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Functional Role of RhoA in Growth Regulation of Primary Hepatocytes

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The expression, activation and involvement in growth regulation of a small GTPase, RhoA, were examined in rat primary hepatocyte cultures. Hepatocytes freshly isolated from liver expressed RhoA protein at high levels. The total level of RhoA protein in the cells decreased markedly within a day in monolayer cultures. Thereafter, RhoA expression recovered as cell-cell attachment occurred during the culture. On the other hand, the level of the active form of RhoA decreased as the culture proceeded. Ca^{2+} depletion in the medium to disrupt cadherin engagement triggered RhoA activation without *de novo* protein synthesis, indicating cadherin engagement regulates RhoA activation in hepatocytes. Hepatocyte growth stimulation by HGF was enhanced by Ca^{2+} depletion or introduction of a constitutively active form of RhoA. The *Clostridium botulinum* C3 enzyme inhibited hepatocyte growth with stimulation by HGF. These results suggest that RhoA has a crucial role in hepatocyte growth control.

Key words: cadherin, HGF, monolayer, primary hepatocyte, Rho, spheroid.

The liver has multiple functions essential to life, including detoxification, carbohydrate and lipid metabolism, and so on. Therefore, for patients with a severe liver disease such as fulminant hepatic failure, liver transplantation is the only way to save their lives. However, an insufficiency of donors has been a serious problem. To date, various attempts have been made to replace the liver. A bioartificial liver support system consisting of a reactor module filled with functional cultured hepatocytes is promising for temporary liver support, and the system had advantages for patients with acute liver disease (*1*). Since cultured primary hepatocytes (parenchymal hepatocytes) are used in the artificial liver module, an effective method of culturing primary hepatocytes to maintain liver function is one of the keys for constructing successful bioartificial liver support systems.

Primary hepatocytes exhibit a different morphology depending on the substratum and conditions used for culture (*2*–*5*). For example, when hepatocytes are cultured on collagen-coated or laminin-coated dishes, they spread to form a two-dimensional flat monolayer. On the other hand, on proteoglycan-coated or positively-charged dishes, the cells first attach weakly to the surface, and then gradually become detached and simultaneously form three-dimensional multicellular aggregates called spheroids. We previously reported a rapid and mass preparation method for hepatocyte spheroids in suspension culture, in which a synthetic polymer was added to the medium to promote cell-cell attachment (*6*–*8*). Hepatocyte spheroids show a well-developed cell adhesion apparatus and bile canaliculi similar to liver tissue *in vivo*, and long-term enhanced liver functions compared with a monolayer culture. Thus, spheroids are promising for the construction of a bioartificial liver (*9*).

It is known that hepatocytes grow actively *in vivo* during regeneration. Epidermal growth factor (EGF), hepatocyte growth factor/scatter factor (HGF/SF), alpha-fibroblastic growth factor (α-FGF), and transforming growth factor-alpha (TGF-α) have been implicated in hepatocyte growth (*10*). However, hepatocytes grow in a very limited way even in the presence of growth factors under culture conditions (*11*, *12*), and the mechanism of growth regulation of cultured hepatocytes is not fully understood. In our previous study, we observed that CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ were highly expressed in hepatocyte spheroids (*5*). When hepatocytes were stimulated with HGF, the activation of Ras did not occur in spheroids in spite of that the HGF receptor (c-Met) was phosphorylated. These results suggest that cell-cell interaction and cell morphology are associated with signal transduction in growth regulation.

In highly differentiated tissues and organs, cell–cell and cell–matrix interactions, and cell morphology play important roles through cell adhesion and cytoskeletal formation. In recent years, it has been reported that Rho-family GTPases (RhoA, Rac1 and Cdc42) are involved in the signaling for cell–cell and cell–matrix adhesion, cytoskeletal formation and cell morphology (*13*). Rho GTPases regulate signal transduction pathways linking plasma membrane receptors to assembly of the filamentous actin cytoskeleton. It is recognized that Rho GTPases regulate many other signal transduction pathways such as those linked to cell polarity, gene transcription and growth regulation (*14*).

