A Novel Function of Thrombin-activatable Fibrinolysis Inhibitor during Rat Liver Regeneration and in Growth-promoted Hepatocytes in Primary Culture*

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Thrombin-activatable fibrinolysis inhibitor (TAFI) exhibits anti-fibrinolytic activity by removing C-terminal lysine residues from fibrin or plasminogen receptor proteins on the cellular surface, and plays an important role in the regulation of fibrinolysis. In this study, we examined the regulation of TAFI in hepatocytes during liver regeneration, and revealed its pivotal role in hepatocyte proliferation. In rat models, partial hepatectomy or carbon tetrachloride (CCl$_4$)-induced acute liver injury suppressed the levels of plasma TAFI activity and hepatic TAFI mRNA, whereas this operation markedly increased both the hepatic plasmin activity and the level of proliferating cell nuclear antigen. In primary cultures of rat hepatocytes, the TAFI mRNA level was decreased under growth-promoting culture conditions. Treatment of the hepatocytes with TAFI siRNA increased the amount of plasmin on the hepatocytes and promoted hepatocyte proliferation. We concluded that TAFI regulates plasmin activity through its enzymatic activity whereby it reduces the plasminogen-binding capacity of the hepatocytes. The TAFI gene expression is down-regulated in hepatocyte proliferation for producing a fibrinolytic microenvironment suitable for cell growth. This is the first report on the role of TAFI in the pericellular fibrinolysis necessary for cellular proliferation.

Thrombin-activatable fibrinolysis inhibitor (TAFI)[3] is a 60-kDa plasma glycoprotein secreted by hepatocytes as an inactive form. Activation of TAFI is known to be mediated by thrombin (1), thrombin-thrombomodulin complex (2), or plasmin (3). Activated TAFI down-regulates fibrinolysis by removing the plasminogen-anchoring structure from fibrin. This fibrin structure contains C-terminal lysine residues, to which plasminogen binds via its lysine-binding site (4). Thus, TAFI is thought to exhibit negative regulatory activity in the binding of plasminogen to fibrin or cell surfaces.

It is well known that plasminogen on fibrin or a cell surface exerts its maximum activity there, because this binding is not only a prerequisite for plasminogen activators to convert plasminogen to plasmin efficiently, but also a guarantee for the plasmin to be protected from inactivation by specific inhibitors, such as α2-plasmin inhibitor (5). As such, plasminogen can fulfill its roles for both thrombolysis and pericellular proteolysis, when it is located on the surface (6).

It has been reported that activation of plasminogen is observed at the early stage of liver regeneration in rats and that plasmin contributes to the priming of hepatocytes for proliferation through the reorganization of extracellular matrix (ECM) components (7). The impairment of liver regeneration and abnormalities in liver repair have been observed in plasminogen-deficient mice, when they have undergone partial hepatectomy (8, 9) or been treated with CCl$_4$ (10). We demonstrated that the plasminogen activator/plasmin system acts to enhance the formation of hepatocyte spheroids (11) and to promote the proliferation of hepatocytes *in vitro* (12). These results strongly suggest that plasmin(ogen) is a positive regulator for liver regeneration.

Although the function of TAFI in fibrinolysis has been well investigated (13), its function, including the regulation of TAFI gene expression in pericellular fibrinolytic events, remains to be investigated. In studies using TAFI-deficient mice, it has been found that such mice develop normally, reach adulthood, and are fertile. No gross physical abnormalities are observed up to 24 months of age (14). On the other hand, abnormalities in the cutaneous wound healing with delayed skin closure due to the altered epithelial migration and colonic anastomosis with bleeding complications are observed in these TAFI-deficient mice (15). Regarding TAFI gene expression, there is a report by Boffa *et al.* (16) showing that the combination of interleukin-1 and interleukin-6, but neither alone, reduces the abundance of TAFI mRNA in cultured human hepatoma (HepG2) cells by decreasing the stability of the mRNA.

In the present study, we investigated the roles of TAFI in liver regeneration. The gene expression of TAFI was found to be strictly controlled by factors responsible for hepatocyte growth such as growth factors or physical conditions. In other words, suppression of TAFI action enhanced the growth of hepatocytes by providing higher plasmin activity on their surfaces. We

[3] The abbreviations used are: TAFI, thrombin-activatable fibrinolysis inhibitor; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CCl$_4$, carbon tetrachloride; ECM, extracellular matrix; EGF, epidermal growth factor; PCNA, proliferating cell nuclear antigen; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

employed the RNA interference technique to clarify the substantial role of TAFI. In TAFI-silenced hepatocytes, plasmin activity on hepatocyte surface increased, and hepatocyte proliferation was enhanced proportionally. Thus we demonstrated for the first time that TAFI plays an important role in the proliferation of hepatocytes *in vivo* through its regulatory effect on the cell surface-bound plasmin.

MATERIALS AND METHODS

Reagents and Antibody—Recombinant human thrombomodulin was kindly provided by Dr. Shingo Niimi (National Institute of Health Sciences, Tokyo, Japan). Antibody against proliferating cell nuclear antigen (PCNA), PC-10, was purchased from Dako-Cytomation (Glostrup, Denmark). Antibody against rat TAFI was kindly provided by Dr. Ann Gils (University of Leuven, Leuven, Belgium). Tranexamic acid was a crystallized pure form obtained from Daiichi Sankyo Co. (Tokyo, Japan) as a gift.

Animal Experiments—All animal experiments were performed in accordance with the Guidelines for Animal Experiments, College of Bioresource Sciences, Nihon University.

Induction of Liver Injury by Carbon Tetrachloride—Acute liver injury was induced by carbon tetrachloride (CCl_4) as described previously (17). Briefly, male Wistar strain rats weighing 150–200 g (Nippon Bio-Suppl. Center, Co., Tokyo, Japan) were provided distilled water containing 0.5 g/liter sodium phenobarbital for 6 days *ad libitum*, and then given an intraperitoneal bolus injection of CCl_4 (0.1 ml of 50% CCl_4/100 g body weight, Nacalai Tesque Inc., Kyoto, Japan). Liver and blood were obtained under anesthesia with diethyl ether after the treatment with CCl_4.

