence (Fig. 3 *d*) or presence of DHCB (Fig. 3 *c*) resulted in limited formation of condensed intermediate filament structures, with only diffuse staining in the majority of cells. No further alterations in intermediate filament architecture were observed by 14 d. Again the specificity of intermediate filament staining was demonstrated by treatment of the cells with colchicine. RA-modulated chondrocytes were cultured for 2 d in the absence of RA, and in the absence (Fig. 3 *e*) and presence (Fig. 3 *f*) of $1 \times 10^{-6}$ M colchicine. The perinuclear rings of fluorescence are characteristic of vimentin-containing intermediate filaments in colchicine-treated cells (21).

### *Time Course of DHCB Effects on Microfilament Organization*

The rate of action of DHCB on microfilament organization was assessed in RA-modulated chondrocytes cultured in the absence of RA and in the presence of DHCB for 2, 24, and 48 h. After 2 h the DHCB-treated cells had lost some intensity of staining and fiber definition (Fig. 4 *b*) when compared with RA-modulated chondrocytes cultured in the absence of both RA and DHCB (Fig. 4 *a*). After 24 h of DHCB treatment the characteristic pattern of stress fiber organization induced by RA was less detectable (Fig. 4 *c*), and had been lost completely by 48 h (Fig. 4 *d*). The RA-induced pattern was replaced with parallel arrays of condensed fibers aligned with the longitudinal axis of the cell and which appeared to be aligned with the same axis of neighboring cells. Changes after 48 h were less substantial, and the stress fiber organization after 7 and 14 d of DHCB treatment showed similar features.

### *Effect of Serum on Microfilament Modification by DHCB*

Analysis of the collagen phenotype of cells cultured with DHCB in medium containing low levels of FBS (1 and 2%), has revealed a complete lack of reexpression of the differentiated phenotype, indicating a requirement for some serum component (6). Therefore the effect of DHCB on microfilament architecture was examined during culture in 2% FBS. The microfilament/stress fiber organization of these cells appeared equally affected by DHCB treatment. After 2 d cells cultured in the absence of DHCB still retained the RA-induced stress fiber organization (Fig. 4 *e*), whilst those cultured in the presence of DHCB (Fig. 4 *f*) showed a pattern of stress fiber organization very similar to that seen in DHCB-treated cells cultured in medium containing 10% serum (Fig. 4 *d*).

## *Discussion*

The immunofluorescent studies presented support the involvement of microfilaments in the modulation of the chondrocyte phenotype, these structures being modified initially by the presence of RA and again during reexpression of the differentiated phenotype by DHCB. In contrast the organization of microtubules was unaffected by either RA or DHCB treatment. Similarly the organization of intermediate filaments, although modulated by RA treatment, was largely unaffected by the presence of DHCB. The modifications in microfilament/stress fiber architecture induced by DHCB occurred within 2 d and preceded both an increase in proteoglycan synthesis and reexpression of type II collagen which occurred after 4 and 6 d of DHCB treatment, respectively (6). This sequence of events and the specificity of DHCB strongly support a causative role for microfilament modification in the reexpression of the differentiated chondrocyte phenotype. The alterations in microfilament organization in the absence of DHCB were less marked and more gradual, and resulted in the synthesis of 10-fold less type II collagen (6). Thus, loss of the RA-induced peripheral filament morphology alone is an inadequate stimulus for reexpression. Clearly, specific structural alterations caused by DHCB are required to free the cells from the residual aspects of the RA-induced phenotype.

These specific alterations and the subsequent induction of reexpression occur in the absence of cell rounding or retraction (6). This dissection of microfilament modification from the events of cell shape change emphasizes the importance of microfilament architecture in the mechanism of reexpression and suggests that shape-dependent changes in chondrocyte phenotypic expression are mediated by this mechanism. This is conceptually supported by a recent study in which it was demonstrated that the induction of procollagenase in cultured fibroblasts was dependent on microfilament disruption but could be independent of spherical morphology. The dissection of these events was achieved by colchicine treatment of cells embedded in collagen which induced spherical morphology without significantly altering the cortical distribution of microfilaments, and without inducing procollagenase synthesis (23).

The present study also demonstrates that DHCB induces essentially identical microfilament rearrangements in both 2 and 10% FBS. This is important because significant DHCB-dependent reexpression is only observed in 10% serum (6). Thus, the failure of 2% serum-treated cultures to reexpress is apparently due to the absence of a required serum factor rather than a deficit in the initial microfilament signal for reexpression.

