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MODULATION OF HEPATOCYTE FUNCTION BY CHANGING THE CELL SHAPE IN PRIMARY CULTURE

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SUMMARY

To study the role of cell shape in control of hepatocyte function, we have developed a system that can quantitatively control the spreading of cultured rat hepatocytes using poly[2-hydroxyethyl methacrylate]. When hepatocytes were cultured in a dish coated with high concentration of poly[2-hydroxyethyl methacrylate] solution, formation of stress fibers were suppressed and they continued to have a compact shape. In the compact-shaped hepatocytes, the ability to induce tyrosine aminotransferase with dexamethasone remained high for longer periods of time, as compared to the hepatocytes that spread following culture in the polystyrene dish. Conversely, the hepatocytes showed more active DNA synthesis when they assumed a flat shape as a result of spreading. When the hepatocytes that had spread following long-term culture in the polystyrene dishes were treated with cytochalasin to induce depolymerization of F-actin, the ability of the cells to induce tyrosine aminotransferase upon stimulation with dexamethasone improved markedly. This effect was not altered by treatment with actinomycin D but was completely suppressed by cycloheximide, suggesting that microfilaments are involved in the post-transcriptional process of tyrosine aminotransferase induction. Thus, there is a possibility that F-actin rather than cell shape might regulate cellular function in primary cultured hepatocytes.

Key words: liver; cytoskeleton; proliferation; tyrosine aminotransferase.

INTRODUCTION

The cells that constitute a variety of tissues have their own functions and possess a shape that is suitable for these cells to express these functions. The cell morphology is determined by the extracellular matrix (ECM) and the cytoskeleton. It is now being found that the ECM and the cytoskeleton are closely related to the cell functions (22). However, little is known about the relationship between cellular morphology and functions. Primary cultured hepatocytes serve as an excellent model for the study of the mechanisms that regulate cell proliferation and differentiation, because their proliferation activity, functions, and morphology change dramatically depending on the conditions of culture. It is known that the differentiation and proliferation of hepatocytes are regulated reciprocally, depending on the density of the cultured cells. That is, their proliferation is enhanced when their density is low, while their differentiation is enhanced when their density is high (19). It is also known that hepatocytes spread and assume a flatter shape when cultured at a low density, as compared to the cells cultured at a high density (12). Therefore, it is now unclear whether the dependency of hepatocyte proliferation and differentiation on the cell density reflects interactions among cells, as speculated by Nakamura et al. (18), or morphological changes of the cells.

Several attempts have been made to answer this question. In most previous studies, however, the cell morphology was regulated by changing the type of ECM used to coat the culture dish or by changing the density of cells in the culture (2,6,7,15,21). With such methods, it was not possible to distinguish the influence of morphological changes from the influence of the change in magnitude of ECM-cell or cell-cell interactions.

We recently developed a system that can quantitatively regulate the spreading activity of hepatocytes, using poly[2-hydroxyethyl methacrylate] (poly-HEMA), which inhibits cell adhesion but is a biochemically inactive molecule. This paper will show that the proliferation and differentiation of hepatocytes are regulated reciprocally with this system, depending on changes in the cell morphology. Furthermore, depending on the basis of an experiment using cytochalasin, this paper will illustrate the possibility that F-actin is involved in the synthesis of tyrosine aminotransferase, a liver-specific enzyme.

MATERIALS AND METHODS

Poly-HEMA coating of tissue culture dishes. Poly-HEMA was kindly supplied by Dr. Fumio Yamamoto (Kurare Co., Tokyo, Japan). The culture dishes were coated with poly-HEMA according to the method reported elsewhere (4). Poly-HEMA was dissolved in 70% ethanol to a concentration of 6%. This solution was diluted with ethanol to several concentrations before it was used to coat the culture dishes. In this paper, the concentrations of poly-HEMA used to coat culture dishes were expressed by the dilution ratios of the 6% poly-HEMA solution. For example, the expression "poly-HEMA (1.0×10^{-3})" means that the 6% poly-HEMA solution was diluted at a ratio of 1:1000. The diluted solution was poured into polystyrene tissue culture dishes (Corning Inc., Corning, New York, USA) and left standing overnight at 37° C. The volume of the diluted poly-HEMA solution was 3 ml for the 60-mm dish, 1 ml for the 35-ml dish, and 0.2 ml/well for the 24-well plate.

