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Cell Adhesion Regulates the Plasminogen Activator Inhibitor-1 Gene Expression in Anchorage-Dependent Cells

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Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of both tissue- and urokinase-type plasminogen activators (t-PA, u-PA). PAI-1 also regulates the attachment of cells to the adhesive glycoprotein vitronectin (VN). PAI-1 gene expression has been observed in various cell types, and many regulatory factors have been identified to play a role in PAI-1 gene transcription. The complete picture of how the PAI-1 gene is expressed when cells adhere to a culture plate has not been fully elucidated. We found that in anchorage-dependent cells, PAI-1 gene was up-regulated when cells were beginning to attach to a culture dish and was down-regulated when cells had attached completely. The PAI-1 gene expression was induced only in adhered cells but not in non-adhered cells. The regulation of PAI-1 protein was also found in both culture medium and cell lysate when cells were attached to a culture dish. Our experiment indicates that vitronectin and fibronectin, as components of ECM, may be the factors involved in the regulation of PAI-1 gene expression. PAI-1, as an inhibitor of the interaction between vitronectin and integrin $\alpha v\beta 3$, may also be a regulator of its own expression.

Key Words: **PAI-1; uPA; t-PA; plasminogen; ECM; adhesion; vitronectin; fibronectin; integrin; $\alpha v\beta 3$.**

PAI-1 is the primary inhibitor of both tissue- and urokinase-type plasminogen activators (t-PA, u-PA) and is thus a primary regulator of plasminogen activation and possibly of extracellular proteolysis (1–3). Since a number of recent observations indicate that PAI-1 also regulates the adhesion of cells to the adhesive glycoprotein vitronectin (VN), PAI-1 may play other roles that are independent of its ability to function as a protease inhibitor (4–8). In the N-terminal domain of vitronectin, the PAI-1 and uPAR each binds to the same region and, adjacent to this region, there is a RGD binding site for αv integrins. By steric hindrance, PAI-1 can inhibit the binding of either uPAR or integrins to the vitronectin (4, 5). Since PAI-1 is a labile molecule, which is easily converted into an inactive form in solution (9), it needs to bind with vitronectin to keep stable and active (10). It has been shown that bound to vitronectin, PAI-1 inhibits vascular smooth muscle cell migration by a mechanism which, independent of its ability to inhibit proteinase activity, acts by preventing binding of the $\alpha v\beta 3$ integrin to vitronectin (4). PAI-1 also inhibits angiogenesis via two overlapping mechanisms that are dependent on its ability either to inhibit proteinase activity or to block integrin binding to vitronectin (11). The PAI-1 seems not only to play a role in inhibiting plasminogen activation, but also to play more roles in the regulation of the interaction between cells and ECM. Hence, PAI-1 may be involved in the effects of embryo development, wound healing, angiogenesis and cancer invasion.

PAI-1 expression has been observed in various cell types and multiple regulatory factors were identified to play a role in PAI-1 transcription. Many different factors such as growth factors (PMA (12), TGF-β (13), EGF (14), VEGF (15), bFGF (16), inflammatory cytokines (IL-1, TNF-α) (17, 18), hormones (corticosteroids (19), insulin (20), hypoxia (21), and p53 (a guard for DNA damage (22) induce synthesis of PAI-1. So far, the transcription of PAI-1 gene influenced by cells adhered to ECM has only been elucidated by the interaction between cells and vitronectin or fibronectin (23, 24). Since the complete picture of how the PAI-1 is expressed when cells adhere to a culture plate has not been fully elucidated, we report here that PAI-1 is regulated in both transcription and translation when cells adhere to a culture dish. The ECM, vitronectin and fibronectin, may be the regulators involved in the mechanism of PAI-1 expression. PAI-1 may also play a very important role in its own expression.

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MATERIALS AND METHODS

Cell culture. Nasal pharyngeal carcinoma (NPC) cell line (NPC-TW01), gastric adenocarcinoma cell line (SC-M1), and human normal fibroblast cell line (HF) were cultured with MEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Leukemia cell line HL-60 was cultured with RPMI-1640 medium (Life Technologies) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Tissue culture dishes and bacteria culture dishes were purchased from BD Biosciences.