Partial Hepatectomy Model—Male Wistar strain rats (150–200 g, Nippon Bio-Suppl. Center, Co.) were subjected to a two-thirds hepatectomy under diethyl ether anesthesia. The residual liver tissue was removed at 0–168 h after the partial hepatectomy or sham operation, flushed with liquid nitrogen, and stored at −80 °C until analysis could be made. Citrated plasma was also prepared at the same time points post operation.

Assay of Aminotransferases—Activities of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by using a commercial kit (Wako, Osaka, Japan).

Measurement of TAFI Activity—Plasma TAFI activity was measured by the method using a synthetic peptide substrate, hippuryl-L-lysine, which was basically described before (18, 19). Briefly, plasma TAFI was activated by a solution containing thrombin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), thrombomodulin, and $CaCl_2$. The activated TAFI was then added to the substrate, hippuryl-L-lysine. Finally, the color developed by the addition of sodium phosphate and cyanuric chloride/1,4-dioxan was measured immediately at 405 nm after the addition of the color reagents. For estimation of the real TAFI activity, the above procedures were performed in the presence or absence of a TAFIa-specific inhibitor, potato carboxypeptidase inhibitor (50 μg/ml, Sigma); the activity was expressed as milliunits/ml, which was calibrated by using a standard curve for pancreatic carboxypeptidase preparation (Sigma).

Experiments on Rat Hepatocytes in Primary Culture—Hepatocytes were obtained from the liver of male Wistar strain rats (150–200 g) by the two-step collagenase perfusion method described by Ichihara *et al.* (20), and the cells were purified by repeated low speed centrifugation (50 *g*). The isolated hepatocytes were used in the following experiments: for the experiment on EGF-stimulated cell growth, the hepatocytes were plated on 35-mm type I collagen-coated dishes (Iwaki, Tokyo, Japan) and precultured for 4 h in Williams' medium E supplemented with 5% calf serum, 10^{-8} M insulin, and 10^{-8} M glucagon, and then incubated for 20 h at 37 °C in serum- and hormone-free Williams' medium E. After 24 h the precultured hepatocytes were cultured in the latter medium containing epidermal growth factor (EGF) for 24 h and then used for the experiments. For the cultures used to examine changes in cell density, different numbers of the isolated hepatocytes were plated on the collagen-coated dishes ($0.1\sim1.0 \times 10^5$ cells/cm^2) and cultured for 24 h.

Silencing of TAFI Gene Expression by RNA Interference—TAFI siRNA (Stealth RNAi, Invitrogen) and control siRNA (Invitrogen) were used in this experiment. The isolated hepatocytes were plated on collagen-coated dishes at a density of 0.5×10^5 cells/cm^2 and precultured as described above. The precultured primary hepatocytes were transfected with 10 nM of those siRNA by using a transfection reagent, Lipofectamine 2000 (Invitrogen). Tranexamic acid (10 mM) was used to remove plasminogen from the hepatocyte surface, on which plasminogen was suspected to have originated from the hepatocytes themselves or fetal bovine serum during the preculture period. Tranexamic acid was added to the culture at 3 h after siRNA transfection. One hour after the treatment, the medium was replaced with Williams' medium E containing 0.1% bovine serum albumin, and the hepatocytes thus treated were cultured for up to 48 h. The efficiency of TAFI silencing was monitored by quantitative reverse transcription-PCR and Western blotting.

Cell Proliferation Assay—For cell proliferation assay, hepatocytes were cultured in the collagen coated 96-well plate (Iwaki). After transfection with siRNA, the hepatocytes proliferation was measured by using Cell Count Reagent SF (Nacalai Tesque Inc.) according to manufacturer's instructions.

Western Blotting—Total liver protein was extracted and prepared by the method described elsewhere (21) for detecting PCNA in the liver. Briefly, the liver was homogenized in phosphate-buffered saline containing 0.5% deoxycholate, 1% SDS, 1% Nonidet P-40, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. The homogenates were centrifuged at 12,000 × *g* for 15 min at 4 °C. Ten micrograms of total protein extract was subjected to SDS-PAGE. For detecting TAFI antigen, culture supernatant harvested from primary cultures of rat hepatocytes was used. After the electrophoresis, proteins that had migrated on the gel were transferred onto a nitrocellulose membrane (Advantec MFS, Inc., Dublin, CA). The membrane was first incubated with the primary antibody, PC-10, a mouse antibody against PCNA (1:200 final dilution), or monoclonal antibody against rat TAFI (20 μg/ml) and then with the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 final dilution). The PCNA and TAFI protein bands

were visualized by using Immunostaining HRP-1000 (Konica Minolta Inc., Tokyo, Japan) and Lumi-Light[PLUS] Western blotting substrate (Roche Diagnostics, Mannheim, Germany), respectively.

Preparation of Plasma Membrane-enriched Fraction—Plasma membrane-enriched fraction was isolated from rat liver according to the method of Hubbard *et al.* (22). Briefly, the liver was homogenized with a Potter homogenizer in a solution of 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride supplemented with a protease inhibitor mixture (Sigma). The homogenate was centrifuged at $280 \times g$ for 5 min, and the supernatant was centrifuged further at $1500 \times g$ for 10 min. The pellet from this step was resuspended in 5 mM Tris-HCl buffer, pH 7.4, containing 1.42 M sucrose, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride with a Dounce homogenizer; and the suspension was then overlaid onto 0.25 M sucrose solution in 5 mM Tris buffer, pH 7.4, and centrifuged at $82,000 \times g$ for 60 min at 4 °C in a swing rotor (MLS-50, Beckman Coulter Inc., Fullerton, CA). The plasma membrane-enriched fraction was taken as a layer formed between these two solutions of different densities.