Isolation and primary culture of rat hepatocytes. Parenchymal hepatocytes from adult rats were isolated according to the methods reported elsewhere

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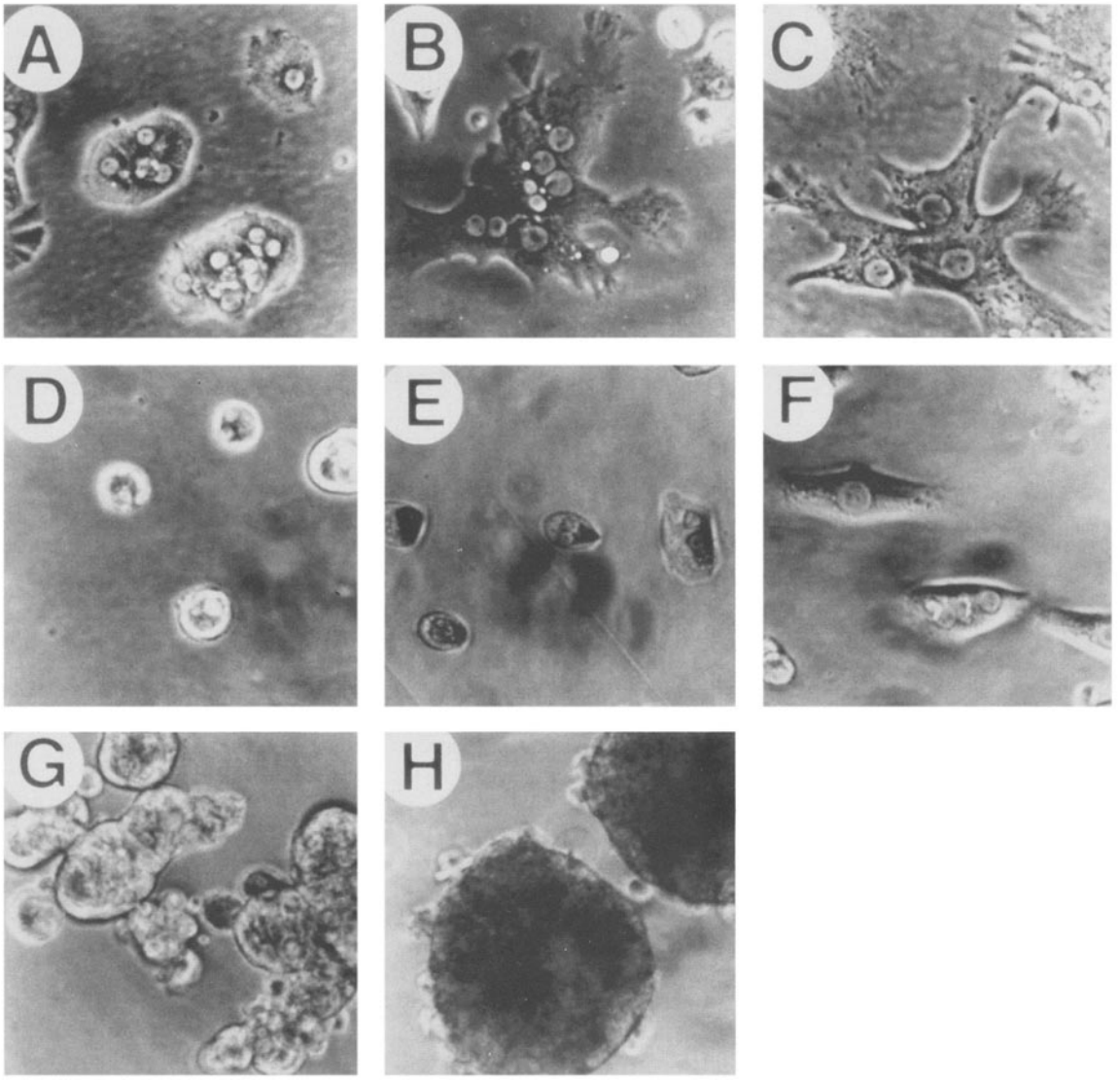

FIG. 1. Effect of poly-HEMA on spreading of hepatocytes. Phase contrast photomicrographs of adult rat hepatocytes cultured on normal polystyrene (*A–C*), poly-HEMA (0.15×10^{-3}) (*D–F*), or poly-HEMA (2×10^{-3}) (*G–H*) for 1 d (*A,D*), 2 d (*B,E,G*) or 3 d (*C,F,H*).