Reagents. PAI-1 monoclonal antibody was purchased from American Diagnostica Inc. (product No. 3785). Integrin $\alpha V\beta 3$ monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (product No. sc-7312). Vitronectin, fibronectin, and cyclo (Arg-Gly-Asp-D-Phe-Val) were purchased from Calbiochem-Novabiochem Corporation (Product Nos. 681105, 341635, and 182015, respectively).

Probe. Probes for Northern blot analysis were synthesized by RT-PCR. The oligonucleotides, PAI-1-5′ (gtctttggtgaagggtctgct) and PAI-1-3′ (ctcgtgaagtcagcctgaaa), were used to amplify the PAI-1 cDNA with 943 bp. The oligonucleotides, GAPDH-5′ (tggtatcgtggaaggactca) and GAPDH-3′ (agtgggtgtcgctgttgaag), were used to amplify the GAPDH cDNA with 370 bp. The PCR conditions for both probes were the same. The initial denaturation phase lasted 5 min at 94°C followed by a 35-cycle amplification phase consisting of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. Amplification was terminated after 7 min at 72°C. After PCR reaction, the DNA fragments were purified with QIA quick gel extraction kit (Qiagen, Inc.). Twenty ng of DNA fragments were labeled with [α-^{32}P]dCTP (Amersham) by the random prime labeling system (Rediprime, Amersham) for Northern blot assay.

Northern blot analysis. Total RNA was isolated from cells cultured in 10 mm dishes with Tri-Reagent (Molecular Research Center, Inc.). Ten to 20 μg of total RNA was separated on 1% agarose/formaldehyde gels as described previously (25) and then transferred onto nylon membrane (Hybond-N +, Amersham). Hybridization was performed at 65°C for 16 h in a hybridization buffer (Rapid-hyb buffer, Amersham). Membrane was then washed once with solution A (5× SSC, 0.1% SDS) at 65°C for 20 min, and twice with solution B (0.1× SSC, 0.1% SDS) at 65°C for 20 min. Finally, the membrane was put into autoradiography.

PAI-1 ELISA assay. Imubind PAI-1 ELISA kit (American Diagnostica Inc., product no. 821) was used for measurement of total PAI-1 antigen in cell extracts and cultured medium.

RESULTS AND DISCUSSION

PAI-1 mRNA Expression Was Induced in Anchorage-Dependent Cells but Not in Anchorage-Independent Cells

In order to investigate the full-spectrum of PAI-1 gene expression in the cell culture system, cells were isolated at different time intervals after they were seeded. Northern blot was performed to analyze the quantity of PAI-1 mRNA expression. In NPC-TW01 cell line, we found that PAI-1 mRNA increased as early as 2 h and continued to increase until 16 h after cells were seeded. After 16 h, when cells had completely attached, the expression of PAI-1 mRNA decreased. Finally, the PAI-1 mRNA declined to the basal level after 40 h (Fig. 1A). This phenomenon was also demonstrated in gastric adenocarcinoma cell line (SC-M1) and human normal skin fibroblast cell line (HF) (Figs. 1B and 1C). All of these cell types mentioned above are anchorage-dependent; on a culture dish, they are adhesive, spreading and flattened. However, in leukemia cell line (HL-60), which the cells are anchorage-independent, suspensible, and round, the expression of PAI-1 mRNA could not be detected at all (Fig. 1D).

