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Induction of Tissue-Type Plasminogen Activator (tPA) and Type-1 Plasminogen Activator Inhibitor (PAI-1) as Early Growth Responses in Rat Hepatocytes in Primary Culture

Shigeyuki Uno, Masaru Nakamura, Taiichiro Seki,[1] and Toyohiko Ariga
Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences, Tokyo 154, Japan

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Early growth response with respect to tissue-type plasminogen activator (tPA) and type-1 plasminogen activator inhibitor (PAI-1) gene expression was studied in rat hepatocytes in primary culture. The genes for tPA and PAI-1 could be categorized as a delayed early growth response (DER) gene and an immediate early growth response (IER) gene, respectively. The expression of tPA was much higher in growth-promoting than in static culture conditions (i.e., cultured at low density and/or on a collagen-coated dish), and that of PAI-1 was regulated in the opposite direction. Experiments using dibutyryl cAMP (dbcAMP) and H-89 showed that the cAMP/A-kinase system might be involved in the induction of the early growth response of tPA and in the augmentation of PAI-1 mRNA induction by dbcAMP. These fibrinolytic components, whose expression is closely associated with hepatocyte growth, may play important roles in pathophysiological events in the liver such as liver regeneration.

PAs are serine proteases that convert the inactive proenzyme plasminogen to its active form, plasmin (1). Plasmin is a broad spectrum endopeptidase; thus, the PA-plasmin system is not only involved in the fibrinolysis, but also in other physiological and pathological processes such as malignant invasion and metastasis, in which breakdown of extracellular matrices is evoked topically (2). Activity of the PA-plasmin system is controlled both by the regulation of PA production and by specific inhibitors.

PAI-1, an approximately 50-kd glycoprotein, is the major physiological inhibitor of PAs. PAI-1 is synthesized by a wide variety of cell types *in vitro*, and its expression has been shown to be regulated by growth factors, tumor-promoting phorbol esters, cytokines, and hormones (3, 4, 5).

Although both PA and PAI-1 are found in several organs *in vivo*, the expression of these fibrinolytic components in normal rat liver is quite low or undetectable (6, 7); however, we have described the induction of tPA and PAI-1 gene expression in carbon tetrachloride-induced liver injury (8). It has also been reported that PAI-1 is expressed by the regenerating rat liver *in vivo* (9), but not expressed by quiescent rat hepatocytes *in vivo* (6,9-11). These results suggest that the PAI-1 gene is an early growth response gene (9). However, the mechanism of the induction of PAI-1 gene expression in hepatocytes or the role(s) of PAI-1, which is produced in response to growth stimuli, is not known. To explore the pathophysiological role of the PA-plasmin system in the liver, we have characterized the tPA and PAI-1 gene expression in an early growth response model using primary cultures of rat hepatocytes.

We report herein the induction of tPA and PAI-1 gene expression in the early growth response of rat hepatocytes in primary culture. The genes for tPA and PAI-1 could be classified as a DER gene and an IER gene, respectively.

MATERIALS AND METHODS

Isolation of rat hepatocytes and primary culture. The hepatocytes were isolated from male Wistar strain rats by a method in which the liver was perfused with collagenase (type I, Sigma Chem., MO. U.S.A.)(12). The isolated cells were plated on collagen-coated culture dishes (100 mm, Iwaki Glass, Chiba, Japan) at a density of 0.5 ×

[1] Corresponding author: Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences, 3-34-1 Shimouma, Setagaya, Tokyo 154, Japan. Fax: +81-3-5431-7895. E-mail: VYB00536@niftyserve.or.jp.

Abbreviations: dbcAMP, N^6, 2′-o-dibutyryladenosine 3′: 5′-cyclic monophosphate; DER gene, delayed early growth response gene; DMSO, dimethylsulfoxide; EGF; epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IER gene, immediate early growth response gene; LPS, lipopolysaccharide; PA, plasminogen activator; PAI-1, type-1 plasminogen activator inhibitor; RT-PCR, reverse transcription-polymerase chain reaction; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; WE, Williams' medium E.

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10^5 cells /cm^2 and cultured in a humidified atmosphere of 5% CO_2 : 95% air as basically described previously (13). The medium for the culture was Williams' medium E (WE; Dainippon Pharmaceutical, Osaka, Japan) supplemented with 5% FBS (JRH Bioscience, KS, Australia), 10^{-8}M insulin (Sigma), 10^{-8}M glucagon (Sigma), and 1.6 n M human recombinant epidermal growth factor (EGF; Higeta Shoyu Co., Ltd., Chiba, Japan) with /without 10 μg/ml cycloheximide (Sigma) at 37°C. The hepatocytes were cultured up to 6 hours, and the total RNA was isolated every 30 minutes after plating.