(23). The isolated cells were cultured at 37° C in 5% CO_2-95% air in L-15 medium supplemented with 5% fetal calf serum (FCS), L-proline (30 mg/ml), N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) (4.766 g/l), amphotericin B (0.25 mg/l), streptomycin (0.1 g/l), and penicillin (0.5 g/l). Hepatocytes isolated from newborn rats according to the method reported elsewhere (17) were also cultured in the same way as mature rat hepatocytes. The cell density in cultures was as follows: 60×10^4 cells/60-mm dish, 21×10^4 cells/35 mm dish, and 4.3×10^4 cells/well of a 24-well dish.

Measurement of the extent of cell spreading. After cells were plated, a photograph of each plate was taken at several points. Fifty areas of the photographic paper, precisely corresponding to areas covered by 50 cells, were cut and weighed. From the weight of the paper thus obtained, the average cell area was calculated.

Measurement of tyrosine aminotransferase (TAT) activity. Cells (60×10^4) were cultured in a 60-mm dish. Unless otherwise specified, dexamethasone (5×10^{-5} *M*, Wako) and dibutyl-cAMP (5×10^{-5} *M*, Wako) were added to the culture 24 h before TAT activity was measured. The harvested cells were disrupted with an ultrasonicator and centrifuged at 15 000 rpm for 10 min. TAT activity in the supernatant was measured by the method of Diamondstone (3). TAT activity was expressed as specific activity per the amount of protein measured according to the method of Lowry et al. (14).

Measurement of DNA synthesis. A cell proliferation kit (Amersham, Buckinghamshire, England) was used to measure the DNA synthesizing activity. Hepatocytes (21×10^4) isolated from 3-d-old rats were cultured in a 35-mm dish. Fifteen hours after the beginning of incubation, a BrdU solution was added to the culture. Twenty-four hours later, the number of cells positively stained with BrdU per 100 cells was counted to yield a labeling index.

Visualization of F-actin in hepatocytes. Cultured cells were fixed in ethanol at −20° C for 20 min. The cells were then washed in phosphate-buffered saline (PBS) and incubated in rhodamine-phalloidin (Sigma Chemical Co., St. Louis, MO), diluted with 5% bovine serum albumin (BSA)/PBS, for 1 h at room temperature. The cells were washed again in PBS and observed with a fluorescent microscope.

RESULTS

Regulation of hepatocyte spreading by poly-HEMA. The hepatocytes cultured on polystyrene (Pst) spread rapidly with time, while

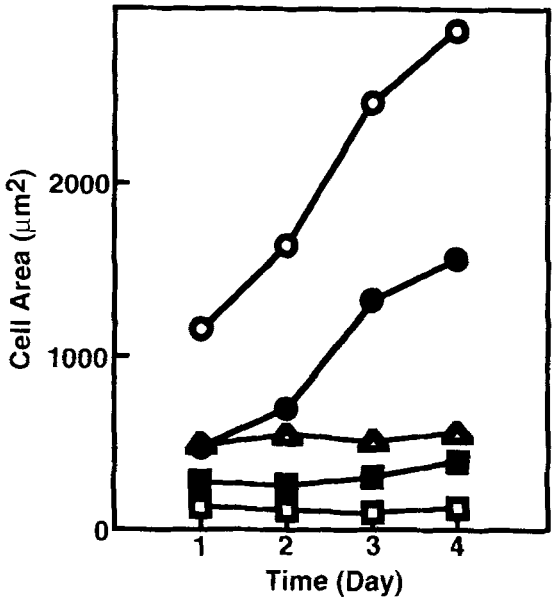

FIG. 2. Effect of poly-HEMA on spreading of hepatocytes. Hepatocytes were cultured on collagen-coated (*open circle*), normal polystyrene (*closed circle*), poly-HEMA (0.05×10^{-3}) (*open triangle*), poly-HEMA (0.15×10^{-3}) (*closed square*), or poly-HEMA (0.35×10^{-3}) (*open square*) dishes. Mean cell area of cells were calculated from weight of phase-contrast photomicrographs of 30 cells.