In considering the modulatory effects of RA on the cyto-

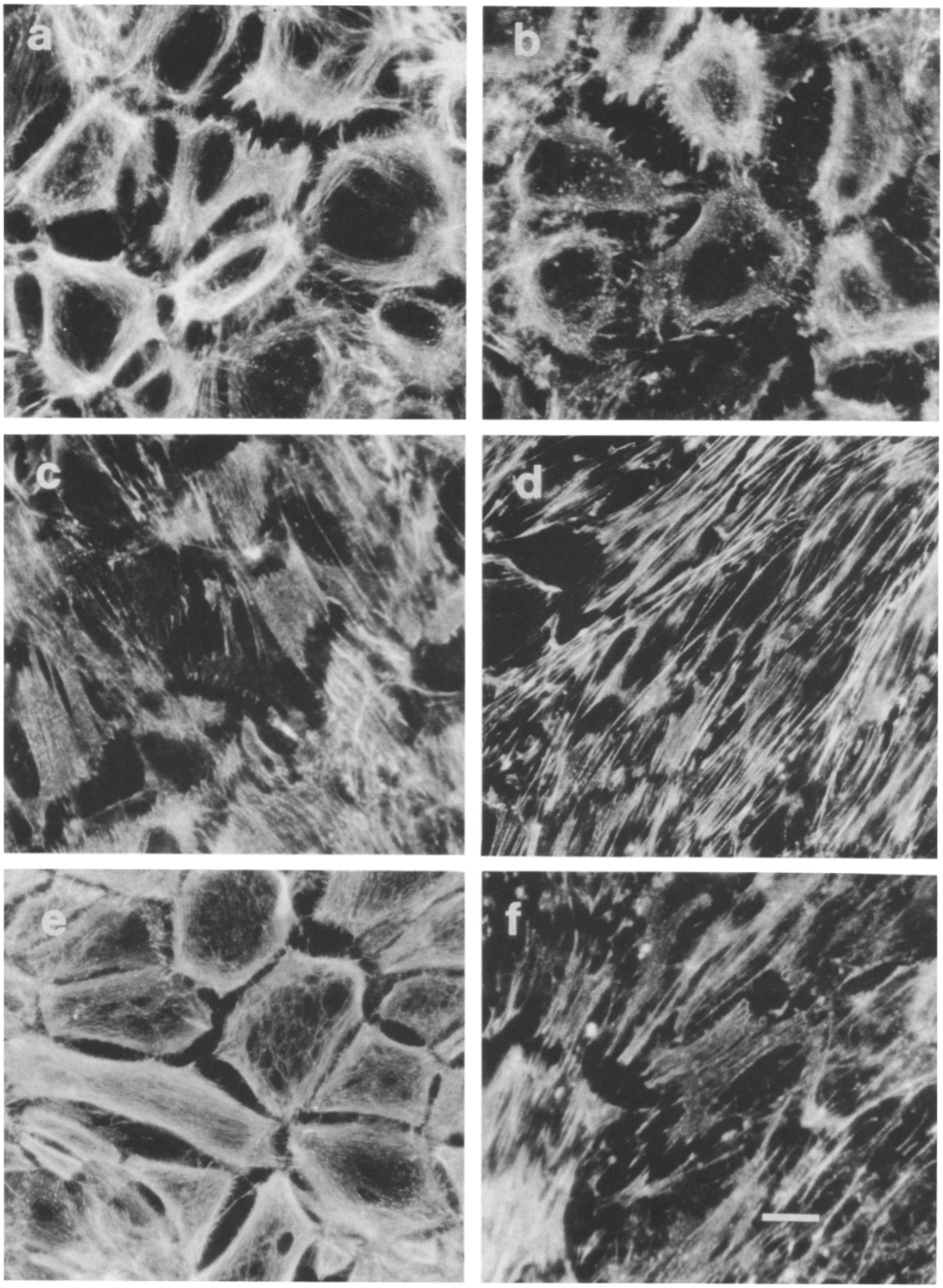


*Figure 4.* Fluorescent staining of actin-containing microfilament bundles in RA-modulated chondrocytes with rhodamine-labeled phalloidin. Cells were cultured in the absence of RA and in the absence (*a* and *e*) and presence (*b*, *c*, *d*, and *f*) of DHCB for 2 (*a*, *b*, and *e*), 24 (*c*), and 48 h (*d* and *f*). Cells shown in (*e*) and (*f*) were cultured in medium containing 2% FBS. Bar, 20 μm.