In the experiments to study effects of N^6,2′-o-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP; Sigma) or H-89 (Seikagaku Co., Tokyo, Japan) on tPA or PAI-1 mRNA expression, the hepatocytes were plated and cultured with these agents for 4 or 6 hours followed by isolation of total cellular RNA.

Isolation of cellular RNA. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (14), and its concentration was determined by spectrophotometry at 260 nm.

Reverse transcription and PCR. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the quantitative changes in the RNAs for tPA, PAI-1 and c-fos. The first strand of cDNA was synthesized, by use of a commercial RT-PCR kit (RT-PCR high, Toyobo Co., Ltd., Tokyo, Japan), in a 5-μl reaction mixture containing 0.25 ng total cellular RNA, and the following PCR was also carried out using the RT-PCR kit and thermal cycler (TC-700, Astec Co., Ltd., Fukuoka, Japan) under suitable conditions for tPA, PAI-1, and c-fos. The primers and conditions used for each mRNA were as follows: the primers for tPA were 5′CCTAAGGGACCAACTGAGGACTGCTATGTT and 5′CAAGGGTGTGAGGTGATGTCTGTGAAGAGT, and a two-temperature step RT-PCR cycle program was carried out at 35 cycles for amplification of tPA cDNA (1 min anealing at 60°C and 2 min extension at 72°C, and 1 min denaturation at 94°C). The primers for PAI-1 were 5′ATGAGATCAGTACTGCGGACGCCATCTTTG and 5′GCACGGAGATGGTGCTACCATCAGACTTGT (15); 35 cycles for amplification of PAI-1cDNA (1 min anealing at 74°C and 2 min extension at 72°C, and 1 min denaturation at 94°C). The primers for GAPDH were 5′ACCACAGTCCATGCCATCAC and 5′TCCACCACCCTGTTGCTGTA (Toyobo); 30 cycles for the amplification of GAPDH cDNA (1 min anealing at 60°C and 2 min extension at 72°C, and 1 min denaturation at 94°C). The primers for c-fos were 5′GGTCATCGGGGATCTTGC and 5′GGGCTCTCCTGTCAAC (16); 30 cycles for the amplification of c-fos cDNA (1 min anealing at 55°C and 2 min extension at 72°C, and 1 min denaturation at 94°C).

The length of the PCR product was checked by ethidium bromide (0.6 μg/ml) gel electrophoresis with DNA molecular weight marker ($\Phi\chi$ 174 RF DNA Hae III Digest,) in a 1.5% agarose gel.

Northern blot analysis. Northern blot analysis was performed as we described previously (8) using the total RNA (20 μg/lane) and rat cDNA probes for tPA (17), PAI-1 (18), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19). The signals were measured quantitatively by a Bio-Imaging analyzer (BAS-2000, Fuji, Tokyo, Japan), and the amount of tPA or PAI-1 mRNA measured was normalized to that of GAPDH mRNA.