Preparation of Cell-associated Plasminogen—Cell-associated plasmin(ogen) was eluted from hepatocytes by using tranexamic acid solution. Cultured hepatocytes were washed with ice-cold phosphate-buffered saline and then incubated at 4 °C for 10 min with phosphate-buffered saline containing 0.1 M tranexamic acid solution. The supernatant containing the eluted plasminogen was stored at −20 °C until used for experiments.

Zymography—Fibrinolytic activity was analyzed by zymography as described elsewhere (23). Cellular proteins, including membrane-associated plasminogen were subjected to SDS-PAGE on a 10% polyacrylamide gel containing 2 mg/ml plasminogen-free fibrinogen for the detection of plasmin activity. After the electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min to remove the SDS and then incubated at 37 °C in buffer (50 mM Tris-HCl, pH 8.3, containing 0.1 M glycine) for 44 h. The gel was stained with 0.075% Coomassie Blue and destained by a solution of 12.5% methanol containing 12.5% acetic acid.

RNA Isolation and Reverse Transcription-PCR—Total RNA was extracted from rat liver tissue by using the single-step method or from primary rat hepatocytes by use of Isogen reagent (Wako). Real-time reverse transcription-PCR was performed by the fluorescent dye SYBR Green I method using the SYBR Premix Ex Taq, Perfect Real Time (Takara Bio, Shiga, Japan) with a LightCycler rapid thermal cycler system (Roche Diagnostics) or StepOne (Applied Biosystems, CA).

The primers used in this study were designed based on the GenBank[TM] information (TAFI, GenBank[TM], NM_053617; GAPDH, GenBank[TM] AF106860) and synthesized by Nihon Gene Research Laboratories (Miyagi, Japan). PCR primers used were as follow: TAFI primers, 5′-AGA AGC AAA AGC AAG GAC CA-3′ and 5′-ATT TTA GAG ACT GCG GCC AA-3′; plasminogen primers, 5′-CCC GAA TGC GAA GAG GAA TG-3′ and 5′-TTG GGG TCT GTG GTG AAG CA-3′; and GAPDH primers, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′.

RESULTS

Expression of TAFI in Rats with CCl_4-induced Acute Liver Injury—We examined the changes in the TAFI level in an animal model of acute liver injury produced by CCl_4 administration to rats (Fig. 1). The plasma levels of ALT and AST released from the injured hepatocytes were significantly increased at 48 h after the CCl_4 administration (Fig. 1*A*). Following the worst indication of injury, the expression of PCNA, a marker of the liver regeneration, was elevated at 72 h (Fig. 1*B*). The TAFI mRNA was significantly decreased at 24–48 h after the CCl_4 administration, and then restored to the basal level by 168 h (Fig. 1*C*). Well consistent with the drop in mRNA level, the plasma TAFI activity was significantly decreased at 48 h after the CCl_4 administration, and then returned to the unsuppressed vehicle level by 168 h (Fig. 1*D*). A moderate gradual increase in the level of TAFI mRNA was also observed in the vehicle-treated control rats (Fig. 1*C*, *vehicle*).

Expression of TAFI during Liver Regeneration after Partial Hepatectomy—We next investigated the change in the TAFI level in rats with 70% partial hepatectomy (Fig. 2). After the hepatectomy, the reduced liver weight was dramatically restored to the preoperative level, at a quick rate from 24 to 48 h, and then slowly from 48 to 168 h (Fig. 2*A*); and PCNA expression was detected clearly during the former accelerated period (Fig. 2*B*). The TAFI mRNA level in the remnant liver tissue significantly decreased just after the partial hepatectomy, and this decrease lasted for 72 h; the mRNA level then increased corresponding to the weight gain, and was restored to the basal level by 168 h (Fig. 2*C*). In the sham-operated rats, the TAFI mRNA level increased ~2-fold at 24 h and decreased thereafter (Fig. 2*C*). The plasma TAFI activity in both hepatectomized and sham-operated rats was well correlated with the mRNA level (Fig. 2*D*).

Localization of Fibrinolytic Factors on Hepatocyte Plasma Membrane after Partial Hepatectomy—To clarify the possible role of TAFI in liver regeneration, we measured plasmin activity localized on the hepatocyte membrane during the regeneration (Fig. 3). Although plasmin activity in the whole liver extract was slightly detected after the partial hepatectomy, that in the plasma membrane-enriched fraction was clearly detected and increased from 24 to 48 h after the partial hepatectomy (Fig. 3, *A* and *B*). These results indicate that plasmin is localized onto the plasma membrane after the partial hepatectomy.

