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Cell Shape and Phenotypic Expression in Chondrocytes (41533)

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Abstract. The relationship between cell shape, proliferation, and phenotypic expression was studied in human chondrocytes. Shape was controlled independent of serum concentration, anchorage, and cell density by alteration of substratum adhesiveness with poly(2-hydroxyethyl methacrylate) (poly[HEMA]). Cells that were held rounded displayed features of the chondrocyte phenotype; i.e., they were round, proliferated slowly, incorporated low levels of [^{3}H]thymidine into DNA, and incorporated large amounts of $^{35}SO_4$ into glycosaminoglycans. In contrast, cells that were held flat were fibrolast-like: they exhibited flattened morphology, more rapid growth, greater incorporation of [^{3}H]thymidine, and less incorporation of $^{35}SO_4$. These studies suggest that cell shape may play an important role in phenotypic expression in chondrocytes.

Attempts to maintain avian embryonic (1) or mammalian (2) chondrocytes in monolayer systems have been frustrated by the dedifferentiation of these cells. Successful maintenance of the chondrocyte phenotype in culture is defined (3) by (i) polygonal, not fibroblastic, morphology; (ii) deposition of metachromatic matrix; (iii) synthesis of type II collagen; and (iv) the quantity and type of sulfated glycosaminoglycans synthesized (4). Certain culture conditions will permit the expression of the chondrocyte phenotype *in vitro*: suspension culture (5), or high initial plating density (6), or supplements of growth hormone (7). Furthermore, it has recently been shown that dedifferentiated rabbit articular chondrocytes can reexpress the differentiated phenotype after suspension culture (8). It occurred to us that an alteration in cell shape was common to all these conditions. We have previously shown that cell shape is correlated with proliferation of normal cells (9–11). We now show that phenotypic expression by chondrocytes is a function of cellular conformation, independent of cell density and anchorage. It was possible to maintain different cell shapes *in vitro* by coating tissue culture dishes with different dilutions of poly(2-hydroxyethyl methacrylate) (poly[HEMA]) and thereby altering substratum adhesivity. The results suggest that cell shape may regulate both proliferation and phenotypic expression in chondrocytes.

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Materials and Methods. *Cell culture.* Pieces of human rib cartilage were obtained from children undergoing surgical correction of pectus excavatum. The tissues were soaked for 10 min in Betadine solution, washed with Ringer's solution, and cleaned of adherent connective tissue. The cartilage was minced to 2-mm^2 pieces and incubated overnight at 37° in 0.2% collagenase (Worthington) in phosphate-buffered saline (PBS) containing penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). The suspension of cells was passed through a 153μm nylon sieve (Tetko) to remove debris and undigested fragments of cartilage. Cells were washed twice with PBS and plated at 2×10^4 cells per 35-mm tissue culture dish in Dulbecco's medium (Grand Island Biologicals) with 10% fetal calf serum (Microbiological), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 m*M*). Cells were prepared for poly[HEMA] experiments by 48 hr preincubation, after which the attached cells were harvested by trypsinization [0.2% trypsin (GIBCO) in PBS] and transferred to poly[HEMA]-coated dishes.

Fibroblasts were grown from explants of human foreskin in Dulbecco's medium supplemented with 10% calf serum (GIBCO), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 m*M*). Seventh passage cells were used in these studies.

Poly[HEMA] dishes. Thirty-five millimeter Falcon tissue culture dishes were coated with various dilutions of a 12% solution poly(2-hydroxyethyl methacrylate) (poly[HEMA]) as

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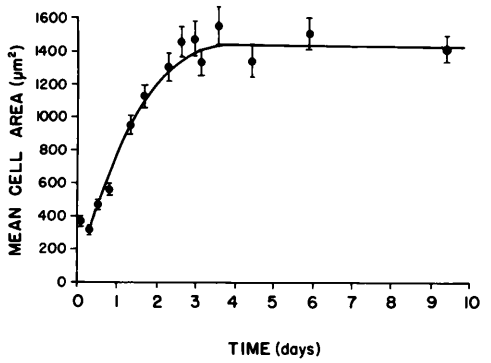

FIG. 1. Kinetics of spreading of human chondrocytes on plastic at 37°. Each point represents the mean area of 60 to 100 cells ± SEM.

previously described (11). The dilutions of 10^{-3} and 8×10^{-8} were chosen for chondrocytes because they permitted sufficient attachment (83 and 22%, respectively) and the most extreme differences in cell shape. Cells were sparsely plated at 2×10^4 cells/1.5 ml/dish in order to minimize cell–cell interactions. Because chondrocytes required some time to establish morphologic equilibrium on uncoated tissue culture dishes (Fig. 1), all experiments were conducted after 48 hr preincubation.