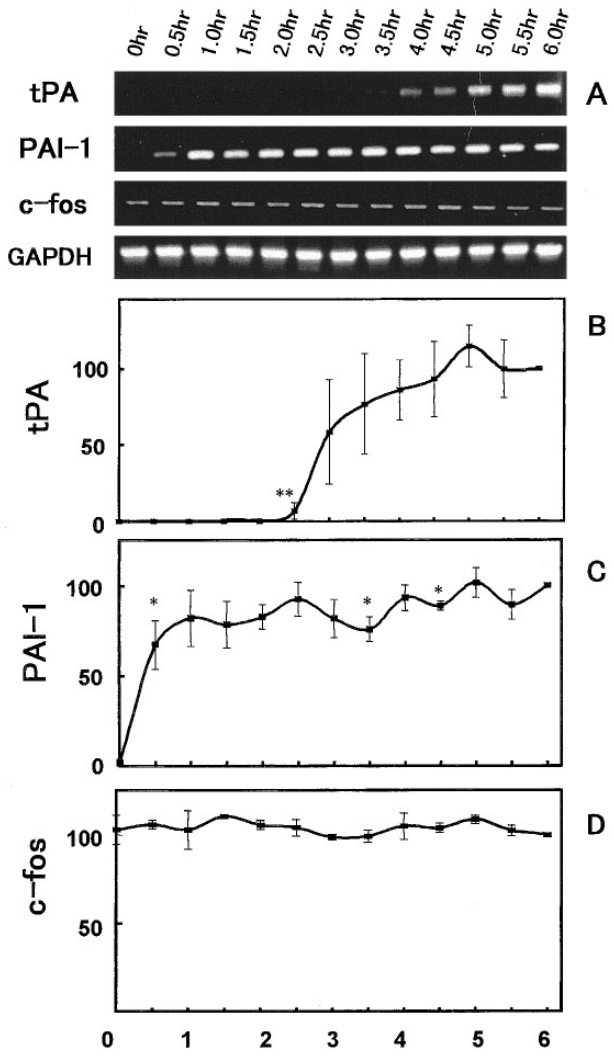

FIG. 1. Time course of the expression of tPA and PAI-1 mRNAs in rat hepatocytes in primary culture. The hepatocytes were plated on a collagen-coated dish at a density of 0.5×10^5 cells/cm^2, cultured for the time indicated, and then processed to isolate cellular RNA. Total RNA (0.25 pg) samples were subjected to RT-PCR as described under Materials and Methods. Quantitative analysis of the signal was performed by a computer assisted analyzer, and the signals were normalized to that of GAPDH. Upper picture (A) shows typical ethidium bromide gel electrophoresis of the RT-PCR products. The lower pictures show the amount of tPA mRNA (B), PAI-1 mRNA (C), and c-fos mRNA (D) presented as the ratio of experimental: 6-hr expression. Each point represents the average ± S.E. of 3 different experiments. *P<0.05, **P<0.01 vs. 6-hour value by Student's paired t-test.

RESULTS

Figure 1 shows the time course of the induction of tPA and PAI-1 mRNAs in the primary cultures of rat hepatocytes. Both tPA and PAI-1 mRNAs were hardly observed in the freshly isolated hepatocytes. PAI-1 mRNA was detected as early as 0.5 hours after the start of cultivation; and 2.0 to 2.5 hours later than that, tPA mRNA began to be expressed (Figure 1, B and C). Messenger RNA for c-fos, a typical immediate early growth response (IER) gene, was fully induced right after the isolation of hepatocytes by collagenase perfusion (Figure 1, A and D). It was reported that mRNA for some protooncogenes is induced during the procedure of hepatocyte isolation by perfusion with collagenase (20). Both tPA and PAI-1 mRNAs were inducible during the time course of the induction of isolated hepatocytes. PA activity was detectable by standard fibrin plate method as early as 4 hours after plating, and gradually increased up to 24 hours (data not shown);