the hepatocytes cultured on poly-HEMA retained a relatively compact shape (Fig. 1). Fig. 2 shows the degree of spreading of hepatocytes cultured on each substrate. The cells cultured in the type-1-collagen coated dishes or the Pst dishes spread markedly on the 2nd and 3rd d of culture, while the spreading of the cells cultured on poly-HEMA was suppressed and the cells retained their initial shape. The suppression of spreading of the cells by poly-HEMA was dose dependent. At a poly-HEMA concentration of 0.35×10^{-3}, the cells spread little and retained their spheroid form until the 4th d. At poly-HEMA concentrations of 0.05×10^{-3} and 0.15×10^{-3}, the cells spread gradually. Thus, it was found that the spread of hepatocytes in cultures can be controlled by different degrees by changing the concentration of poly-HEMA that coats the culture dishes.

F-actin in hepatocytes cultured on poly-HEMA. To determine the mechanism of regulation of cell spread by poly-HEMA, we then studied the distribution of F-actin, which is known to be closely involved in the cell morphology (Fig. 3). Stress fibers, mainly consisting of F-actin, began to be noted on about the 3rd d after the beginning of culture, when the cells began to spread markedly. On the other hand, the cells cultured on poly-HEMA had formed few stress fibers even on the 4th d of culture. Thus, formation of stress fibers appeared to be suppressed in the cells showing a spheroid morphology cultured on poly-HEMA.

TAT induction and DNA synthesis in hepatocytes cultured on poly-HEMA. TAT (EC 2.6.1.5.) is a liver-specific enzyme that has been extensively studied. It is often used as an indicator of the degree of hepatocyte differentiation. To study the relationship between cell morphology and the expression of liver function, we examined hepatocytes cultured on Pst or poly-HEMA for the induction of TAT by dexamethasone (Dex) (Table 1 and Fig. 4). The TAT activity of hepatocytes cultured on Pst decreased with time. The decrease in Dex-stimulated TAT induction was particularly marked on the 1st through 3rd d of culture when the cells spread sharply. The hepatocytes cultured on poly-HEMA continued to have high TAT activity levels for relatively long periods of time. Fig. 4 graphically shows the Dex-stimulated TAT induction rate. For the hepatocytes cultured on Pst, the Dex-stimulated TAT induction rate decreased sharply from the