[^{3}H]Thymidine incorporation. After 48 hr preincubation, [*methyl*-^{3}H]thymdine (6.7 Ci/mmole, New England Nuclear Corp.) was added to three sets of dishes in 0.5 ml fresh medium at a final concentration of 1 μCi/ml. The incorporation of [^{3}H]thymidine into DNA over 14 hr was measured for attached cells as well as for those cells suspended in overlying medium. Supernatant media containing suspended cells were centrifuged. The pelleted cells as well as the cells attached to the dishes were washed twice with PBS, twice with cold methanol, and twice with cold 5% trichloroacetic acid. The cells were dissolved with 0.5 ml of 0.3 *N* NaOH. Ten milliliters of Instagel (Packard, Inc.) was added and the radioactivity measured. Values are expressed as the means of triplicate measurements.

^{35}Sulfate incorporation. ^{35}Sulfate incorporation into glycosaminoglycans by attached cells was evaluated by the method of Meier and Solursh (12). After 56 hr incubation, $H_2{}^{35}SO_4$ (New England Nuclear) was added to three sets of dishes in 0.5 ml fresh medium per dish to a final concentration of 2 μCi/ml. After 6 hr, the dishes were washed three times with PBS, fixed for 10 min in Kahle's solution, rinsed three times with PBS, and incubated at 37° with shaking in 1 ml of hyaluronidase (Worthington) at a concentration

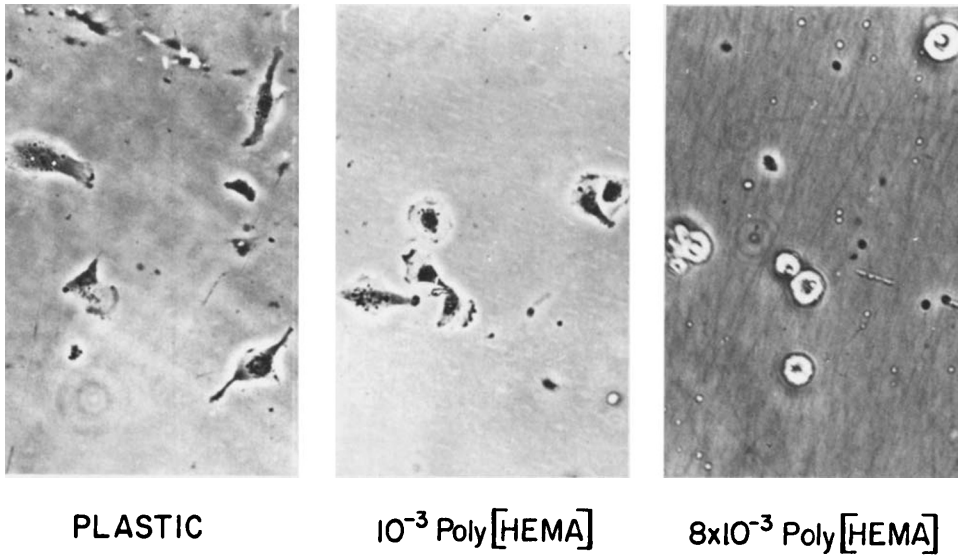

FIG. 2. Effect of poly[HEMA] on spreading of chondrocytes after 60 hr incubation. Dilution of poly[HEMA] indicates the dilution of 12% stock solution used to coat the dishes. Photographs are 100× magnification.

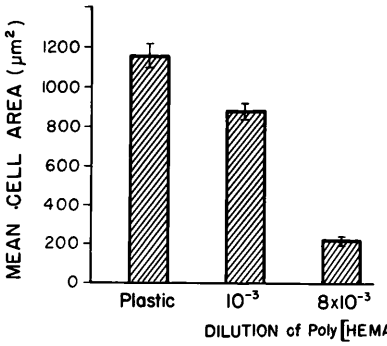

FIG. 3. Effect of poly[HEMA] on mean cell area after 60 hr incubation.

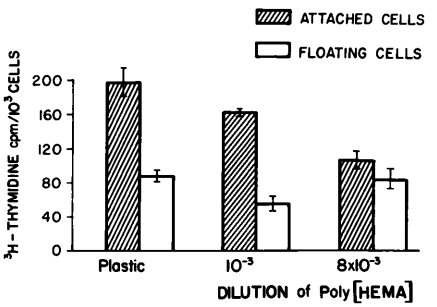

FIG. 4. Effect of poly[HEMA] on incorporation of [^{3}H]thymidine by chondrocytes after 60 hr incubation. The values for floating cells reflect baseline incorporation that is not related to cell division.

of 0.5 mg/ml in 0.5 *M* phosphate buffer (pH 5.63). The supernatant solutions which contained extracellular hyaluronidase-sensitive material were counted in 10 ml Instagel (Packard). Nonadherent cells were not evaluated. Values are expressed as the mean of triplicate measurements.