We describe here the expression, activation and involvement in growth regulation of RhoA in cultured primary hepatocytes. The expression of total and the active form of RhoA differed between monolayers and

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spheroids of hepatocytes. The expression of RhoA was induced by cell-cell adhesion mediated by E-cadherin in a Ca^{2+}-dependent manner, and RhoA was transiently activated on the inhibition of cell–cell adhesion by the removal of Ca^{2+}. Furthermore, the generation of active RhoA was essential for hepatocyte growth promotion induced by HGF.

MATERIALS AND METHODS

Isolation and Culture of Rat Primary Hepatocytes—Adult rat hepatocytes were obtained from male Sprague-Dawley rats (6–7 weeks old; Japan SLC, Shizuoka) by the collagenase perfusion method (*5*). Cells were seeded at a density of 5×10^5 cells per 35 mm-diameter dish in 2 ml of Williams' medium E (WE) (Invitrogen, San Diego, CA, USA) supplemented with 0.1 μM $CuSO_4 \cdot 5H_2O$, 25 nM Na_2SeO_3, 1.0 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM insulin (Sigma-Aldrich, St. Louis, MO, USA), 1.0 μM dexamethasone (Wako Pure Chemical Industries, Osaka), 20 ng/ml of epidermal growth factor (EGF) (Sigma), 48 ng/ml of gentamicin sulfate (Sigma), and 100 μg/ml of chloramphenicol (Wako). For monolayer cultures of hepatocytes, collagen type I-coated plastic dishes (Catalog No. 4000–010; Iwaki Glass Works, Chiba) were used as the substratum. For spheroid cultures of hepatocytes, cells were cultured in positively-charged Primaria® dishes (Catalog No. 3801; Becton Dickinson, Bedford, MA, USA) or in untreated dishes (Catalog No. 1000–035X; Iwaki Glass Works) in the presence of 0.1% Eudragit S100 (a copolymer of methacrylic acid and methylmethacrylate; Röhm Pharma, Darmstadt, Germany) (*6*). In some experiments, 2 mM EGTA (Sigma) and/or 10 ng/ml of HGF (R&D Systems, Minneapolis, MN, USA) were added to the medium.

SDS-PAGE and Western Blot Analysis—Rho GTPase proteins were detected by Western blot analysis essentially as described previously (*15*) after separation on a 12% SDS–polyacrylamide gel (*16*). The proteins were detected with anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rac1 (BD Transduction Laboratories, Lexington, KY, USA), or anti-Cdc42 (BD Transduction) antibodies. The specific antibodies were detected with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) using an ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Measurement of GTP-Bound RhoA—Active-form RhoA was detected as described previously (*17*) using the RhoA-binding domain of Rhotekin (RBD) expressed as a GST-fusion protein. For the construction of a GST-RBD expression vector, mouse total RNA was prepared from mouse liver cells using a total RNA extraction kit (Amersham Biosciences), and reverse transcribed with an enzyme (ReverTra Ace®; Toyobo, Osaka) to generate cDNAs. The cDNA fragment of RBD comprising amino acids 7–89 was then amplified by PCR using 5′-ATCCTGGAGGACCTCAATATGC-3′ and 5′-GCCTGTCTTCTCCAGCACC-3′, as primers. Then, the cDNA fragment encoding RBD was amplified again by PCR using 5′-GGTGGATCCATCCTGGAGGACCTCAATATG-3′ (*Bam*HI site is underlined) and 5′-GATGAATTCTAGCCTGTCTTCTCCAGCACC-3′ (*Eco*RI site is underlined) to add restriction enzyme sites at the ends. The RBD cDNA fragment was ligated into the pGEX-6P plasmid (Amersham Biosciences) to express RBD as a GST fusion protein. Production of RBD-GST fusion proteins and measurement of the active form by the pull-down assay procedure were performed as described previously (*17*).