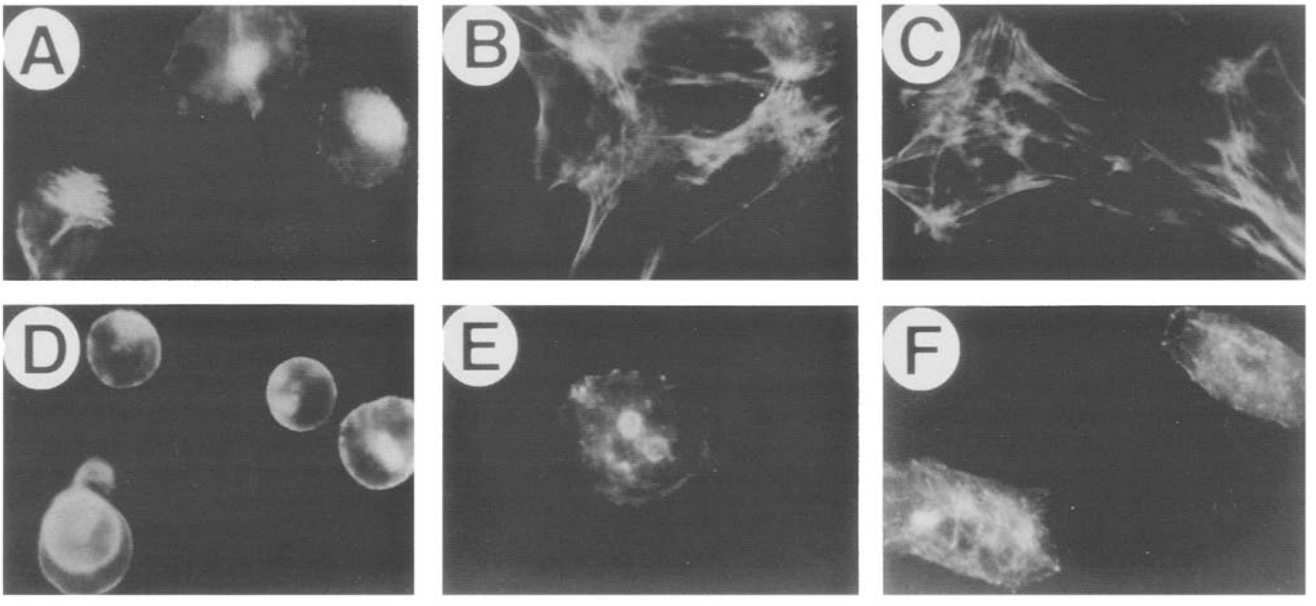

FIG. 3. Effect of poly-HEMA on formation of stress fiber. Hepatocytes cultured on polystyrene (*A–C*) or poly-HEMA (*D–F*) coated dishes were fixed after 1 d (*A,D*), 2 d (*B,E*), or 3 d (*C,F*) and stained with rhodamine-conjugated phalloidin.

TABLE 1

TAT ACTIVITY OF HEPATOCYTES CULTURED ON PST AND POLY-HEMA[a]

Time (Day)	Polystyrene Dish		PHEMA 0.1 × 10^{-3}		PHEMA 0.35 × 10^{-3}	
	Control	Dex + cAMP	Control	Dex + cAMP	Control	Dex + cAMP
1	13.0 ± 0.2	61.5 ± 7.5	15.3 ± 0.3	68.9 ± 1.1	15.8 ± 0.2	72.1 ± 0.8
3	10.8 ± 2.1	20.5 ± 3.0	14.0 ± 0.7	44.1 ± 1.0	15.6 ± 0.7	62.1 ± 1.2
5	9.2 ± 1.2	14.8 ± 0.5	10.4 ± 1.4	29.6 ± 1.7	14.6 ± 0.7	52.8 ± 2.4
7	7.6 ± 1.5	9.5 ± 1.4	9.9 ± 0.2	18.5 ± 1.0	12.0 ± 1.4	33.3 ± 0.6

[a]Tyrosine aminotransferase (TAT) activity was measured 24 h after addition of Dex and cAMP at the time indicated. Equal volume of ethanol was added in the control experiments. Average activity ± SE was calculated from four experiments for each. Pst = polystyrene.

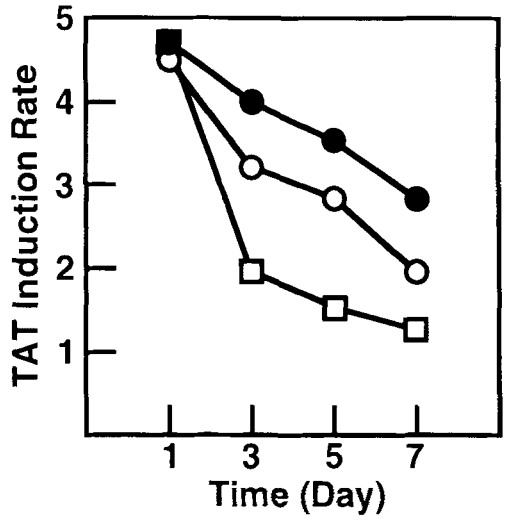

FIG. 4. Effect of poly-HEMA on TAT induction rate by Dex. TAT induction is expressed as the rate of the activity 24 h after addition of dexamethasone and cAMP to the activity of cells without induction. Hepatocytes were cultured on normal polystyrene (*closed circle*), poly-HEMA (0.1 × 10^{-3}) (*open circle*), or poly-HEMA (0.35 × 10^{-3}) (*open square*).