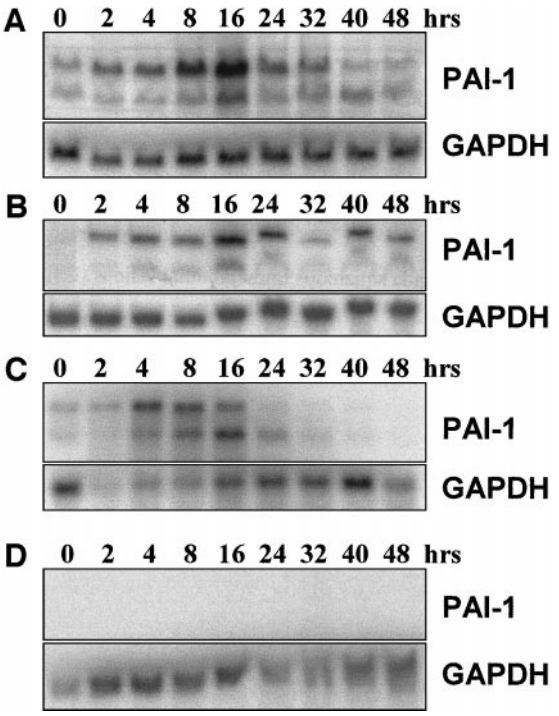

FIG. 1. Northern blots of PAI-1 and GAPDH expression in the cells: (A) NPC-TW01, (B) SC-M1, (C) HF, and (D) HL-60 at different time intervals after cells were seeded.

Induction of PAI-1 Expression in NPC-TW01 Cells Was Directed by the Adhesion of Cells to the Culture Dish

There are two possible reasons that explain why PAI-1 gene expression began to increase 2 h and continued to increase until 16 h after cells were seeded. The first possibility is that in the process of seeding cells, the culture environment was changed when cells were transferred from used medium to fresh medium. The augmentation of PAI-1 gene expression might be the result of some changing medium components. Another possibility might be with the activation of certain signals during the process when cells are attached to a culture dish. To explore whether fresh cultured medium would influence the PAI-1 gene expression, we compared NPC-TW01 cells cultured with fresh medium and used medium. The used medium was the same one cultured with NPC-TW01 cells for 24 h. Although the cells in the used medium attached to a

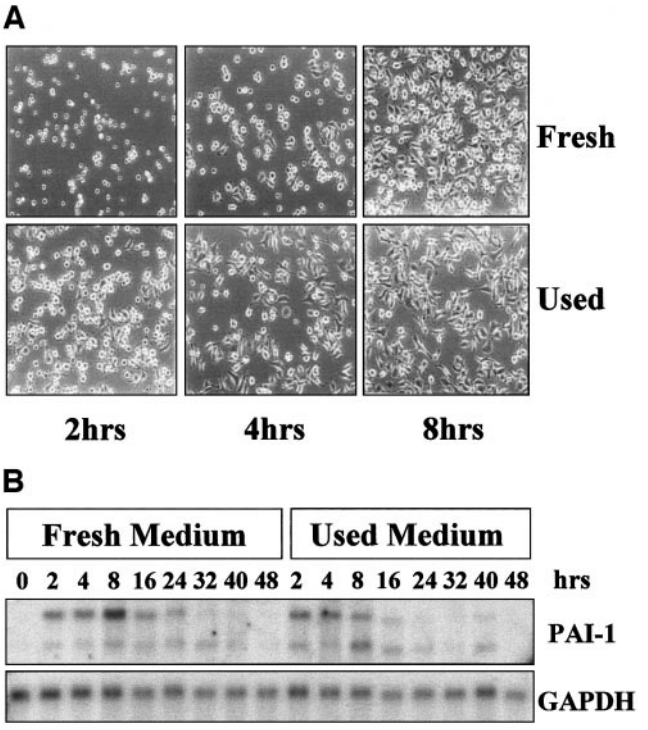

FIG. 2. The morphology of NPC-TW01 cells 2, 4, and 8 h after cells were seeded with fresh or used medium, which had been used for culturing NPC-TW01 cells for 24 h. (B) Northern blots of PAI-1 and GAPDH expression 2, 4, and 8 h after cells were seeded with fresh or used medium.

culture plate earlier than in the fresh medium (Fig. 2A), we found that the PAI-1 mRNA levels were both increased as early as 2 h after cells were seeded in fresh and used medium, and then decreased after 16 h (Fig. 2B). This result ruled out the possibility that the regulation of PAI-1 gene expression was the result of the replenishment of certain components in the medium. Furthermore, we compared the cells adhered on tissue culture dish with those non-adhered on bacterial culture dish (Fig. 3A). We found that the PAI-1 mRNA expression was up-regulated in adhered cells but not in non-adhered cells (Fig. 3B). This result indicated that the attachment of cells to culture dish activated certain signals causing up-regulated PAI-1 gene expression.