Construction of a Mutant RhoA Expression Vector—Mouse RhoA cDNA was amplified by reverse transcription-PCR using 5′-ATAGGATCCACCATGGCTGCCATCAGGAA-3′ (*Bam*HI site is underlined) and 5′-CCCGAATTCTCACAAGATGAGGCACCCA-3′ (*Eco*RI site is underlined), as primers, and total RNA from mouse liver cells as a template. The resulting fragment encoding a full-length mouse RhoA cDNA was ligated into the pcDNA4 plasmid (Invitrogen). The expression vector for constitutively active RhoA (Val14) (*18*) was generated by PCR using forward (5′-CGGACTCTAGCGTTTAAACTTAAGCTTGGTACCGAG-3′) and reverse (5′-GCAAGCATGTCTTACCACAAGCTACATCACCAA-3′) primers. The resulting cDNA fragment was ligated into pcDNA4. The DNA sequences of the inserts were confirmed with a DNA sequencer.

Transfection of Plasmids into Cultured Primary Hepatocytes—Hepatocytes were seeded at a density of 2×10^5 cells per well onto collagen type I–coated 24-well plastic plates (Catalog No. 4820–010; Iwaki Glass Works). Cells were cultured in 1.0 ml of WE medium and the medium was renewed just before transfection. The plasmid DNA (0.5 μg per well) was transfected into cells using a transfection reagent (Lipofectamine 2000™; Invitrogen). After incubation for 4 to 5 h, the medium was replaced with a fresh batch, followed by incubation for 24 h.

Treatment of Cultured Primary Hepatocytes with Clostridium botulinum C3 ADP-Ribosyltransferase—Onto a 24-well collagen-coated plate, hepatocytes were inoculated at a density of 1×10^5 cells/well and cultured in 1.0 ml of WE medium. One microgram of C3 enzyme in 50 μl of PBS and 2 μl of protein transfection reagent (Chariot™; Active Motif, Carlsbad, CA, USA) in 50 μl of sterile water were mixed, and then the mixture was stood for 30 min at room temperature to allow the complex to form. After a change of medium, the cells were incubated at room temperature for 30 min. Then, 100 μl of the C3 enzyme complex was added to cells in 100 μl of WE medium, followed by incubation at 37°C in 5% CO_2 for an hour. Finally, 1.0 ml of WE medium was added to each well and the cells were cultured under 5% CO_2 for 24 h.

Other Analyses—Protein concentrations were determined by the bicinconinic acid method using bovine serum albumin as a standard. DNA synthesis was evaluated as the incorporation of ^{3}H-thymidine (1 μCi/ml; Amersham Biosciences) as described previously (*5*).

RESULTS AND DISCUSSION

Figure 1 shows the typical morphology of hepatocytes under different culture conditions. Hepatocytes formed a flat monolayer on a collagen-coated dish within a day. On a Primaria dish or with the addition of Eudragit on an untreated dish, cell aggregates formed within 2 days and spheroid formation was completed by day 4. Eudragit promoted rapid spheroid formation compared with Primaria dishes (Fig. 1B).

In general, integrins and cadherins are involved in cell-matrix and cell-cell adhesion, respectively, and these