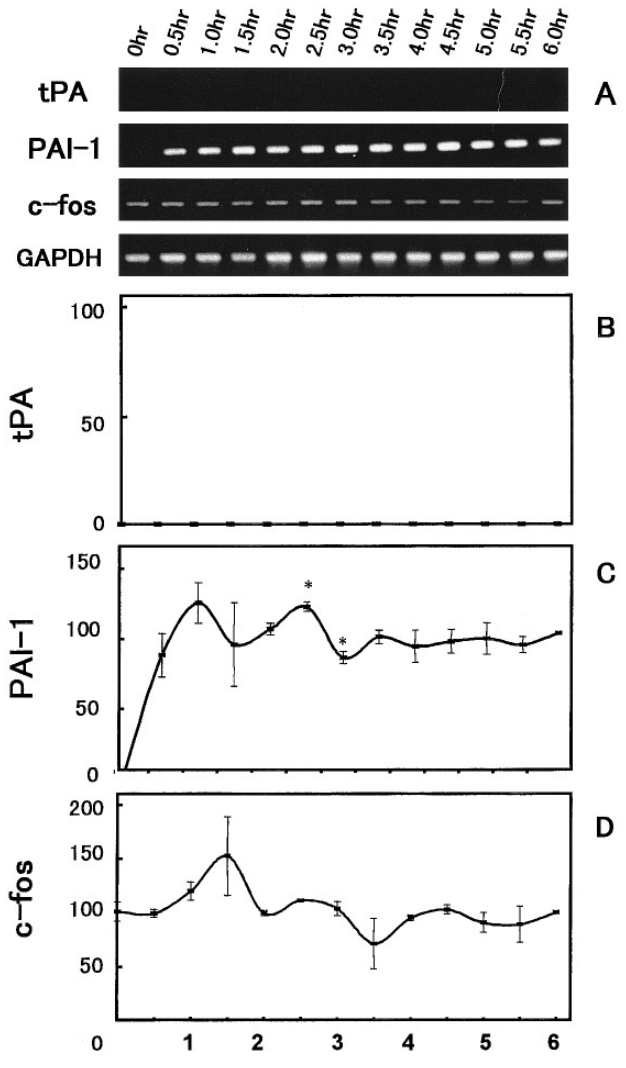

FIG. 2. Effect of cycloheximide on the expression of tPA and PAI-1 mRNAs. The hepatocytes were plated and cultured as described in Figure 1, but in the presence of 10μg/ml cycloheximide. tPA and PAI-1 mRNA expression was measured by the method using RT-PCR as described under Materials and Methods. Each point represents the average ± S.E. of 3 different experiments. *$P<0.05$ vs. 6-hour value by Student's paired t-test.

PA activity was dominant over PAI-1 activity. Two functionally distinct forms of PAI-1 has been identified, these have been termed "active" and "latent" (21, 22). The latent PAI-1 is the predominant species in the culture media *in vitro* and platelets (23-25). Thus the latent PAI-1 may also be predominant species over active PAI-1 in the conditioned medium of rat hepatocytes in primary culture.

To know if *de novo* protein synthesis was required for the induction of tPA and PAI-1 mRNAs, the hepatocytes were cultured in the presence of cycloheximide (Figure 2). PAI-1 mRNA was expressed within 60 min after the start of cultivation, and its level was not influenced by the inhibition of *de novo* protein synthesis by cycloheximide (Figure 2, A and C). On the other hand, expression of the tPA mRNA was completely abolished by this agent (Figure 2, A and B). The expression of c-fos mRNA was observed throughout the culture with cycloheximide (Figure 2, A and D). We recently studied the regulation of tPA and PAI-1 gene expression (S. Uno, M. Nakamura, Y. Ohmagari, S. Matsuyama, T. Seki, T. Ariga, in submission) and showed stimulation of their transcript production (26) by cyclic AMP in rat hepatocytes in primary culture. Cyclic AMP is also supposed to play important roles both in the proliferation of hepatocytes and regeneration of liver; the concentration of cAMP in the liver is known to increase just before liver regeneration (27, 28), and to stimulate DNA synthesis in cultured hepatocytes and the cell cycle in the regenerating liver (27, 29). With these reports taken into consideration, we studied the effects of cAMP and H-89, the latter of which is an inhibitor for cAMP-dependent protein kinase (A-kinase), on the early growth response of tPA and PAI-1 genes in the hepatocytes in primary culture.

Figure 3 shows the effect of dibutyryl cAMP (dbcAMP), H-89, and DMSO, which was used as a vehicle for H-89, on the early growth response of tPA and PAI-1 genes. Early growth response of tPA was significantly depressed by H-89 (to 50% of that of the vehicle). PAI-1 induction was also depressed by H-89, but the reduction was not significant. Both tPA and PAI-1 mRNAs were augmented by dbcAMP, and their levels reached about 2.5-times and 3.0-times higher, respectively, than those of the agent-free control culture. The augmentation of tPA mRNA expression by dbcAMP was partially depressed by H-89 (see the column of dbcAMP+H89 in Figure 3), whereas that of PAI-1 mRNA by dbcAMP was depressed by it to a level lower than the control one. These results suggest that the cAMP/A-kinase system is involved in the early growth response of tPA mRNA, and partially involved in the augmentation of tPA mRNA by dbcAMP. On the contrary, cAMP/A-kinase system is thought to be more important in the augmentation of PAI-1 mRNA expression by dbcAMP, than in the early growth response of the PAI-1 gene, since the early growth response of the PAI-1 gene was only slightly affected by H-89 alone.

Figure 4 shows the effects of collagen, an extracellular matrix, and the density of hepatocytes on the early growth response of tPA and PAI-1 gene expression. In the cultures at the low density (0.5×10^5cells/cm^2), tPA mRNA expression was augmented about 3-4-fold in the presence of collagen, whereas in those at the high density (1.5×10^5cells/cm^2), the expression was hardly changed by culturing on collagen. The tPA mRNA expression with cells on collagen was augmented by growth factors (I+G+E>E>control). In the high-density case, tPA mRNA expression was hardly influenced by growth factors nor by culturing on collagen.