Down-regulation of TAFI mRNA Expression in Rat Hepatocytes Cultured under Growth-promoting Conditions—Because the TAFI mRNA level was decreased in the regenerating liver, we investigated the regulation of TAFI expression in rat hepatocytes in primary culture under growth-promoting conditions. We first examined the effects of EGF on the expression of TAFI mRNA in the hepatocytes in primary culture (Fig. 4*A*). EGF reduced the TAFI mRNA level in the hepatocytes to ~50% of its level in the EGF-free control hepatocytes. The influence of the cellular density on the expression of TAFI was studied next. The TAFI mRNA level in hepatocytes was significantly decreased by lowering the cellular density in cultures; *e.g.* the hepatocytes plated at a density of 0.1×10^5 cells/cm² expressed the mRNA at a level only 40% of that by the hepatocytes plated
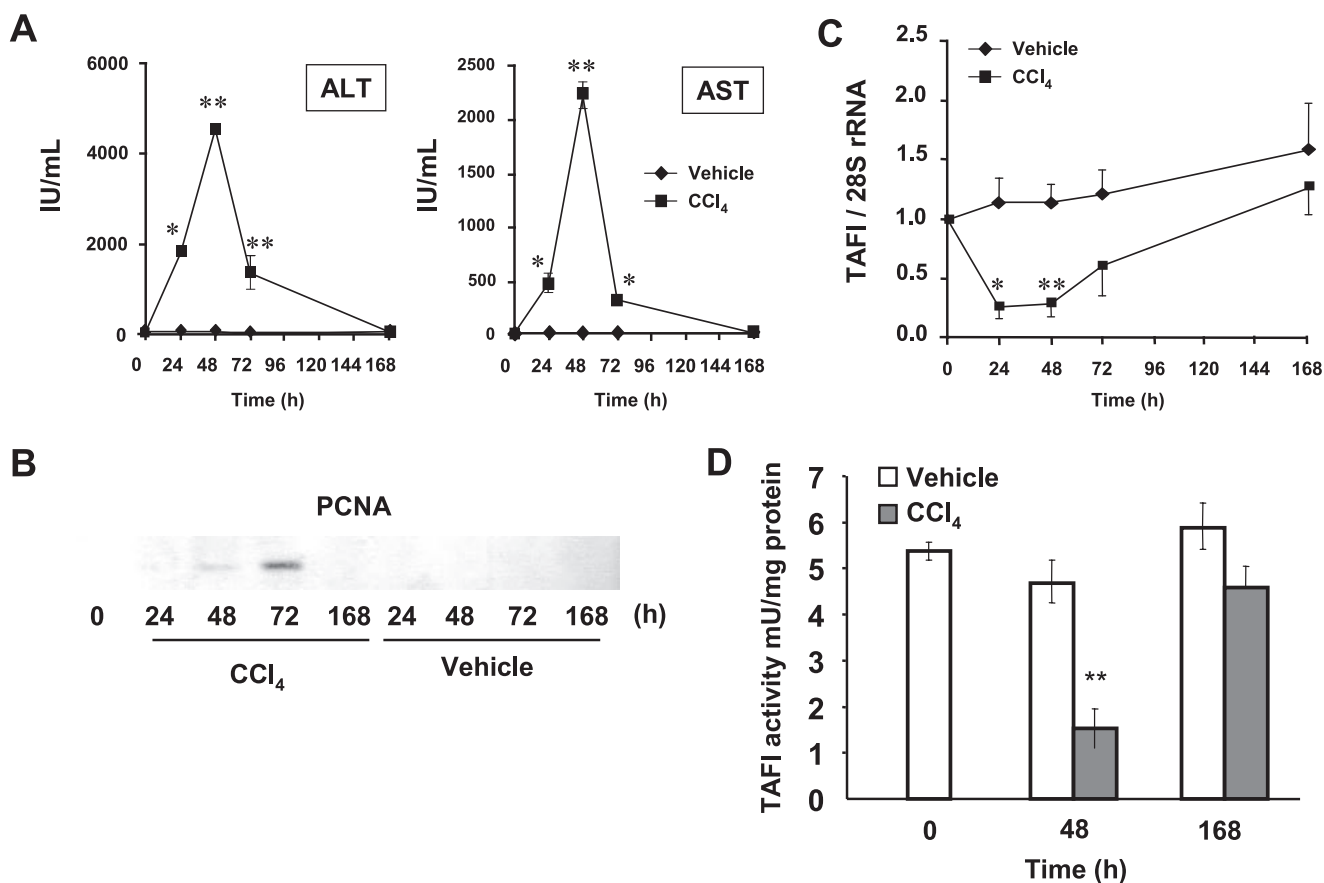
FIGURE 1. **Expression of TAFI and PCNA in rat liver with or without CCl$_4$-induced acute injury.** *A,* plasma ALT and AST activities of CCl$_4$-administered rats. Each *point* represents the mean ± S.E. of three rats (*, $p < 0.05$; **, $p < 0.01$ *versus* vehicle). *B,* expression of PCNA in the liver after CCl$_4$ administration. *C,* TAFI mRNA levels in the livers of CCl$_4$-administered rats and in those of vehicle-treated rats. The amount of TAFI mRNA detected was normalized with GAPDH mRNA and expressed as a ratio against that at time 0. Each *point* represents the mean ± S.E. of three rats (*, $p < 0.05$; **, $p < 0.01$ mean significance as determined by Student's paired *t* test *versus* vehicle). *D,* plasma TAFI activity of rats with acute liver injury with CCl$_4$. TAFI activity was normalized with total plasma protein. Each *bar* represents the average ± S.E. of 3–5 rats (**, $p < 0.01$).

at the 10-fold higher one of 1.0×10^5 cells/cm^2 (Fig. 4*B*). We also investigated the influence of type-I collagen (collagen for short) on the expression of TAFI mRNA. Isolated hepatocytes were plated onto an uncoated dish or a collagen-coated dish at a density of 0.5×10^5 cells/cm^2. The level of TAFI mRNA was significantly decreased by culturing hepatocytes on a collagen-coated dish in comparison with that of hepatocytes cultured on an uncoated dish, indicating that the gene expression of TAFI in hepatocytes is down-regulated under a growth-promoting condition (Fig. 4*C*).

TAFI Silencing Increases Hepatocyte Proliferation—To verify substantial role of TAFI in hepatocyte proliferation, we performed TAFI gene silencing on primary hepatocytes by using a TAFI gene-specific siRNA. With TAFI siRNA treatment, TAFI gene expression in the hepatocytes at 24 h was suppressed by 80% of that in the hepatocyte with the control siRNA (Fig. 5*A*). TAFI mRNA in the control siRNA-treated cells was decreased markedly during the culture for 48 h. Because we observed such a marked decrease in the expression of TAFI mRNA even in the untreated primary cultured hepatocytes (data not shown), this would be a natural phenomenon for the TAFI gene in the primary cultures of hepatocyte (at least as for this gene). In these gene-suppressive situations the TAFI siRNA transfection was still effective,

and we could observe a stimulated cell growth under such a strikingly suppressed TAFI gene. As can be seen in Fig. 5*B*, the decrease in TAFI protein content in the culture medium was more prominent in the TAFI siRNA-transfected hepatocytes than in the control siRNA-transfected cells. Especially, the protein in the transfected cell medium was mostly undetectable, whereas that in the control cell medium was unexpectedly high. This would be due to the high levels of gene expression from 0 to 24 h.