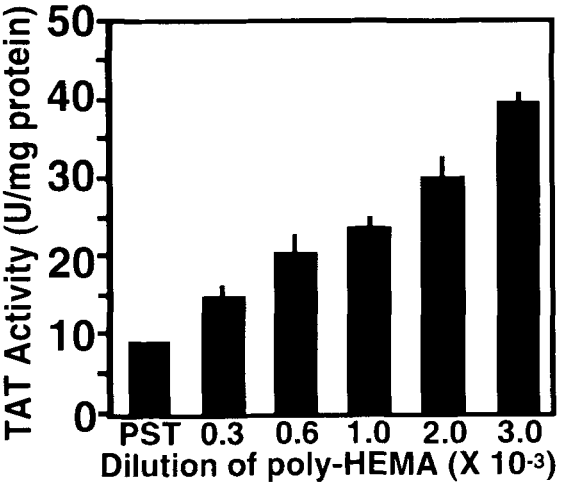

FIG. 5. Aggregation of hepatocytes causes a tyrosine aminotransferase (TAT) induction. Hepatocytes were cultured in the normal polystyrene (Pst) dishes or dishes coated with high concentrations of poly-HEMA solution for 3 d and then assayed for TAT activity.

1st to 3rd d. For the hepatocytes cultured on poly-HEMA, the decrease was less sharp. As the concentration of poly-HEMA increased, the TAT induction rate was maintained for longer periods of time. These results indicate that TAT induction, stimulated by glucocorticoids, depends on cell morphology.

When the concentration of poly-HEMA was further increased, the hepatocytes formed multicellular aggregates (Fig. 1 *G,H*). With the increasing of concentrations of poly-HEMA, the size of the aggregates were gradually increased (data not shown). When the spread of cells was suppressed by low concentrations of poly-HEMA in the absence of interactions among the cells, only the Dex-stimulated TAT induction increased, the basal activity remained unchanged (Table 1). However, when the cells formed spheroidal aggregates, their basal activity increased markedly (Fig. 5). This suggests that as the organization of hepatocytes advances, additional TAT induction occurs. It has been reported that expression of liver function is maintained at high level in the aggregate culture of hepatocytes (10,11,13,23).

We then examined hepatocytes for changes in their proliferating activity following changes in their shape. Hepatocytes of newborns, which are known to have an autonomous growth potential, were cul-

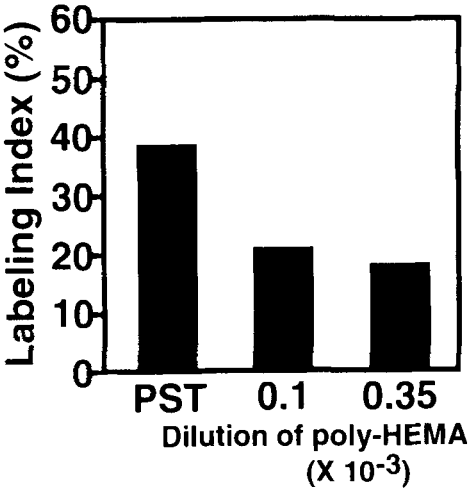

FIG. 6. Effect of poly-HEMA on DNA synthesis of neonatal rat hepatocytes. Neonatal hepatocytes 50 h after plating on polystyrene (Pst) or poly-HEMA were labeled with BrdU for 24 h. BrdU incorporation in the nucleus was detected by immunocytochemistry and quantitated as nuclear labeling index (%).

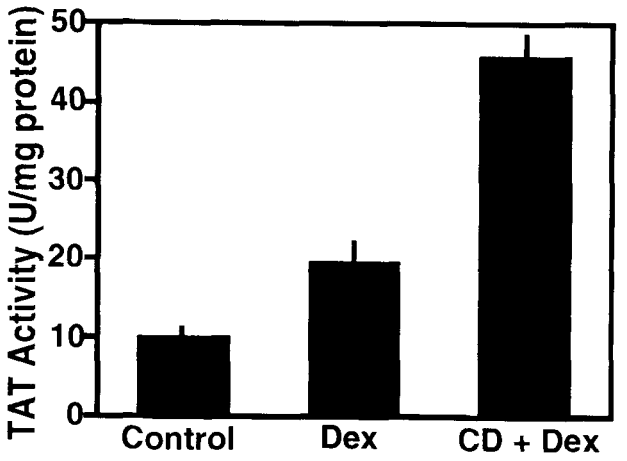

FIG. 7. Effect of cytochalasin D on tyrosine aminotransferase (TAT) induction by Dex. Hepatocytes at 2 d after plating were treated with 2×10^{-5} *M* of cytochalasin D (CD + Dex) or equal volume of ethanol (Dex) for 6 h and assayed for TAT activity after further 24 h culture with Dex. Equal volumes of ethanol were added instead of cytochalasin D solution and Dex solution in the control experiment (Control).