skeletal organization and phenotypic expression of chondrocytes it is important to note that the mechanism of action of this compound remains unclear. The suppressive effect of RA treatment on the formation of intermediate filaments is probably a result of the contact inhibition of growth exhibited by these cells. Previous studies have shown vimentin synthesis and intermediate filament formation to be highly responsive to cell density in monolayer culture. Bovine epithelial cells grown in sparse monolayer culture contained an extensive intermediate filament network as detected by immunofluorescence, however the same cells grown in high-density monolayer culture contained only very limited intermediate filament structures (3). Thus cell–cell contact may directly suppress filament formation in RA-modulated chondrocytes. Control cells that do not exhibit contact inhibition of growth, and therefore may be generally less responsive to cell contact stimuli, possess more extensive networks of intermediate filaments.

The means by which RA is able to induce change in stress fiber organization may be related to its ability to modify the glycosylation of chondrocyte fibronectin, increasing the ratio of complex-type carbohydrate chains to high-mannose oligosaccharides (8). This represents a shift towards fibroblast fibronectin, which contains only complex-type carbohydrates and might explain the increased ability of RA-treated chondrocytes to bind fibronectin to their surfaces (11). This increased binding ability might in turn be expected to effect the complex relationship between actin, vinculin, fibronectin, and the fibronectin receptor (10, 20) resulting in increased regions of focal contact or adhesion plaques, hence causing a reordering of the actin containing stress fibers. Such changes in cell adhesion/shape or microfilament organization may subsequently contribute to the mechanism of phenotypic modulation.

The addition of fibroblast-derived fibronectin to chick embryo chondrocyte cultures has been shown in previous studies to enhance cell adhesion and spreading, and increase the rate of phenotypic modulation (26). This appears to be a specific effect of cellular fibronectin, because plasma fibronectin enhances adhesion without causing detectable modulation (25). Thus RA may be accelerating both the processes of cell adhesion and phenotypic modulation that occur more gradually during serial monolayer culture (7). Indeed it has been observed in the current study that RA-treated cells show enhanced adhesion in primary culture. However it should be noted that the pattern of stress fiber organization in these cultures is very different from that seen in subculture-modulated chondrocytes, the latter consisting of very brightly staining fibers aligned in parallel and running throughout the cell (unpublished observations). In addition important differences in collagen phenotype exist between these kinds of modulated chondrocytes (4).

A more direct role for RA in phenotypic modulation has been proposed in a recent report in which it was demonstrated that RA was able to modulate the phenotype of chick sternal chondrocytes maintained in a spherical conformation by culture in methyl cellulose (12). The authors conclude that RA exerts its modulatory effect in a direct manner, independent of changes in cell shape or adhesion. The results presented here do not contradict this conclusion, and it is probable that in the current monolayer study, RA is operating through a combination of direct and indirect effects. However the study by Horton and Hassell does not rule out the possibility that phenotypic influence is mediated by the binding of RA-modulated fibronectin to spherical chondrocytes. Indeed a role for microfilament/surface fibronectin interactions in the modulation of the chondrocyte phenotype is supported by studies in which chondrogenesis of proximal limb bud cells in hydrated collagen gels was inhibited by fibronectin-containing gels (22), and released from this inhibition by treatment with cytochalasin D (27).

The effects of DHCB on cytoskeletal organization have been shown to be relatively specific; glucose transport being unaffected by concentrations of DHCB that caused marked changes in microfilament structures (2). Other studies have demonstrated the ability of cytochalasins to inhibit or reduce the rate of actin polymerization and interfilament cross-linking (9, 15). It has also been reported that the disruption of microfilaments by cytochalasin B causes the release of fibronectin from the surface of cells in culture (1), presumably as a result of the disruption of the actin–vinculin–fibronectin complexes that have been located at focal contact points in growth arrested cells (20). We have recently demonstrated by immunofluorescence that RA-modulated rabbit chondrocytes accumulate increased amounts of surface-bound fibronectin relative to control cells and that DHCB treatment results in a rapid loss of this bound fibronectin (unpublished observations). Thus DHCB treatment of RA-modulated chondrocytes can induce subtle changes in fibronectin distribution without inducing overt changes in cell shape.

In furthering the understanding of these mechanisms of phenotypic regulation it will be important to discriminate between changes in cell shape, cell adhesion, cell–cell contact, and cytoskeletal organization. The results presented here have allowed some dissection to be made between the effects of cell shape and microfilament organization. Further analysis of the sequence of changes following DHCB-induced microfilament modification, and the identification of the site of action of the required serum component should allow a better understanding of this mechanism of regulation and its biological significance.

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