Involvement of Rho/Rho kinase pathway in regulation of apoptosis in rat hepatic stellate cells

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IT IS NOW WELL KNOWN that hepatic stellate cells (HSCs) play a central role in the development of hepatic fibrosis (9). In response to liver damage, HSCs “activate” to a myofibroblast-like phenotype with enhanced proliferation, fibrogenesis, and contractility. Current evidence demonstrates that the mechanisms of spontaneous resolution of rat hepatic fibrosis involve HSC apoptosis, suggesting that HSCs may also play a pivotal role in the resolution of hepatic fibrosis (14, 16). Moreover, gliotoxin, which induces HSC apoptosis, has been shown to reduce experimental hepatic fibrosis in rats (41). Thus to know the mechanisms of HSC apoptosis is important to clarify the pathophysiology and establish the therapeutic strategy for hepatic fibrosis. However, little is known regarding this matter.

The ras-like guanosine triphosphate-binding protein Rho has emerged as an important regulator of the actin cytoskeleton and, consequently, cell morphology (30). Regarding Rho and HSCs, Yee (43) first demonstrated that Rho directs activation-associated changes in HSC morphology via regulation of the actin cytoskeleton. Rho is known to have a variety of putative effectors. Among them, the serine/threonine kinase named Rho kinase has been shown to mediate multiple Rho effects, including formation of actin stress fibers and focal adhesion. Using a specific inhibitor of Rho kinase, Y-27632 [(R)-(+-)trans-N-(4-pyridyl)-4-(1-aminoethyl)cyclohexane carbamoyl], Rho kinase has been shown to mediate contraction, migration (20), and proliferation (17) as well as activation-associated morphological changes in HSCs. Moreover, we have demonstrated that lipid mediators of lysosphosphatidic acid (LPA) and sphingosine 1-phosphate, which are known to enhance Rho and Rho kinase activity, stimulate contractility and cell attachment to the extracellular matrix with modulation of cell morphology in HSCs (13, 42).

Although increasing evidence suggests a link between Rho and apoptosis, contradictory results have been reported in this context (1, 5, 7, 19). The controversy may be explained, at least in part, by the difference in the cells examined in those experiments. In this study, we directed our focus on the role of the Rho pathway in the regulation of HSC survival. C3, an inhibitor of Rho, increased histone-associated DNA fragmentation and caspase 3 activity with enhanced condensation of nuclear chromatin in rat cultured HSCs. Moreover, Y-27632, an inhibitor of Rho kinase, had the same effects, suggesting that inhibition of the Rho/Rho kinase pathway induces apoptosis in HSCs. On the other hand, the same effects, suggesting that inhibition of the Rho/Rho kinase pathway causes HSC apoptosis. In this study, we directed our focus on the role of the Rho/Rho kinase pathway in the regulation of HSC survival and found that inhibition of the Rho pathway induces apoptosis in HSCs.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were fed a standard pellet diet and water ad libitum and used in all of the experiments. All animals received humane care in compliance with...
Cell isolation and culture. HSCs were isolated from the rats, weighing 300 to 400 g, using a metrizamide (Sigma) gradient centrifugation, as previously described (12). The isolated cells were seeded on uncoated plastic tissue culture dishes (Falcon, Lincoln Park, NJ) at a starting density of $1 \times 10^4$ cells/cm$^2$ and cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (GIBCO, Grand Island, NY). The confluent cells were subcultured as previously described (31), and experiments were performed between 2 and 6 wk after isolation (between 1 and 3 passages).

Hoechst staining. HSCs were plated on Lab Tek slides (4 wells; Nalge Nunc International, Naperville, IL). After treatment, Hoechst staining was performed as previously described (23) and examined by fluorescence microscopy.

Cell death detection enzyme-linked immunosorbent assay. A cell death detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Indianapolis, IN) was used to quantitatively determine cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death. Briefly, after treatment, HSCs were lysed with 200 μl of lysis buffer and incubated for 30 min at room temperature. Then 20 μl of supernatant were transferred into the streptavidin-coated microtiter plate, and 80 μl of the immunoreagent were added to each well. After incubation at room temperature for 2 h, the solution was decanted, and each well was rinsed three times with incubation buffer. Color development was carried out by adding 100 μl of 2,2’-azino-di(3-ethylbenzthiazoline sulfonate (ABTS) solution, and absorbency was measured at 405 nm against ABTS solution as a blank.

Caspase 3 activity assay. HSCs cultured in 100-mm-diameter dishes were harvested and pelleted by centrifugation. The medium supernatant was discarded, and the cell pellet was washed in 1 ml of ice-cooled phosphate-buffered saline. Caspase 3 (DEVDase) activity was determined by a colorimetric CaspACE kit (Promega, Madison, WI) following the manufacturer’s instructions.