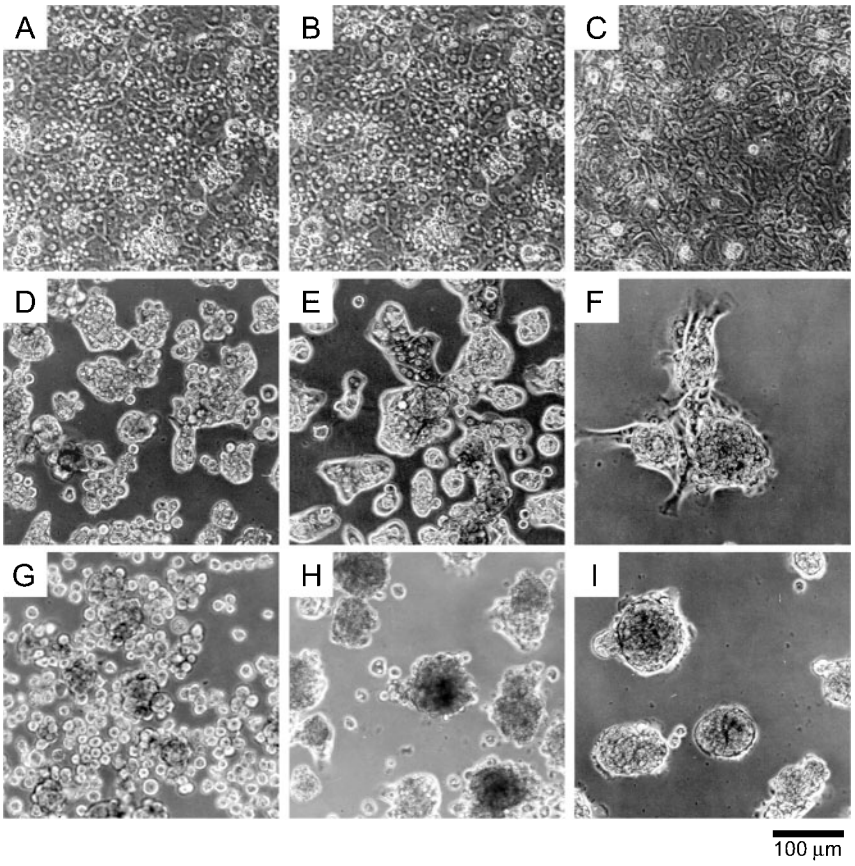

Fig. 1. **Morphological observation of cultured primary hepatocytes.** Rat primary hepatocytes isolated from liver were cultured on a collagen-coated dish (A–C), on a Primaria dish (D–F), or in the presence of Eudragit on an untreated dish (G-I). The cells were observed on day 1 (A, D, G), day 2 (B, E, H), and day 4 (C, F, I).

interactions regulate the functions of small GTPases such as RhoA, Rac1 and Cdc42 in epithelial cells (*19*, *20*). In this regard, we first analyzed the expression levels of these small GTPases in monolayers and spheroids of hepatocytes. Figure 2 shows the total expression levels of small GTPases. Certain amounts of Rho family GTPase proteins were detected just after isolation (0 day). The levels decreased for the first day and then gradually recovered during the culture. The first decrease in expression was evident in the monolayer cells (Fig. 2A), and spheroids showed much higher levels of Rho GTPase proteins. These results suggest that an increase in cell-cell interaction induces the expression of total Rho GTPases. Since cell-cell interaction occurred rapidly under the conditions for spheroid formation even on day 1, spheroids might exhibit higher expression levels of Rho GTPases.

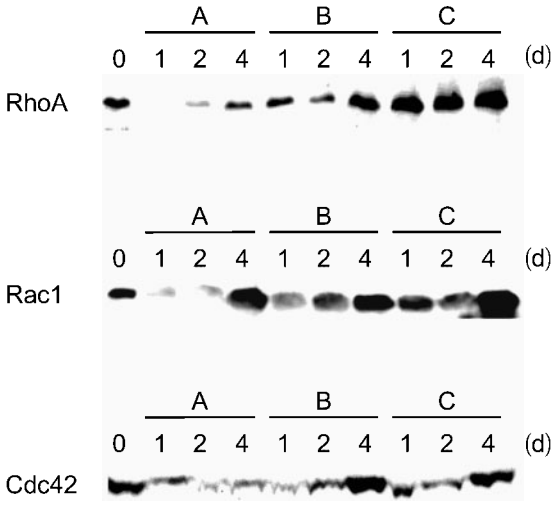

Fig. 2. **Expression of Rho family GTPase proteins in hepatocyte cultures.** Western blot analysis for the detection of Rho family GTPases (RhoA, Rac1 and Cdc42) was performed for hepatocytes cultured as a monolayer and spheroids. A monolayer on a collagen-coated dish (A), and spheroids on a Primaria dish (B) or in the presence of Eudragit on an untreated dish (C) were formed. One hundred micrograms of total cell protein was applied to a SDS-PAGE gel.