The TAFI gene-silencing effect on hepatocyte growth was determined to be stimulative as can been seen in the microscopic observation (Fig. 5*C*) and by cell counting (Fig. 5*D*). The pictures taken at 48-h culture show that there is a sparse population of control cells, but is a dense population of TAFI siRNA-transfected cells (Fig. 5*C*). On the cell growth, both cultures of control cells and TAFI siRNA-transfected cells were comparable with each other, and did not grow in the first 24 h, even though the growth-inhibitory TAFI was suppressed at these times as shown above (see Fig. 5, *A* and *B*). However, the cell growth was augmented in the next 48 h, and TAFI siRNA-transfected cells significantly surpassed the control cells in their cell counts measured optically (Fig. 5*D*).

As shown in Fig. 6, the primary cultured hepatocytes generated plasmin on their surfaces, and its activity increased along
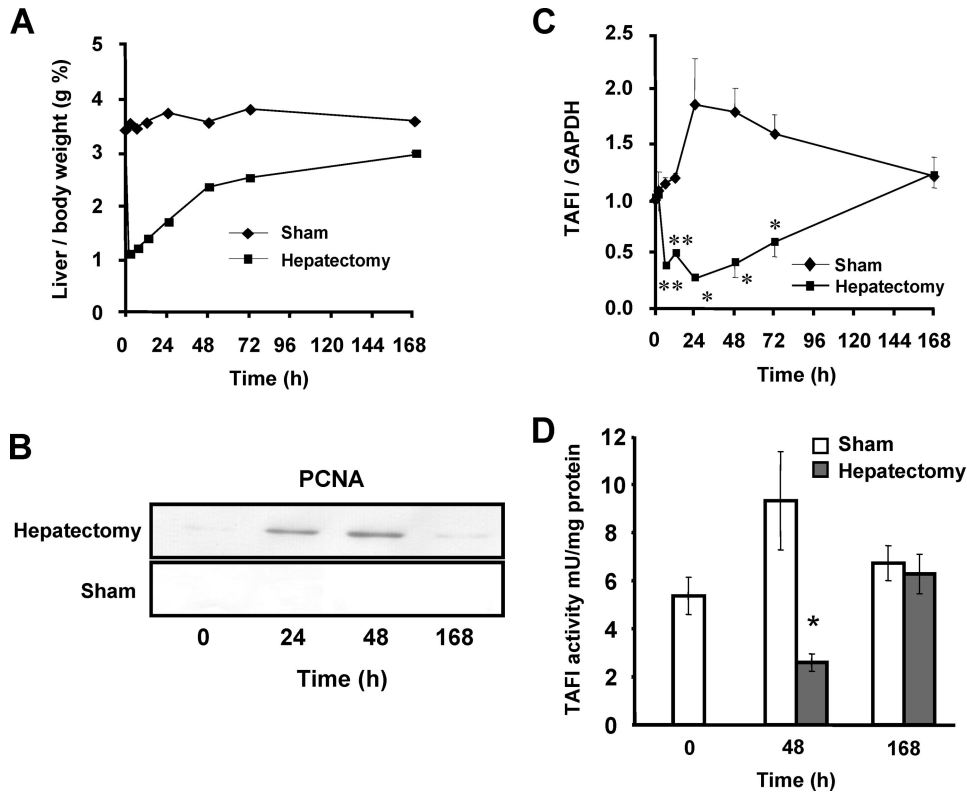
FIGURE 2. **Expression of TAFI in the regenerating liver, and its activity in plasma in rats with partial hepatectomy.** *A*, restoration of liver weight by 168 h after 70% partial hepatectomy. *B*, expression of PCNA in the liver after 70% partial hepatectomy. *C*, TAFI mRNA levels in the liver of rats with partial hepatectomy or sham operation. TAFI mRNA was normalized with GAPDH mRNA and expressed as a ratio against that at time 0. Each *point* represents the mean ± S.E. of three rats (*, $p < 0.05$; **, $p < 0.01$). *D*, TAFI activity in plasma from rats those were treated by the sham operation or partial hepatectomy. TAFI activity was normalized with total plasma protein. The data represent the average ± S.E. of 3–5 different rats (**, $p < 0.01$).

The liver TAFI mRNA and plasma TAFI activity, once elevated by the operation, decreased very quickly, in accordance with the liver regeneration. Therefore, we speculated that the growth signaling in hepatocytes negatively regulates their expression of TAFI. Numerous factors such as hormones and physical conditions surrounding hepatocytes are known to be involved in controlling hepatocyte proliferation. EGF, transforming growth factor-α, and heparin-binding EGF-like growth factor, which are the ligands of EGF receptor, are well known as potent mitosis-inducing factors for hepatocytes both *in vivo* and *in vitro* (27). Hepatocyte proliferation capacity is also tightly regulated by cellular density (28). When hepatocytes are cultured at a high cellular density, the specific liver functions such as albumin production and urea synthesis are highly maintained, but the response to growth factors such as EGF and hepatocyte growth factor is quite low (29). Actually, the expression of c-met, an hepatocyte growth factor receptor, is relatively low in hepatocytes cultured at a high cell density

with the culture time. As can be seen in the zymogram and quantitated results (Fig. 6, *A* and *B*), the cell-associated plasmin activity was clearly stronger in the TAFI-silenced cells than in the control cell.

DISCUSSION

TAFI is the zymogen of plasma carboxypeptidase B secreted from the liver, specifically from the hepatocytes, into the circulation. Activated TAFI inhibits fibrinolysis by reducing the number of plasminogen molecules binding to fibrin. The roles of TAFI in fibrinolysis have been well delineated by numerous studies reported previously (13, 24); however the role of TAFI in cellular fibrinolysis has not yet been fully clarified. Here we first described the growth-related regulation of TAFI and its role in hepatocyte proliferation via cellular fibrinolysis during liver regeneration.