Trypan blue staining. HSCs were cultured in 35-mm-diameter dishes. After treatment, cell survival analysis was performed as described previously (22). Detached cells were collected by centrifugation, and the remaining adherent cells were trypsinized. Both cell fractions were combined, and cell viability was determined by trypan blue staining.

Immunoblot analysis. After experimental treatment, the medium was discarded, and HSCs were incubated in 2% sodium dodecyl sulfate and 60 mM Tris, pH 6.8, boiled for 5 min, and then disrupted by sonication. Samples containing the same amount of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% acrylamide) under reducing conditions and then transferred to a sheet of polyvinylidene difluoride membrane (Amersham). To block nonspecific binding, the membrane was soaked in blocking agent derived from skim milk (Blockace; Snow Brand Milk Product, Sapporo, Japan) for 1 h at room temperature. Then, it was incubated with primary antibody for 1 h at room temperature. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham) and recorded in a chemiluminescence recording system (model LAS 1000; Fuji-film, Tokyo, Japan). To determine p53, Bel-2, Bax, or Rho expression, anti-p53 from BD Biosciences, anti-p53 (Pab240), anti-Bax (N20), or anti-Rho (26C4) from Santa Cruz Biotechnology was used as primary antibody, respectively. Densities of bands were quantitated with an Image Gauge (Fuji-film).

Determination of activity of Rho. The activity of Rho was determined by Rho Activation Assay Kit (Upstate, Lake Placid, NY) following the manufacturer’s instructions.

RESULTS

Inhibition of Rho induces HSC apoptosis. C. botulinum exoenzyme C3 catalyzes the specific ADP ribosylation and inactivation of Rho and has been used to probe Rho function. The serum-containing culture medium was replaced by the serum-free medium, and 24 h later C3 (2 μg/ml) was added to the medium of HSCs. Then, 24 h after the addition of C3, nuclear morphology was assessed by Hoechst 33258 staining. As shown in Fig. 1A, the condensation of nuclear chromatin appeared to be enhanced by C3. Thus we next quantitatively determined cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death in hepatic stellate cells (HSCs).

![Fig. 1. Effects of Clostridium botulinum exoenzyme C3, an inhibitor of Rho, on nuclear morphology (A) or histone-associated DNA fragmentation (B) in hepatic stellate cells (HSCs). A: HSCs were cultured without serum for 24 h and then with (right) or without (left) C3 at 2 μg/ml for an additional 24 h. Nuclear morphology was assessed by Hoechst 33258 staining. B: HSCs were cultured in the same time course as above with C3 at 0.2 or 2 μg/ml. Histone-associated DNA fragmentation was determined using a cell death detection enzyme-linked immunosorbent assay kit. Columns and bars represent means ± SD of 3 experiments.](http://ajpgi.physiology.org/Downloadedfile/10.23063/ajpgi.on June 22, 2017)
cell death with a cell death detection enzyme-linked immunosorbent assay kit. In the same time course, the treatment of HSCs with 0.2 or 2 μg/ml C3 significantly caused a three- or four- to sixfold increase in histone-associated DNA fragmentation, respectively, as shown in Fig. 1B. In this study, cells between 2 and 6 wk after isolation were used, and no differences in response to C3 in histone-associated DNA fragmentation were found irrespective of times of passage between 1 and 3. These results suggest that the inhibition of Rho induced HSC apoptosis.

Because caspase 3 plays a key role in various forms of apoptosis, we investigated whether this enzyme was involved in the increased DNA fragmentation in HSCs induced by the inhibition of Rho. After a 24-h incubation of HSCs in serum-free medium, the cells were treated with 2 μg/ml C3 for an additional 24 h, and caspase 3 activity was determined. As shown in Fig. 2A, 24-h treatment with C3 resulted in a threefold increase in caspase 3 activity. Moreover, cotreatment of Z-VAD-FMK (50 μM) completely abrogated C3-induced DNA fragmentation, as demonstrated in Fig. 2B. Therefore, these results indicate that HSC apoptosis caused by the inhibition of Rho involved caspase 3 activation.

Finally, the amount of cell death was determined by trypan blue staining. After a 24-h incubation of HSCs in serum-free medium, the cells were treated with 2 μg/ml C3 for an additional 48 h, and trypan blue staining was performed with adherent and detached cells. As shown in Fig. 3, 48-h treatment with C3 significantly increased HSC death.