Next, the amount of active-form RhoA in hepatocytes was determined (Fig. 3). RhoA protein cycles between inactive GDP-bound and active GTP-bound forms *via* a process regulated by guanine nucleotide exchange factors and GTPase-activating proteins in addition to the intrinsic GTPase activity of RhoA protein. Active-form RhoA was expressed at a high level in freshly isolated hepatocytes (0 day). The level was maintained on day 2 in monolayer hepatocytes, but the active RhoA disappeared as the culture proceeded. In hepatocyte spheroids, the expression level of active RhoA was very low throughout the culture period. For Madin-Darby canine kidney (MDCK) cells, it has been reported that the formation of cell-cell adherens junctions mediated by E-cadherin results in a decrease in active RhoA but an increase in active Rac1 (*20*). Adult hepatocytes also express E-cadherin exclusively (*21*), and adherens junctions develop between the cells as the culture proceeds. Thus, our data

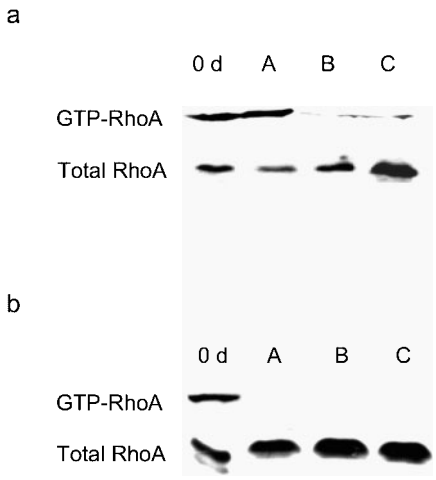

Fig. 3. **Expression of active RhoA in hepatocyte cultures.** Active RhoA was collected on day 2 (a) or day 4 (b) by the pull-down procedure using RBD-GST fusion proteins, and detected by Western blot analysis. Total cell protein was obtained from hepatocytes cultured as monolayers and spheroids. A monolayer on a collagen-coated dish (A), and spheroids on a Primaria dish (B) or in the presence of Eudragit on an untreated dish (C) were formed.

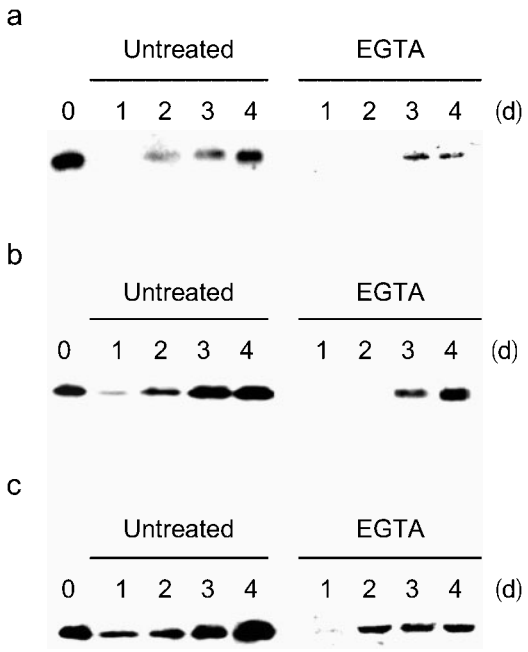

Fig. 4. **Time-dependent expression of RhoA in hepatocyte cultures after Ca^{2+} depletion for 24 h.** Hepatocytes were cultured with 2 mM EGTA for 24 h (EGTA) or without treatment (Untreated). Total RhoA protein from the cells was analyzed by Western blotting. A monolayer on a collagen-coated dish (a), and spheroids on a Primaria dish (b) or in the presence of Eudragit on an untreated dish (c) were formed.

on the expression level of active-form RhoA are well consistent with those for MDCK cells.