To prove whether the increment of PAI-1 gene expression will influence the quantity of PAI-1 protein, we quantified the PAI-1 protein concentration simultaneously in both cultured medium and cell lysate with ELISA assay. In NPC-TW01 cell cultured medium, we found that the PAI-1 protein concentration was up-regulated both in adhered and non-adhered cells as early as 2 h after cells were seeded, then down-regulated after 8 h ($P < 0.01$ by ANOVA analysis). The PAI-1 protein concentration in the medium cultured with adhered cells was about two times higher than that with non-adhered cells 8 h after cells were seeded (Fig. 4A) ($P < 0.05$ in the individual times of 2, 4, and 8 h by *t* test analysis). In cell lysate, PAI-1 augmentation was also seen ($P < 0.01$ by ANOVA analysis) 8 h after cells were seeded and the protein expression in the adhered cells was 5 to 7 times more than that in the non-adhered cells 4 to 16 h after cells were seeded (Fig. 4B) ($P < 0.05$ in the individual times of 4, 8, and 16 h by *t* test analysis). In adhered cell lysate, the dramatic increment of PAI-1 protein was correlated with the induction of PAI-1 mRNA expression. However, in the medium of adhered cells, only slightly increased amount of PAI-1 protein was observed.

Many Factors Were Involved in the Induction of PAI-1 Expression during the Cells' Attachment to the Culture Dish

In order to investigate the factors that may be involved in cell adhesion and the induction of PAI-1 gene expression, we added two essential ECM components, vitronectin and fibronectin, into the culture medium. We found that both vitronectin and fibronectin slightly up-regulated the PAI-1 gene expression 2 h after cells were seeded. We also discovered that the PAI-1 gene expression augmented by vitronectin was inhibited by its inhibitor, cyclo (Arg-Gly-Asp-D-Phe-Val) cRGDfV (Fig. 5A). The cyclo (Arg-Gly-Asp-D-Phe-Val), cRGDfV, specifically blocks vitronectin by conjugating with integrin $\alpha v\beta 3$. This result indicated that both vitronectin

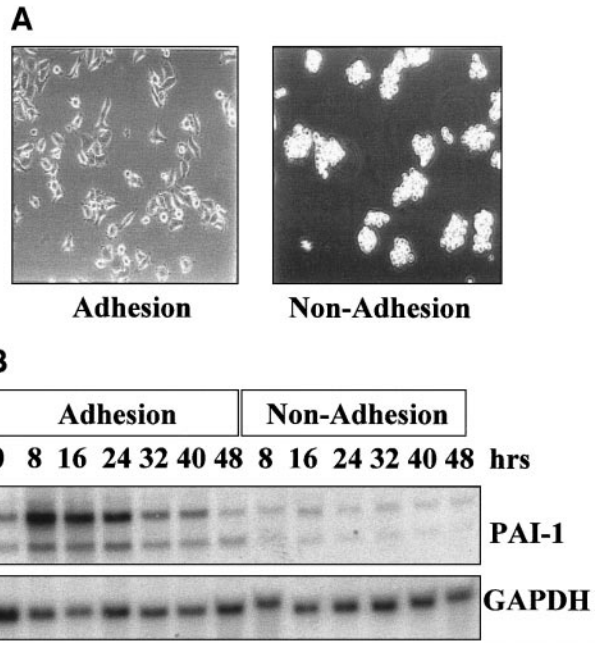

FIG. 3. The morphology of NPC-TW01 cells, which adhered to a tissue culture plate or non-adhered to a bacteria culture plate, 8 h after cells were seeded. (B) Northern blots of PAI-1 and GAPDH expression of the cells, which were seeded on to a tissue culture plates (attached) and a bacteria culture plates (non-attached) at different time intervals after cells seeded.