**Inhibition of Rho kinase induces HSC apoptosis.** We next examined whether the process of HSC apoptosis caused by inhibition of Rho might involve Rho kinase, one of the immediate downstream effectors of Rho. The specific Rho kinase inhibitor Y-27632 was used to address this question. HSCs cultured in serum-free medium for 24 h were treated with 3 or 30 μM Y-27632 for an additional 24 h. Figure 4A shows that treatment of HSCs with Y-27632 for 24 h significantly induced histone-associated DNA fragmentation in a concentration-dependent manner; 30 μM Y-27632 increased histone-associated DNA fragmentation two- to threefold. Then, we investigated whether caspase 3 was involved in the HSC apoptosis induced by the inhibition of Rho kinase. Y-27632 is taken up by cells within a few minutes (15), although C3 is relatively cell impermeant and needs prolonged incubation times to achieve functionally significant intracellular levels (1). In fact, the alteration in HSC morphology by Y-27632, shrinking and losing stress fibers (42), was determined within 30 min after the addition (not shown). Thus we examined the effect of Y-27632 on caspase 3 activity in HSCs as early as 30 min after its addition and performed a time course study. As demonstrated in Fig. 4B, Y-27632 increased caspase 3 activity at 30 min after its addition. On the other hand, the significant enhancement of DNA fragmentation by Y-27632 was first detected at 2 h after its addition; caspase 3 activation preceded DNA fragmentation. This finding suggests that HSC apoptosis induced by Rho kinase inhibition is mediated by caspase 3 activation.

**Rho/Rho kinase stimulator LPA inhibits HSC apoptosis in serum-free medium but does not alter in serum-containing medium.** Because LPA is known to activate the Rho/Rho kinase pathway, we next examined whether LPA might affect HSC apoptosis. HSCs were cultured in the presence or absence of serum for 24 h, and 10 μM LPA or 2 μg/ml C3 was added to the medium. Twenty-four hours after the addition of LPA.

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**Fig. 2.** Effect of C3 and Z-VAD-FMK, a pancaspase inhibitor, on caspase 3 activity (A) or histone-associated DNA fragmentation (B) in HSCs. HSCs were cultured without serum for 24 h, and then C3 at 2 μg/ml with or without Z-VAD-FMK (Z-VAD) at 50 μM was added to the culture medium of HSCs. Twenty-four hours after the addition, caspase 3 activity or histone-associated DNA fragmentation was determined using a colorimetric CaspACE kit or a cell death detection enzyme-linked immunosorbent assay kit. Columns and bars represent means ± SD of 3 experiments.

**Fig. 3.** Effect of C3 on HSC death determined by trypan blue exclusion. HSCs were cultured without serum for 24 h, and then C3 at 2 μg/ml was added to the culture medium of HSCs. Forty-eight hours after the addition, the attached cells were trypsinized and combined with detached cells, and survival was determined by trypan blue exclusion. Columns and bars represent means ± SD of 3 experiments.
or C3, histone-associated DNA fragmentation was determined. As depicted in Fig. 5, the treatment of HSCs with LPA in the serum-free condition caused a 33% reduction in histone-associated DNA fragmentation. In contrast, 10% fetal calf serum also reduced histone-associated DNA fragmentation of HSCs by 44%, and in this culture condition of HSCs with 10% fetal calf serum LPA did not alter histone-associated DNA fragmentation. C3 increased DNA fragmentation irrespective of the presence of serum in the medium. These results indicate that LPA inhibited HSC apoptosis in the absence of serum but not in the presence of serum.

Coincidence of Rho activity and HSC apoptosis. Rho expression in HSCs was previously demonstrated by cell staining (43), but the expression of its active form was not determined. In this study, we directly measured amounts of GTP-bound Rho (GTP-Rho), an active form of Rho, by immunoblotting. In the case of C3 treatment, it ADP-ribosylates Rho at Asn14, a amino acid residue located within the effector domain of Rho (36) and functionally inactivates Rho by blocking the interaction of Rho with effector molecules (26). Thus C3 inhibits the ability of GTP-Rho from binding to rhotein, and as a result the amount of GTP-Rho detected by Rho pull-down assay with rhotein is reduced, reflecting a decrease in Rho activity. Previously, the treatment of rat-cultured HSCs with 2 μg/ml C3 for 6 h was determined to inhibit Rho activity (13). Thus HSCs pretreated in serum-free medium for 24 h and with 2 μg/ml C3 for 6 h were then treated with or without 10 μM LPA and 10% fetal calf serum for 5 min, and GTP-Rho was measured. In the serum-free condition, LPA caused an increase, and C3 caused a decrease in Rho activity. In contrast, in HSCs coadded with fetal calf serum, LPA did not cause any change, whereas C3 caused a decrease in Rho activity (Fig. 6). The levels of Rho activity appeared to coincide conversely with histone-associated DNA fragmentation in the presence or absence of fetal calf serum, C3, or LPA, suggesting that Rho activity may play a role in the regulation of HSC survival.