To clarify the involvement of cadherin in the morphological change, and in the expression and activity of RhoA, we then applied a so-called Ca^{2+}-switching procedure to hepatocyte cultures. Ca^{2+}-switching has often been used for the analysis of cadherin engagement (*22*). In the present study, Ca^{2+} in the medium was chelated by the addition of 2 mM EGTA (equimolar as to the Ca^{2+} concentration in the medium) for the first 24 h culture so that the cell-cell interaction mediated by Ca^{2+}-dependent E-cadherin was inhibited by the Ca^{2+} depletion, and then the medium was changed to that containing a normal concentration of Ca^{2+}. In the monolayer culture, although the cells attached to and spread on the dish surface, a boundary between cells was observed during the Ca^{2+} depletion, indicating that cell-cell interaction was inhibited by the treatment. Spheroid formation was also inhibited or delayed on EGTA treatment either by Eudragit or on Primaria dishes. Since cell viability and albumin production were not significantly affected, the depletion did not profoundly affect the hepatocytes (data not shown). Figure 4 shows the expression of RhoA protein in monolayer and spheroid cultures with the depletion of Ca^{2+}. After this treatment for 24 h, RhoA protein disappeared from the monolayers and spheroid cells on Primaria dishes, and then gradually reappeared during culture in the normal medium. This suggests that cadherin-mediated cell-cell interaction stimulates RhoA gene expression. On the other hand, in spheroids induced by Eudragit, RhoA was not completely abolished with Ca^{2+} depletion and recovered much faster upon switching to a normal Ca^{2+} concentration. Since it is possible that the cell-cell attachment between hepatocytes in spheroids is mediated not only by Ca^{2+}-dependent E-cadherin but also by the extracellular matrix or polymer in Eudragit spheroids, the inhibition of cell-cell interaction might be incomplete. In fact, small cell aggregates were formed in the presence of Eudragit even under the conditions of Ca^{2+} depletion (data not shown).

To determine whether or not cadherin engagement controls the activity of RhoA, as observed for MDCK cells (*20*), the amounts of the active-form and total RhoA expressed were determined after various periods of Ca^{2+} depletion in monolayer cultures of hepatocytes (Fig. 5a). Active-form RhoA was not detected before Ca^{2+} depletion, but it appeared after only 30 min treatment, and 12 h treatment gave similar RhoA activity. However, active-form RhoA disappeared on 24 h treatment. The total RhoA protein level was highest before the EGTA treatment and gradually decreased in the absence of Ca^{2+}. This shows that the cadherin engagement and the activation of RhoA are inversely correlated, as observed in MDCK cells (*20*). We then determined whether or not activation of RhoA requires *de novo* synthesis of RhoA protein. As shown in Fig. 5b, active-form RhoA induced by Ca^{2+} depletion was also detected in the presence of a translational inhibitor, cycloheximide. Therefore, it is assumed that inactive RhoA (possibly GDP-bound RhoA) was converted directly to the active GTP-form of RhoA with the depletion. The high-level expression of inactive RhoA induced by cell-cell interaction in hepatocytes may be considered to provide a pool for immediate conversion to active-form RhoA. Recently, it was reported that p190RhoGAP activates the GDP-form of RhoA *via* cadherin signaling and that the activation is triggered by tyrosin-phosphorylation of p190RhoGAP by a Src family

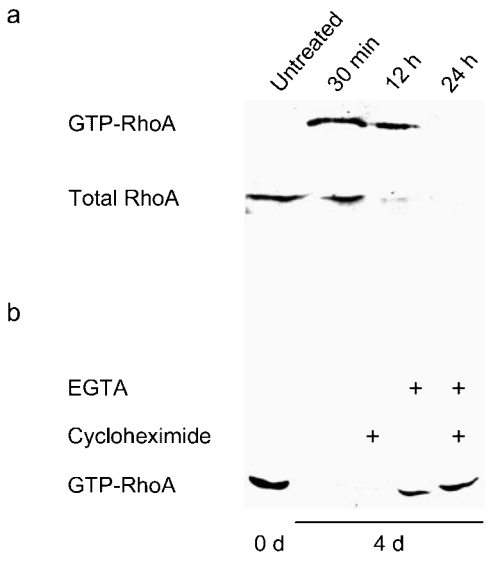

Fig. 5. **Activation of RhoA on Ca^{2+} depletion.** (a) Hepatocytes were cultured on a collagen-coated dish for 3–4 days, and then the medium was replaced with that containing 2 mM EGTA. The cells were incubated for 30 min, 12 h or 24 h. After the Ca^{2+} depletion, the cells were harvested and used for analyses. (b) Hepatocytes were cultured for 4 days in normal medium, and then transferred to the medium containing 2 mM EGTA in the presence or absence of 5 mM cycloheximide. The cells were incubated for 30 min and then harvested.