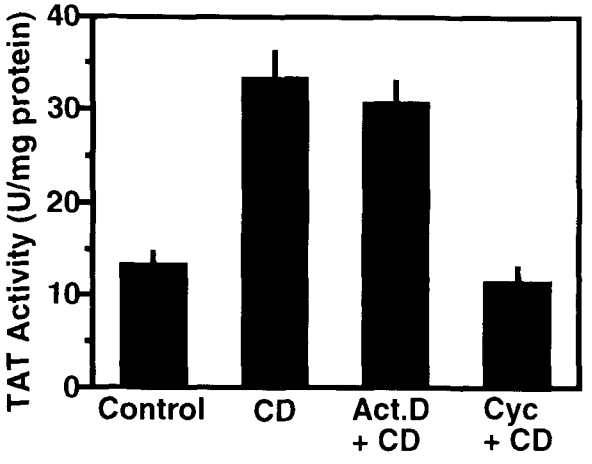

FIG. 8. Effects of actinomycin D and cycloheximide on tyrosine aminotransferase (TAT) induction by depolymerization of F-actin. Twenty-four hours after addition of Dex (5×10^{-5} *M*) on the 4th d of culture, the cells were treated with cytochalasin D (2×10^{-5} *M*) for 12 h (CD). The cells were cultured for another 12 h and then assayed for TAT activity. An equal volume of ethanol was added to the culture as a control for the cytochalasin solution (Control). Actinomycin D (Act.D + CD) or cycloheximide (Cyc + CD) were added to the culture 30 min before the addition of cytochalasin D.

tured in Pst dishes or in dishes coated with two concentrations of poly-HEMA and labeled with BrdU. Twenty-four hours later, the percentage of cells that had taken up BrdU into their nuclei (i.e., the labeling index) was determined (Fig. 6). The labeling index was highest for cells that were cultured on Pst; (i.e., cells that were cultured under a condition most favorable for the spread of cells). The index was lowest for the cells that were cultured on the higher concentration of poly-HEMA. Taken together, these results indicate that the proliferation and differentiation of hepatocytes can be reciprocally controlled by the change in their shape.

The effect of cytochalasin on TAT induction by Dex. The experiments described above revealed that stress fibers do not develop in the compact-shaped hepatocytes cultured on poly-HEMA (Fig. 3), and that TAT induction by Dex is maintained at high levels for relatively long periods (Fig. 4, Table 1) in these hepatocytes. We then investigated the relationship between these two findings. If the decrease in Dex-stimulated TAT induction following the spread of hepatocytes cultured on Pst is an outcome of stress fiber formation, the reactivity to Dex should be recovered by depolymerization of F-actin. In fact, following treatment with cytochalasin D (2×10^{-5} *M*) for 6 h on the 4th d, their reactivity to Dex returned to the level recorded immediately after the beginning of culture (Fig. 7). The treatment with cytochalasin for 6 h depolymerized stress fibers without affecting cell shape (data not shown). When cytochalasin B was used for the same experiment, the results were the same as those yielded by the experiment using cytochalasin D (data not shown). Only the reactivity to Dex was altered by cytochalasin treatment; the basal activity remained unchanged for at least 36 h after treatment with cytochalasin D (data not shown).