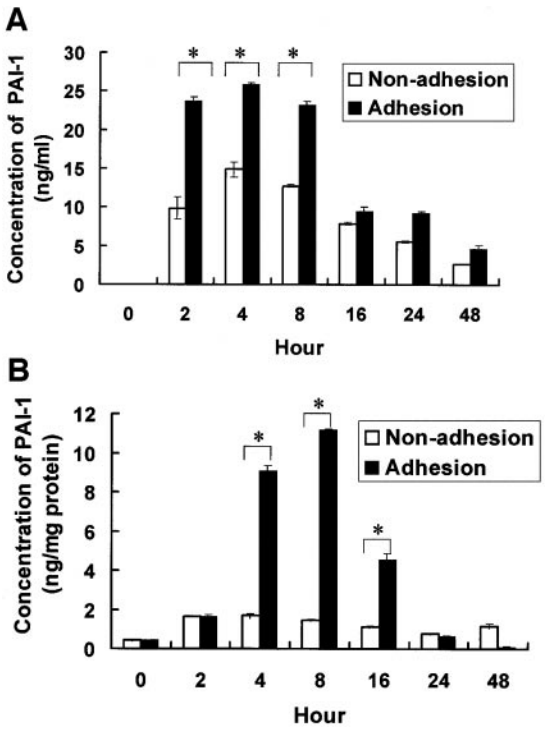

FIG. 4. PAI-1 protein concentrations determined by ELISA assay in the medium (A) and cells lysate (B) of NPC-TW01 cells cultured at different intervals after cells were seeded. *: Means biostatistically significant through t test analysis ($P < 0.05$).

and fibronectin were involved in the activation of PAI-1 gene expression when cells adhere to a culture plate. Since it is known that PAI-1 plays a role in regulating the binding and signal transduction between vitronectin and integrin $\alpha v\beta 3$, we added inhibitors of vitronectin, integrin $\alpha v\beta 3$ and PAI-1 to see which factor is directly involved in PAI-1 regulation. We added $\alpha v\beta 3$ antibody, PAI-1 antibody, and cRGDfV as the inhibitors of integrin $\alpha v\beta 3$, PAI-1, and vitronectin, respectively, into the cultured medium. The results revealed that PAI-1 gene expression was not influenced by medium containing $\alpha v\beta 3$ antibodies or cRGDfV but was up-regulated when PAI-1 antibody was added into the medium (Fig. 5B). This result indicated that some mechanism might be activated to increase PAI-1 gene expression by blocking the function of PAI-1 itself.

In our study, we discovered that the PAI-1 expression was activated when cells were seeded on a cell culture plate but not activated when they were seeded on a bacteria culture plate. The surface of cell culture plate is hydrophilic and contains a variety of negatively charged functional groups that support cell attachment and spreading. The ECM distribution in the serum-containing medium may conjugate with the hydrophilic surface of the culture dish and interact with its receptor on cell membrane, such as integrin. It has been reported that the activity of PAI-1 promoter was augmented up to 5-fold as a function of integrin $\alpha v\beta 3$/vitrnectin interaction in human ovarian cancer cells (24). It was also known that fibronectin alone was sufficient to initiate PAI-1 gene transcription in renal epithelial cells (23). In our study, the addition of fibronectin or vitronectin was found to up-regulate PAI-1 gene expression. This coincided with the previous study. However, when we only added inhibitors of vitronectin or integrin $\alpha v\beta 3$, there was no obvious decrease of the PAI-1 gene expression. The result suggested that many other components in ECM might also play a role in the regulation of PAI-1 gene when cells adhere to a culture dish. Further study is needed to elucidate the detailed mechanism.