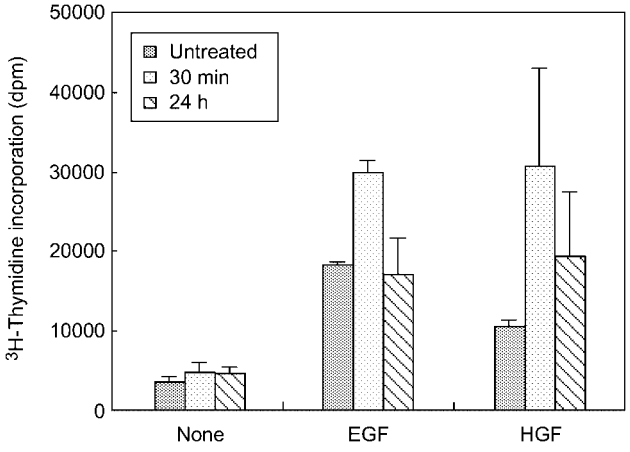

Fig. 6. **Growth response to EGF or HGF stimulation with Ca^{2+}-depletion.** Hepatocytes were cultured on a collagen-coated dish for 2 days (for 24 h treatment) or 3 days (for 30 min treatment), and then the medium was replaced with that containing 2 mM EGTA. After the treatment, the medium was changed to the normal one and ^{3}H-thymidine incorporation was measured for 24 h.

protein kinase (*23*). A similar mechanism may be involved in the activation of RhoA in hepatocytes.

Since the involvement of RhoA in cell-growth control has been reported (*24*, *25*), we studied the relation between hepatocyte growth and changes in RhoA activity due to cadherin engagement and disruption. For this purpose, cell growth stimulation by EGF and HGF was performed after 30 min- or 24 h-Ca^{2+} depletion. After 30 min-Ca^{2+} depletion, ^{3}H-thymidine incorporation during the following 24 h culture in the presence of Ca^{2+} was increased 1.8-fold by EGF and 2-fold by HGF, compared

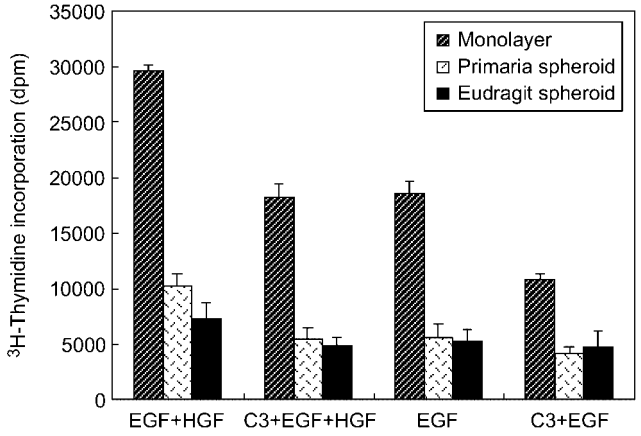

Fig. 7. **Effect of *C. botulinum* C3 enzyme on the growth response to EGF or HGF stimulation.** After hepatocytes had been cultured for 2 days (monolayer on a collagen-coated dish) or 4 days (spheroids on a Primaria dish or in the presence of Eudragit on an untreated dish), C3 enzyme was added to the medium. From day 3 (monolayer) or day 5 (spheroids), ^{3}H-thymidine incorporation was measured for 24 h in the presence of growth factors.