To identify the particular process of TAT induction in which F-actin is involved, the effect of actinomycin D (a transcription inhibitor) and cycloheximide (a translation inhibitor) were determined. After 4 d of culture, the hepatocytes, which had lost their reactivity to Dex almost completely, were treated with Dex for 24 h. Subsequently, Dex was removed from the culture and cytochalasin D was added to induce depolymerization of F-actin. This resulted in about a 2.5-fold increase in TAT activity. This effect of cytochalasin D was little affected by treatment with actinomycin D at a concentration high enough to completely suppress TAT induction, while it was almost completely suppressed by cycloheximide (Fig. 8). These results suggest that F-actin is involved in the posttranscriptional processes of TAT induction.

DISCUSSION

Several studies were recently conducted to determine the relationship between the morphology of hepatocytes and their proliferation and differentiation. However, since all of these studies used ECM to induce morphological changes in the cells, it was difficult to definitely determine whether the results are attributable to ECM activity or to morphological changes in the cells (2,6,7,15,21). In the present study, in which poly-HEMA (a biochemically inactive polymer) was used to coat the culture dishes, it was clearly demonstrated that the proliferation and differentiation of hepatocytes can be reciprocally regulated by changing the shape of the cells. Cells with a spheroid form manifested higher levels of liver function and had lower growth potential. Cells that had spread had lower levels of liver function and a higher growth potential. These features of hepatocytes resemble those of chondrocytes (5).

It is known that the proliferation and differentiation of hepatocytes are reciprocally regulated by the density of the cultured cells; that is, their differentiation is promoted at high densities, while their proliferation is promoted at low densities (19). It has also been reported that a culture with a high cell density can be mimicked by adding a "cell surface modulator" extracted from the liver cell membrane to a culture with a low cell density (18). Therefore, it has been thought that the dependency of hepatocyte proliferation and differentiation on cell density represents dependency on the degree of cellular interactions mediated by cell surface modulators. It is, how-

ever, true that changes in cell density cause morphological changes in cells (12). Moreover, adequate stress fiber formation is not seen even after 4 d of culture when the cells are cultured at a high density (data not shown). The present study revealed that even when the cell density is too low to cause contact of cells with each other, the proliferation and differentiation of hepatocytes can be reciprocally regulated by changing their shape. Therefore, we claim that the function of hepatocytes is regulated by not only cell-cell interaction but also their shape in primary culture.

The molecular mechanism of the cell-shape-associated regulation of cellular function is still unknown. However, it was found that the hepatocytes that had lost their reactivity to Dex during long-term incubation began to show TAT induction by Dex again when F-actin was depolymerized. These data suggest that F-actin is involved in the Dex-stimulated TAT induction. However, we cannot rule out the possibility that cytochalasin-caused morphological changes of the hepatocytes recovered their reactivity to Dex. Our finding that the cytochalasin-stimulated TAT induction was not affected by a transcription inhibitor but was suppressed by a translation inhibitor suggests the involvement of F-actin in the posttranscriptional processes. It is known that cytoskeleton plays a role in the mRNA localization (8,20). Furthermore, it was recently found that poly-A^+ RNA can bind to the neuronal cytoskeleton (1). It seems likely, therefore, that interactions between microfilaments and mRNA regulate the translation of the mRNA that are involved in liver function or growth of hepatocytes.

It is known that expression of ECM molecules is dynamically changed during liver regeneration (16). It is, therefore, likely that reconstruction of the cytoskeleton by ECM causes morphological changes in the hepatocytes during liver regeneration. Hepatocytes stop expressing liver function when they begin to proliferate, and that once the liver has resumed its original size, the hepatocytes stop proliferating and begin to express liver function again. The reciprocal control of "differentiation or proliferation" by cell shape changes may be associated also with liver regeneration *in vivo*, not only *in vitro*.

Various methods have been devised to maintain the function of cultured hepatocytes at high levels for long periods of time so that these cells can be used for hybrid artificial livers (9). Most of these methods were unsuccessful, however, because it was difficult to keep cells normal for long periods. The present study revealed that dysfunction of the hepatocytes is attributable to excessive spreading of cells, and that retaining a compact shape of hepatocytes is useful in keeping these cells normal for long periods of time. Recently, a method was developed that can strictly regulate cell morphology by printing laminin in any desired form onto a noncontact-type substrate (21). If such a method is confirmed, cultured hepatocytes may be used for hybrid artificial livers. Further studies are required for elucidation of the relationships between the function and cell shape at the molecular level.

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