Cell counts. Supernatant media containing nonadherent cells were collected from three sets of dishes at the end of the experiments. After centrifugation, the pelleted cells, as well as the dishes, were incubated for 10 min in 0.5 ml 0.25% trypsin in PBS. The cells were counted with a Coulter counter. Incorporation data are expressed as counts per minute of ^{3}H or ^{35}S per 10^3 attached cells. In one case, [^{3}H]thymidine incorporation by floating cells was calculated as background uptake by nondividing cells.

Morphometrics. Cell area was assessed at the conclusion of each experiment by tracing cell outlines of unfixed cells projected onto a Zeiss-Kontron MOP digitized analyzer. These measurements were made on replicate dishes; values are expressed as mean cell area ± SEM for 50–100 cells. Photographs were taken with a phase-contrast inverted Nikon microscope at 100× magnification.

Results. Chondrocytes that had been incubated for 60 hr on dishes coated with 10^{-3} or less poly[HEMA] were flat; cells on dishes with 8×10^{-3} poly[HEMA] were attached but rounded (Figs. 2, 3). Maintenance of cells in the rounded configuration in this manner resulted in the expected decrease in [^{3}H]thymidine incorporation (Fig. 4). This decrease was significant ($P < 0.05$) and the amount of radioactivity incorporated by attached round cells was equivalent to the background uptake over 14 hr by floating, nondividing cells in all dishes. The rounded chondrocytes showed greater incorporation of $^{35}SO_4$ into glycosaminoglycans, a phenotypic marker, than did the flattened cells ($P < 0.05$; Fig. 5). This shows that round cells are not metabolically inhibited in a generalized manner, but produce more of the cartilage-specific extracellular matrix.

Control experiments with human foreskin fibroblasts were performed to demonstrate that $^{35}SO_4$ incorporation and its modulation by cell shape represented phenotypic expression specific to the chondrocytes. Although [^{3}H]thymidine incorporation was reduced when fibroblasts were maintained in the spherical configuration, as expected from previous work (11), $^{35}SO_4$ incorporation was low and independent of cell shape (Figs. 6–8).

Discussion. These experiments show that flat human chondrocytes synthesize DNA at

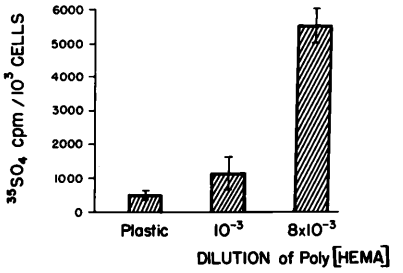

FIG. 5. Effect of poly[HEMA] on $^{35}SO_4$ incorporation into glycosaminoglycans by chondrocytes. Dilution of poly[HEMA] indicates the dilution of 12% stock solution used to coat the dishes.

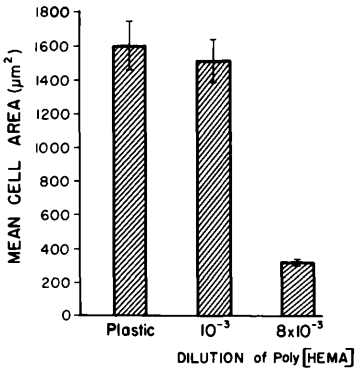

FIG. 6. Effect of poly[HEMA] on spreading of human fibroblasts after 60 hr incubation. Each value represents the mean area of 70 cells ± SEM. Dilution of poly[HEMA] indicates the dilution of 12% stock solution used to coat the dishes.

a relatively high rate and produce sulfated glycosaminoglycans at a low rate. Cells that had been maintained at low density as attached spheres, on dishes coated with poly[HEMA], incorporated less thymidine yet incorporated more sulfate into glycosaminoglycans. Control experiments with human fibroblasts provide evidence that the shape-dependent production of sulfated glycosaminoglycans is a specific function of chondrocytes and not a general property of cells. Thus, changes in cell shape alone can modify function specific to a cell's phenotype. That matrix production could be directly influenced by cell proliferation rates is consistent with the view that differentiation follows cessation of cell division (6).

Having advanced the argument that cell shape plays an important role in growth control and in gene expression, we caution that "cell shape" may be a misleading term. We use it because it is what we measure. While the measurements of shape are correlated with both cell proliferation and gene expression, it seems likely that some as yet unknown process associated with change in cell shape effects the appropriate biochemical change. For example, a very small change in tension of certain parts of the cytoskeleton may produce effects on cell proliferation rates or lead to large changes in rate of protein synthesis.