It has been reported that TAFI is reduced in patients with liver diseases. For example, in chronic liver diseases, the activity of TAFI in plasma is reduced (25), and its antigen level is significantly decreased in patients with cirrhosis (26). These results suggest that the reduction in the TAFI level leads to the hyperfibrinolytic state in such pathogenic livers. In this report, we demonstrated that both hepatic TAFI expression and plasma TAFI activity are markedly reduced in experimental rat liver-injury models produced by either CCl_4 administration (Fig. 1) or partial hepatectomy (Fig. 2).

(30). Reciprocally, hepatocytes cultured at a low cell density possess a high proliferation capacity. Nagaki *et al.* (31) reported that, in the presence of EGF and insulin, the DNA synthesis of hepatocytes, which were cultured at a low cell density, reached a level of about 10 times higher than that of hepatocytes cultured at a high cell density. In addition, the ECM is known as a key regulator of hepatocyte proliferation (32). The ECM in the normal liver contains non-fibril-forming collagens such as types IV and VI; however in the injured liver, they shift to fibril-forming collagens such as type I and type III (33). When hepatocytes are cultured on a type I collagen-coated dish, their proliferation increases in comparison with those cultured on the uncoated dish (34). In the present study, we demonstrated that these hepatocyte growth-regulating factors, such as EGF, cellular density and type I collagen, are all novel regulators of TAFI gene expression (Fig. 4). The reduction in TAFI gene expression observed during liver regeneration would therefore have resulted either from the general loss of differentiated hepatic functions or conversely by an activation mechanism involving the down-regulation of TAFI by the above-mentioned hepatic growth stimuli.

There have been some observations suggesting that TAFI functions in cellular fibrinolysis. The treatment of U937 monocytes with a TAFI preparation reduces the binding of plasminogen on the surface of these cells (4). Thioglycollate-induced leukocyte recruitment is more effectively promoted in TAFI-
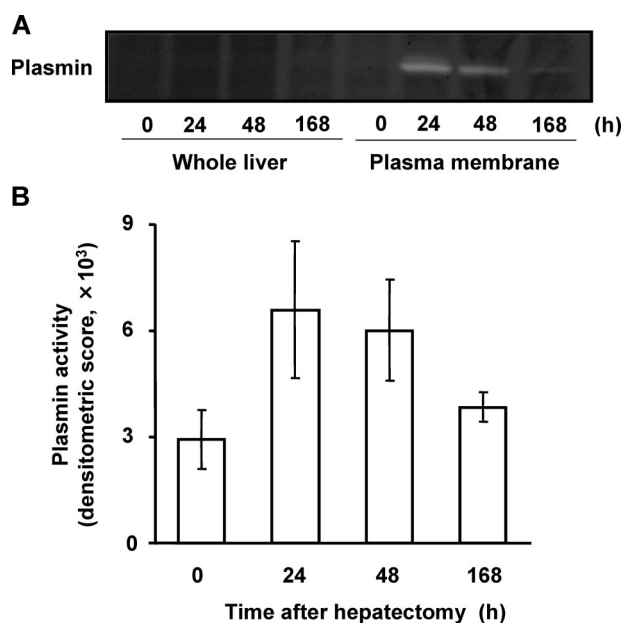
FIGURE 3. **Localization of plasmin on hepatocyte plasma membrane after partial hepatectomy.** *A*, localization of plasmin in the liver after partial hepatectomy. Whole liver extract (*Whole liver*) and plasma membrane-enriched fraction (*Plasma membrane*) were prepared from the liver of rats that had received a partial hepatectomy or sham operation at the time points indicated. Both liver samples were subjected to zymography performed on a 10% SDS-polyacrylamide gel containing plasminogen-free fibrinogen, and plasmin activity was detected. *B*, optical densities representing the plasmin activity in the plasma membrane-enriched fraction are shown. The data represent the average ± S.E. of four different rats.

deficient mice than that in wild-type mice (36). On the other hand, fibroblast migration is suppressed, and in turn impairment of wound healing is manifested in these mice (14). However, direct observations of TAFI function in cellular fibrinolysis, which reduces the plasminogen-binding capacity of the cell surface, have not been previously reported. As to the relationship between plasmin and TAFI in the liver of partially hepatectomized rats, we found that these factors were regulated reciprocally; *i.e.* the increased plasmin activity in the plasma membrane-enriched fraction was accompanied by a decrease in the TAFI level not only in its gene expression, but also in its activity in plasma (Fig. 3).

To directly clarify the role of TAFI in hepatocyte proliferation, we prepared TAFI-deficient hepatocytes by using the RNA interference technique. The hepatocytes with a silenced TAFI gene showed increased plasmin activity on their surface (Fig. 6), and their proliferation was promoted (Fig. 5). The plasmin in hepatocyte seems to play with its proteolytic activity, by which the pericellular fibrinolysis may proceed (6). In this role, the activation of growth factors (3), removal of fibronectin for the remodeling of ECMs (37), and activation of inactive proenzymes would be involved (38). Activation of plasminogen can be observed at the early stage of liver regeneration, and it is known that such reaction accompanies with an increased expression of uPA activity as early as 1 min after the hepatectomy (7). We know that in the liver of partially hepatectomized animals the gross organ changes in plasmin activity and related extracellular matrices degradation and/or remodeling are operated mostly within 24 h (7), but sometimes changes last long; *e.g.* high amounts of laminin present in Ito cells at 8 days after
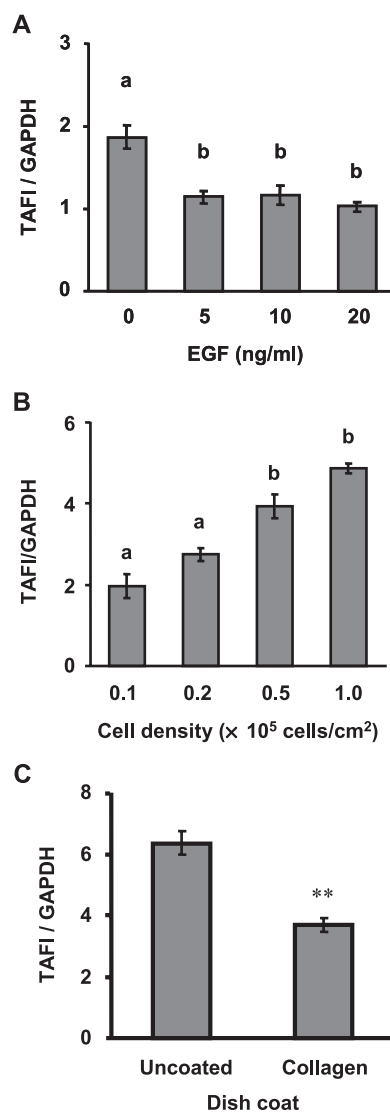