C3 or Y-27632 does not affect p53, Bcl-2, or Bax expression in HSCs. The p53 tumor suppressor gene is crucial in some forms of apoptosis. Thus we examined whether the p53 protein level was altered in HSCs by the inhibition of Rho/Rho kinase pathway. Figure 7A shows that 24-h treatment of HSCs with C3 at 2 μg/ml or Y-27632 at 30 μM did not affect p53 protein level in HSCs. This result suggests that HSC apoptosis induced by the inhibition of Rho/Rho kinase pathway was not mediated by p53.
Next, the possible mechanism by which the inhibition of Rho/Rho kinase pathway induces HSC apoptosis was examined by quantifying any associated changes in cellular content of proteins that enhance or inhibit apoptosis. As shown in Fig. 7B, immunoblot analysis of Bcl-2 or Bax extracted from HSCs showed that 24-h treatment with 2 μg/ml C3 or 30 μM Y-27632 did not alter each level. Thus it is unlikely that Bcl-2 or Bax plays a role in HSC apoptosis induced by the inhibition of the Rho/Rho kinase pathway.

DISCUSSION

In the present study, C3, an inhibitor of Rho, increased histone-associated DNA fragmentation in HSCs with enhanced condensation of nuclear chromatin. C3 also increased caspase 3 activity in HSCs. Because caspase 3 is a central caspase in the proapoptotic cascade and can be used as an alternative assay to assess apoptosis (25), it is reasonable to assume that the inhibition of Rho induces HSC apoptosis. Moreover, evidence that Y-27632, an inhibitor of Rho kinase, enhanced caspase 3 activity and then histone-associated DNA fragmentation in HSCs suggests that Rho kinase is involved in the signaling pathway of Rho-mediated HSC apoptosis. Although the findings that the inhibition of Rho mediates apoptosis in various cells have been recently accumulating (3, 11, 25, 27, 32), the effector molecule of Rho is not Rho kinase in all cases (11, 27). Thus it is suggested that Rho-mediated apoptosis may have distinct signaling pathways, and the significance of those pathways remains to be clarified.

A role of Rho/Rho kinase in the regulation of HSC apoptosis was confirmed by the additional evidence that LPA, a stimulator of Rho/Rho kinase, decreased...
HSC apoptosis. Although LPA is known as a survival factor in various cells (3, 6, 10, 21, 24, 35, 40), the involvement of G_{i}, but not Rho, has been reported in its mechanism (3, 6). Of interest is the fact that LPA inhibits HSC apoptosis in serum deprivation but not in the presence of serum. The evidence that the serum has Rho activators, for example LPA itself (4, 38), would explain the lack of LPA effect in the presence of serum. This finding also suggests that HSC apoptosis induced by serum deprivation (16, 39) may be due, at least in part, to the absence of Rho activators in the culture medium.

Previously, increased cell spreading was shown to promote cell survival in endothelial cells (2). In HSCs, we (42) reported that, by use of the electric cell-substrate impedance sensing system, LPA increased the cell-attached area and Y-27632 decreased it. In this study, the decrease in cell spreading by Y-27632 was found within 30 min and preceded the DNA fragmentation. Thus we speculate that the regulation of cell spreading and attachment by Rho and Rho kinase might determine HSC fate.

Although several factors that regulate HSC apoptosis have been recently identified, such as soluble Fas (CD95/APO-1) ligand, transforming growth factor-β, tumor necrosis factor-α, nerve growth factor, benzodiazepine, gliotoxin, tissue inhibitor of metalloproteinase-1, or disruption of integrin-mediated cell adhesion (8, 18, 29, 33, 34, 39, 41, 44), the intracellular signaling pathway(s) has not been unveiled. It is unlikely that Rho/Rho kinase might be involved in the intracellular signaling pathway(s) of HSC apoptosis regulated by those reported factors. Because those factors affected p53, Bcl-2, or Bax expression, whereas the inhibition of Rho/Rho kinase pathway did not, the pattern of alteration in p53, Bcl-2, or Bax expression is distinct among those factors, suggesting that the mechanisms of HSC apoptosis could consist of multiple pathways.

Y-27632 was shown to inhibit experimental hepatic fibrosis in rats (28, 37). Although its exact mechanism of inhibition of fibrogenesis in the liver remains to be elucidated, the resolution of hepatic fibrosis is associated with inactivation of HSCs (28, 37). In cultured HSCs, their proliferation, one of the main features of HSC activation, was inhibited by Y-27632; DNA synthesis was reduced with 10 μM Y-27632 by ~30–40% (17). In contrast, Kawada et al. (20) reported that 10 μM Y-27632 did not alter DNA synthesis in those cells. Although the difference between these two studies is yet to be explained, we would like to point out that Y-27632 induced HSC apoptosis at as little as 3 μM in this study. The effect of Y-27632 on apoptosis appears more specific than the effect, if any, on proliferation in HSCs. Thus we speculate that the induction of HSC apoptosis might be the mechanism of resolution of hepatic fibrosis in rats by Y-27632. This matter should be investigated further.

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