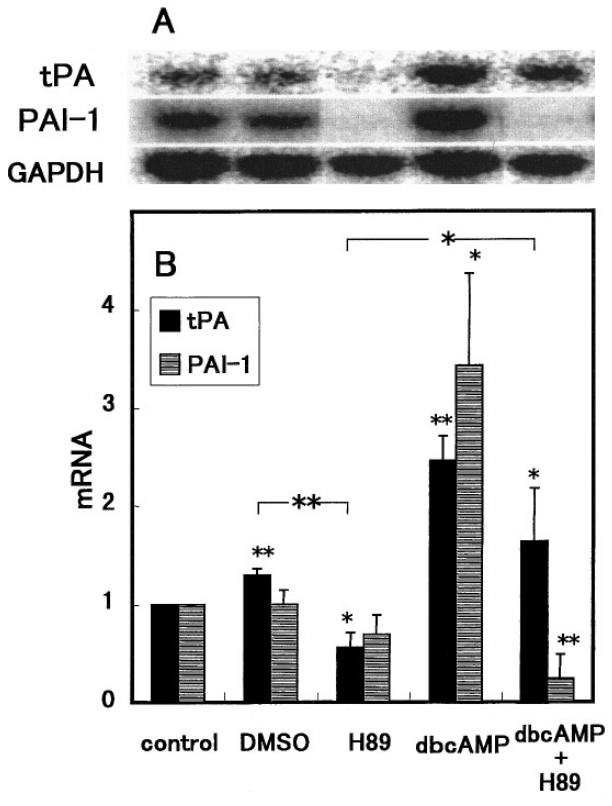

FIG. 3. Effects of cAMP and H-89 on the induction of tPA and PAI-1 mRNAs in rat hepatocytes in primary culture. The hepatocytes were cultured on collagen-coated dishes in the presence of cAMP (10^{-5}M), or H-89 (30 μg/ml) for 4 hr, followed by the isolation of cellular RNA. The total cellular RNA (20 μg) samples were subjected to Northern blot analysis as described under Materials and Methods. The amount of tPA and PAI-1 mRNA in these cells was normalized to that of GAPDH and expressed as the ratio against that expressed by control cells. Each bar represents the average ± S.E. of 3-5 different experiments. *P<0.05, **P<0.01 vs. the control value control (or as indicated by brackets) by Student's paired t-test.

In the case of PAI-1 mRNA, its expression was higher in the high-density cultures than in the low-density ones. PAI-1 mRNA expression was reduced slightly by culturing the low-density cells in the presence of EGF (+E) or the combination of EGF, insulin, and glucagon (+I+G+E), with or without collagen coating. The same tendency for hormonal suppression of PAI-1 mRNA was also observed in the cultures at high density.

DISCUSSION

Rat hepatocytes in primary culture have been shown to maintain the normal features, functions, and growth regulation of the hepatocyte observed *in vivo* (30). Therefore, we employed rat hepatocytes in primary culture as an *in vitro* model system to characterize the early growth response of the cells, specifically in the induction of tPA and PAI-1 gene expression that occurs *in vivo.*

Early growth response genes are induced in the regenerating liver, and may play important roles in regulating the cascade of events through cell proliferation. IER genes represent diverse functional classes and include transcription factors, growth factors, signal transduction regulators, and other type of proteins (31-36). These genes are induced within minutes after the partial hepatectomy in a protein synthesis-independent manner. DER genes are induced within a few