FIGURE 4. **Expression of TAFI mRNA by rat hepatocytes in primary culture under various conditions.** *A*, isolated hepatocytes were cultured in the presence of various doses of EGF as indicated for 24 h. TAFI mRNA was normalized to GAPDH mRNA. Each *bar* represents the average ± S.E. of four different experiments. Values annotated with different letters are significantly different from each other as determined by the Tukey's test after one-way analysis of variance. *B*, isolated hepatocytes were plated at the different cell densities as indicated, and cultured for 24 h. Each *bar* represents the average ± S.E. of three different experiments. Values annotated with different letters are significantly different as determined by the Tukey's test after one-way analysis of variance. *C*, isolated hepatocytes were inoculated onto culture dishes coated or not with type-I collagen (collagen), and cultured for 24 h. Each *bar* represents the average ± S.E. of three different experiments. **, $p < 0.01$.

hepatectomy (39). However, we have had little knowledge on the cellular changes of plasmin and its major regulator TAFI in these liver samples. Then we studied them here using both animal model, which was analyzed for 7 days after hepatectomy, and primary cultured hepatocytes. In this study, uPA activity was detected as early as 2 h after partial hepatectomy and gradually increased (data not shown). Thus uPA is thought to be involved in the activation of plasminogen bound on membrane, as has been reported (6). The changes in plasmin activity on membrane were thought to be solely due to the localization of plasmin(ogen) on the hepatocyte membrane, because TAFI should not be responsible for the localization of uPA; uPA
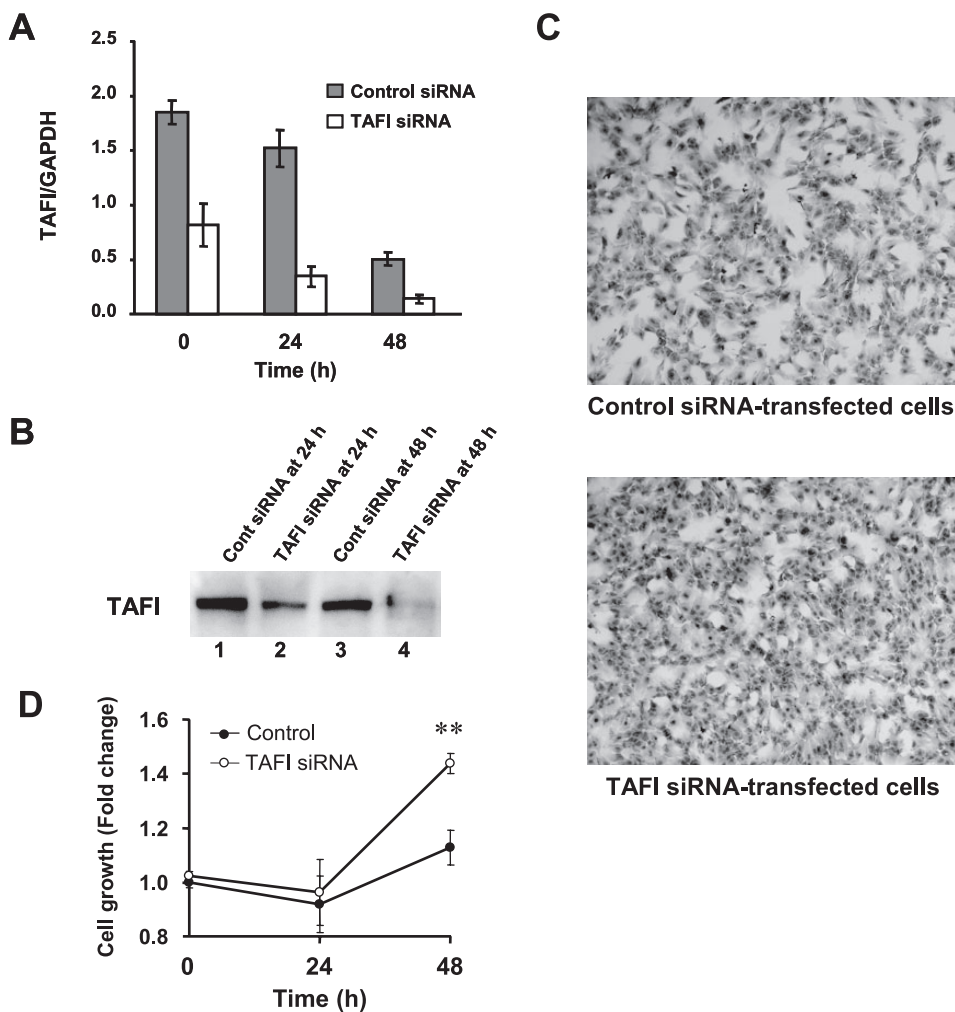
FIGURE 5. **Effect of TAFI-silencing on hepatocyte proliferation.** *A*, expression of TAFI mRNA was monitored by quantitative reverse transcription-PCR. As described under "Materials and Methods," *0 time* is designated to be the time after 4 h from siRNA transfection, thus TAFI mRNA level of the transfected hepatocytes would already be suppressed at 0 time. *B*, TAFI protein was detected in the cultured media by Western blotting. *C*, micrographs of siRNA-transfected hepatocytes. The *photographs*, taken at 48 h after TAFI-silencing (original magnification, ×40), show that there are many more nuclei stained *black* in the TAFI-silenced culture (see the *lower picture*) than that in the control culture (the *upper one*). *D*, hepatocyte proliferation was measured at each time points by the cell count reagent SF. Each *bar* represents the average ± S.E. of five different experiments, assuming the cell population at 0 time is 1.0. The *ordinate* (cell growth) scaled as -fold changes from the population at 0 time.

atocyte growth-promoting factors, with the consequence being increased plasmin activity at the hepatocyte surface, leading to hepatocyte proliferation.