The concept of cell shape helps to explain previous studies which demonstrated that spherical chondrocytes in suspension exhibit more differentiated properties than do flat cells in monolayer (5, 8). The studies with suspension cultures had raised the question of a relationship between attachment to substratum and differentiated phenotype. The current studies of rounded chondrocytes attached to dishes coated with poly[HEMA] eliminate the possibility that anchorage of cells to the substratum *alone* can regulate growth and differentiation. Monolayer growth in the presence of serum is very unlike the environment *in vivo* for chondrocytes where the cells are spherical and in an avascular milieu. In addition, chondrocytes do not synthesize large amounts of the cell surface glycoprotein, fibronectin (12). Yet, when grown *in vitro* in the presence of fibronectin, chondrocytes assume the dedifferentiated, fibroblastic morphology and biochemical phenotype (14, 15). The presence of fibronectin in serum may explain the dedifferentiation of chondrocytes in monolayer cultures (16). Recent studies have shown that the fibronectin effect on chondrocyte dedifferentiation is not seen in agarose cultures where a flattened morphology is not possible (8). Thus, changes in cell shape can account for phenotypic expression.

Similarly, colonies of chondrocytes in monolayer at high cell density had been shown to display heterogeneity of cell shape, rate of proliferation, and production of metachromatic matrix (6). The cells in the center of the colonies are more crowded and hence less spread than those at the periphery. These central cells are the cells that produce metachromatic matrix. Elimination of the variables of cell density and cell-to-cell contacts in the

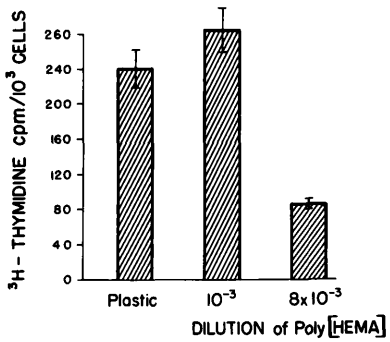

FIG. 7. Effect of poly[HEMA] on [³H]thymidine incorporation by fibroblasts after 60 hr incubation.

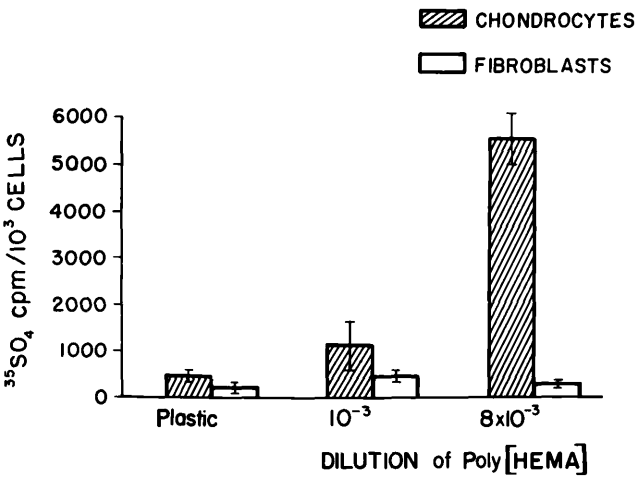

FIG. 8. Effect of poly[HEMA] on $^{35}SO_4$ incorporation into glycosaminoglycans by fibroblasts, compared to chondrocytes. Dilution of poly[HEMA] indicates the dilution of 12% stock solution used to coat the dishes.

present communication reveals that cell shape is sufficient to explain the phenomenon of variability in chondrocyte properties in tissue culture.

The control experiments demonstrated that the coupling of cell shape and the production of sulfated glycosaminoglycans is a property of differentiated chondrocytes but not of fibroblasts. Although the sulfated glycosaminoglycans have not been isolated and characterized, it is unlikely that their increased production by round chondrocytes is a nonspecific effect; were it due to general phenomena, such as increased sulfate pools or nonspecific sulfation, the fibroblasts would be expected to show the same changes in response to cell shape. We have shown, furthermore, that rabbit ear perichondrocytes but not fibroblasts can be induced to make sulfated glycosaminoglycans by changes in cell shape (17). This *in vitro* result is consistent with the observations that perichondrium is a relatively differentiated tissue that can make neocartilage when transplanted to an avascular site (17).

The correlation between cell shape and phenotypic expression provides a more basic interpretation of phenomena reported in other systems *in vitro*. Bennett *et al.* demonstrated that rat mammary tumor cells began to produce milk proteins as the cells become crowded (18). They also showed that the crowded, differentiated cells are more spherical than cells at low density. Emerman *et al.* showed that mouse mammary epithelial cells grown on a collagen membrane produce casein after the membrane is released from the plastic substratum (19). By this technique, the membrane contracts to one-eighth of the original area, thereby altering the shape of the overlying cells. More recently, the morphology of these cells has been linked to their ability to respond to their hormonal milieu (20). Therefore, the coupling of conformational signals and phenotypic expression may operate more generally in cell biology.

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Note added in proof. Archer *et al.* (Cell Diff **11**: 245–251, 1982) have shown that there is a similar shape-dependent relationship in the differentiation of chick embryonic mesoblasts into chondroblasts.

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