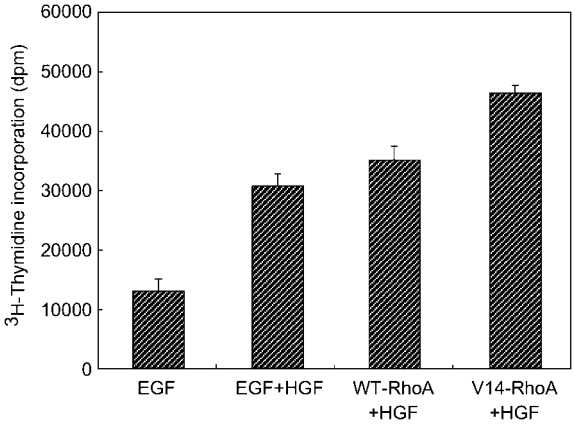

Fig. 8. **Effect of dominant active RhoA on the growth response to HGF stimulation.** After hepatocytes had been cultured on collagen-coated dishes for 24 h, the expression vector for a constitutively active mutant (V14-RhoA) or wild type RhoA (WT-RhoA) was transfected into the cells. At 48 h post-transfection, ^{3}H-thymidine and HGF were added to the medium, and then ^{3}H-thymidine incorporation was measured for 24 h.

to that without the depletion (Fig. 6). On the other hand, less (for HGF) or no (for EGF) growth stimulation was observed after Ca^{2+} depletion for 24 h. With the 30 min treatment, active RhoA appeared, whereas it disappeared with the 24 h treatment (Fig. 5a). Therefore, it is suggested that the activation of RhoA mediated by cadherin disruption is important in hepatocyte growth regulation. In addition, we have to point out that the stimulation by growth factors may be affected at least partly by the down-regulation of receptors, since it has been reported that the HGF receptor (c-Met) was co-internalized with cadherin on Ca^{2+} depletion in MDCK cells (*26*, *27*).

To clarify the relation between RhoA signals and cell growth regulation, hepatocytes in monolayers and spheroids were stimulated by growth factors in the presence of a specific RhoA inhibitor, C3 enzyme (*28*) (Fig. 7).

Growth stimulation by HGF and/or EGF was repressed almost to half the level in the presence of C3 enzyme in monolayers. Although the cells in spheroids expressed active RhoA at a low level (Fig. 3), and grew poorly even with EGF and HGF stimulation, C3 enzyme also repressed the ^{3}H-thymidine incorporation by spheroid cells (Fig. 7). This suggests that active RhoA has an important role in hepatocyte growth. To confirm this, an expression vector encoding a constitutively active RhoA was introduced into hepatocytes cultured as monolayers. Overexpression of wild type RhoA slightly increased hepatocyte growth and constitutively active RhoA stimulated ^{3}H-thymidine incorporation almost 1.5-fold compared to the control (Fig. 8).

It has been reported that a decrease in active RhoA caused growth arrest in Ras-transformed fibroblasts; active Ras induced the expression of cycline-dependent kinase inhibitor $p21^{Waf1/Cip1}$, when RhoA was inhibited (*24*, *25*). In monolayer hepatocytes, we detected trace amounts of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, and the levels of the two CDK inhibitors were increased in spheroids (*5*). Therefore, it is unlikely that the inactivation of RhoA causes the induction of CDK inhibitors in a monolayer culture. In contrast, the growth arrest of spheroid cells may be mainly due to the high levels of CDK inhibitors, although the direct involvement of RhoA in the regulation of CDK inhibitors has not been made clear in hepatocytes. The present results indicate that the activation of RhoA has a crucial role in the stimulation of hepatocyte growth.

Hepatocytes in the lobules of normal liver are attached to each other, orderly arranged, growth-arrested, and highly differentiated. In regeneration processes such as partial hepatectomy, hepatocytes proliferate in response to stimulation by growth factors such as HGF, and then cease growing when the liver regains its original size. In hepatocytes cultured as spheroids, highly developed cell-cell interactions, a poor growth response to HGF stimulation, and good liver functions have been observed. Thus, hepatocyte spheroids may mimic conditions *in vivo* in normal liver. On the other hand, since hepatocytes cultured as monolayers exhibit good growth ability compared to spheroids, they may be similar to regenerating liver, at least in an early culture period. Thus, the following mechanism of hepatocyte growth regulation in the regeneration of liver can be assumed; activation of RhoA is caused by disrupted cadherin attachment between hepatocytes at the site of regeneration in the liver, and so hepatocytes become sensitive to stimulation by growth factors.

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