On the other hand, TAFI is known as a regulator of the complement system. For example, TAFI inactivates C5a and C3a by removing C-terminal arginine residues from their structures (46). It has been reported that liver TAFI mRNA is induced by lipopolysaccharide (47). Renckens *et al.* (48) also reported that there was clear induction of both hepatic TAFI mRNA and plasma TAFI protein in an abdominal sepsis model in mice produced by the intraperitoneal injection of *E. coli*. These reports indicate that TAFI is an acute-phase reactant. In our experiments, a moderate increase in TAFI mRNA was observed in the vehicle-treated control rats (Fig. 1*C*) and sham-operated animals (Fig. 2*C*). These increases are therefore thought to occur as a part of the acute-phase response toward the abdominal surgery (sham operation) or to the intraperitoneal injection of olive oil as the vehicle. More interestingly, it has been reported that the liver injury caused by *E. coli* is not serious in TAFI-deficient mice, but serious in wild-type mice (48). Because the administration of *E. coli* to TAFI-deficient mice does not influence hemostatic parameters such as plasma levels of thrombin-antithrombin complexes and D-dimer,

receptor does not possess C-terminal lysine residue. Furthermore, membrane-bound plasminogen activator activity is not influenced by serine protease inhibitors such as plasminogen activator inhibitor-1 (6). We have also previously reported that hepatocyte surface-bound plasminogen regulates hepatocyte proliferation via activation of the plasminogen by uPA (40). The other function is its signaling, which is proposed to begin with plasminogen/plasmin-binding to cell surface receptors, resulting in cell migration and cytokine release (41, 42). Recently, Nicholl *et al.* (43) demonstrated that plasmin induces smooth muscle cell proliferation via the ERK1/2 signaling cascade. Plasmin also induces the expression of the cysteine-rich angiogenic inducer 61 through the activation of protein activated receptor and increases the growth of fibroblasts (44). In hepatocytes, plasmin regulates the expression of pro-apoptotic protein Bim (EL) via the ERK1/2 signaling pathway (45). These results suggest that, in liver regeneration, TAFI is down-regulated by hep-

the liver injury in TAFI-deficient mice can be attributed to the enhanced activity of complement components. In complement-deficient mice, the liver regeneration after partial hepatectomy or the liver restoration after CCl_4-induced injury is markedly delayed (49). It is also known that C5 induces hepatocyte growth factor, cyclin E, or cyclin D1 expression via C5a receptors and stimulates hepatocyte proliferation (35). Taken together, the reduced expression of TAFI during liver regeneration might be favorable or even essential to the hepatocytes for their proliferation under the conditions thus provided. Namely, the down-regulation of TAFI would give rise to the elevation of complement activities and the up-regulation of plasminogen binding to hepatocytes. Actually, our *in vivo* experiment using TAFI knockout mice indicated that TAFI deficiency accelerates liver regeneration after partial hepatectomy (data not shown).

In summary, the TAFI level in hepatocytes was suppressed during liver regeneration, and this down-regulation may be
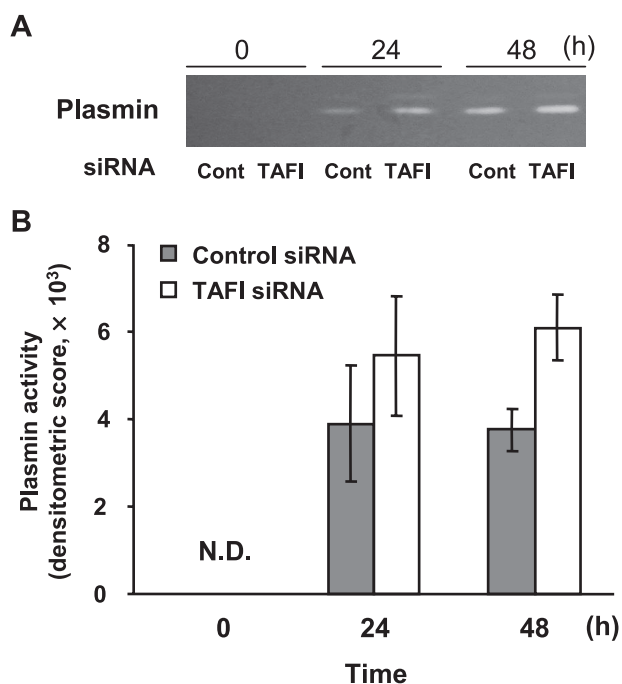
A

B

FIGURE 6. **Effect of TAFI silencing on the localization of plasmin on the hepatocyte surface.** *A*, plasmin activity of the rat hepatocytes transfected with control siRNA (*Cont*) or TAFI siRNA (*TAFI*). Cell-associated plasmin was eluted by treating the hepatocytes with tranexamic acid, and the eluate was subjected to zymography. *B*, plasmin activity observed on the zymogram was quantified by computer-assisted densitometry. The data represent the average ± S.E. of three different experiments. *N.D.*, not determined, because the plasmin activity was under estimable levels in these cells.

crucial for producing a hyperfibrinolytic state with increased activity of plasmin on the hepatocyte surface. In addition, the growth-stimulating factors negatively regulated TAFI expression in rat hepatocytes in primary culture. This is the first report to describe the role of TAFI as a negative regulator in hepatocyte proliferation and liver regeneration.

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