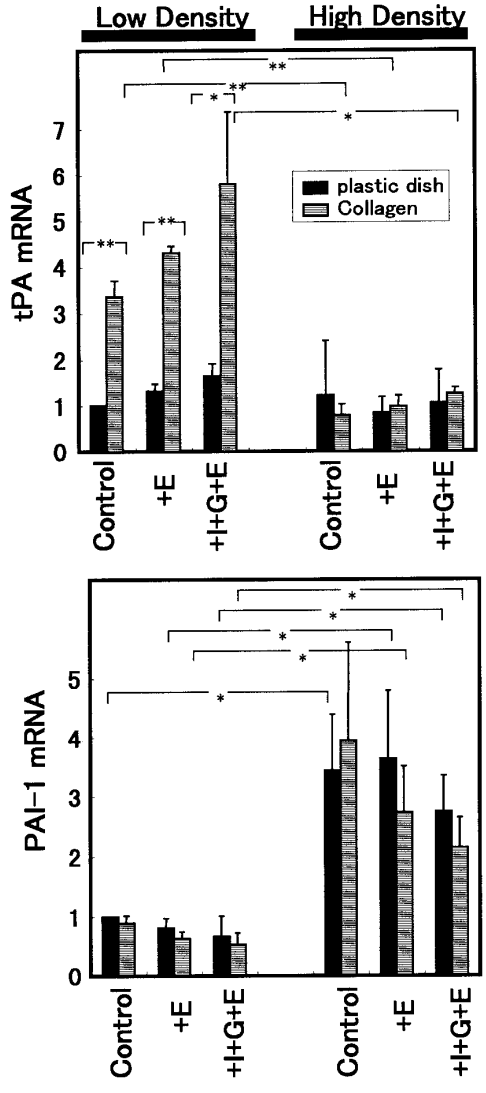

FIG. 4. Effects of serum, growth factors, cell density, and extracellular matrix on the early growth response expression of tPA and PAI-1 mRNA in rat hepatocytes in primary culture. The hepatocytes were plated on collagen-coated dishes or plastic dishes at a density of 0.5×10^5 cells/cm^2 (low density) or 1.5×10^5 cells/cm^2 (high density) and cultured in growth factor-free medium (WE; control), WE containing epidermal growth factor (+E), or WE containing insulin, glucagon, and EGF (+I+G+E) for 6-hr. The cells were harvested at the times indicated, and then the tPA and PAI-1 mRNA expression was measured by Northern blotting as described in Fig. 3. Each point represents the average ± S.E. of 4-6 different experiments. *P<0.05, **P<0.01, as indicated by brackets, by Student's paired t-test.

hours after hepatectomy, and their transcription requires protein synthesis.

The gene for PAI-1 was reported to be one of the IER genes in the liver *in vivo*, because it was expressed by hepatocytes after the partial hepatectomy (9). In this study, we observed that PAI-1 gene was also induced as an IER gene in the hepatocytes in primary culture. More importantly, tPA, a target enzyme of PAI-1, was also induced in the hepatocytes in response to their growth; and such induction was shown to proceed in a protein synthesis-dependent fashion. This is the first evidence for the tPA gene behaving as a DER gene.

Cyclic AMP has been thought to play important roles in the regulation of tPA and PAI-1 gene expression in hepatocytes in primary culture (26, 37). Indeed, the expression of both of these mRNAs was augmented 2.5 to 3.0-fold by the incubation of hepatocytes with dbcAMP for 6 hours. From the results of our experiments using H-89, the cAMP/A-kinase system seemed to be important in both the early growth response induction of tPA mRNA and augmentation of PAI-1 mRNA.

Collagen induces anchorage-dependent hepatocyte growth in primary culture (20). In this study, tPA mRNA induction was also augmented in such cultures; i. e., hepatocytes plated on a collagen-coated dish at low density expressed tPA mRNA at a higher level than the hepatocytes plated at the same density on a plastic dish or the hepatocytes plated at high density, irrespective of the substratum. Interestingly, PAI-1 mRNA expression was oppositely regulated in response to the culture conditions; at higher density, greater expression, and in collagen-coated dishes, lesser expression.

A number of growth factors including HGF (38) and TNFa (39) have been implicated as having a role in liver regeneration, but the exact mechanisms regulating this complicated process are largely unknown. On the contrary, a component of the PA-plasmin system is known to be involved in the processing of factors governing hepatocyte proliferation and liver regeneration *in vitro* (40); both tPA and uPA activate an inactive hepatocyte trophic growth factor, pro-HGF (41, 42), to become active HGF (43-46), and such activation is prevented by PAI-1 (43, 44). Plasmin activates the latent TGFβ to active TGFβ (47), which has a function as a potent inducer of production of PAI-1 (48). HGF is also reported as an inducer of PAI-1 in Hep G2 hepatoma cells (49). Thus the tPA produced by hepatocytes in the way of their early growth response may stimulate the liver regeneration through HGF activation, and PAI-1 produced concomitantly by hepatocytes may represent a negative feedback in regulation of the regeneration process (9). Actually, tPA mRNA/activity, but not uPA, and PAI-1 mRNA are thought to be induced in the rat liver injury caused by carbon tetrachloride (8); uPA activity/mRNA was not detectable even in liver of LPS-treated animals (6).

In this study, we characterized the early growth response of tPA and PAI-1 gene expression in hepatocytes in primary culture. These fibrinolytic components, whose expression is closely associated with hepatocyte growth, may play important roles in pathophysiological events in the liver such as liver regeneration. Studies to elucidate the pathophysiologic role(s) of tPA and PAI-1 produced by hepatocytes are now underway in both *in vitro* and *in vivo* systems in our laboratory.

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