Previous reports demonstrated that active PAI-1 is a steric hindrance inhibitor to the interaction between vitronectin and integrins, and that the interaction between vitronectin and integrin $\alpha v\beta 3$ can induce the PAI-1 gene expression (24). We speculated that the PAI-1 itself might be a negatively feedback regulator for its gene regulation. The increase of PAI-1 protein concentration blocked the interaction between integrin $\alpha v\beta 3$ and vitronectin and then further decreased the signal that was evoked from their binding. The less active the PAI-1 was, the more vitronectin was released, causing vitronectin to interact more easily with integrin $\alpha v\beta 3$, and inducing the signals that up-regulated the PAI-1 gene expression. Our experiment revealed that the PAI-1 gene expression was reduced in the presence of high concentration of PAI-1 protein. Furthermore, after adding PAI-1 antibody in the me-

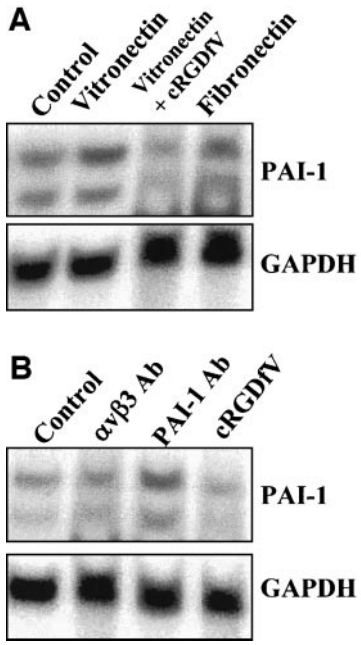

FIG. 5. Northern blots of PAI-1 and GAPDH expression in NPC-TW01 cells 2 h after cells were seeded with serum-containing medium added with: (A) vitronectin (1 μg/ml); vitronectin (1 μg/ml) + cRGDfV (1 μg/ml); fibronectin (1 μg/ml), and (B) integrin $\alpha v\beta 3$ antibody (2 μg/ml); PAI-1 antibody (5 μg /ml); cRGDfV (1 μg/ml).

dium to block the functional activity of PAI-1, we discovered that the PAI-1 gene expression was increased. This result suggested the PAI-1 might play a very important role in regulating its own expression through its interaction between ECM and integrins.

The PAI-1 protein concentration of cell lysate in adhered cells was 5 to 7 times higher than that in non-adhered cells. The significant difference in range between adhered and non-adhered cells was correlated with the result of PAI-1 mRNA expression. However, the concentration of PAI-1 protein secreted into the medium from adhered cells was only two times higher than that from non-adhered cells. This discrepancy may be due to the biological instability of PAI-1 protein when it is in solution (9). The lack of cysteine residues (and hence disulfide bonds) in PAI-1 protein may in turn account for its biological instability in solution. The normal concentration of PAI-1 protein in human plasma ranges from 6 to 80 ng/ml with a geometric mean at 24 ng/ml. Although the plasma concentration of PAI-1 is low, its half-life in blood is relatively short (≦10 min). This suggests that the biosynthetic rate of PAI-1 protein is high. Moreover, its concentration rapidly increases in response to a variety of agents or changes in physiological state, indicating that the amount of PAI-1 protein in plasma is subject to dynamic regulation (26).

Cultured cells provide an *in vitro* paradigm to evaluate molecular mechanisms associated with proliferative control since they require both growth factors and substrate for the adhesion to progress throughout the division cycle (27–29). It has been found that PAI-1 mRNA was induced and accumulated at mid-G1 phase after cells respond to mitogenic stimulation in quiescent state (30). In our study, we found PAI-1 mRNA was transcribed when cells adhered to a culture plate even in a used medium. Furthermore, we revealed that non-adherent cells, even in a serum-containing medium, did not express PAI-1 mRNA at all. We suggest that initiation of PAI-1 gene expression of a cell is anchorage-dependent and serum only prolongs the PAI-1 gene expression before cells enter into cell cycle. The expression of PAI-1 gene may play an important role in the complicated process of cell adhesion and may be closely associated with the growth control of anchorage-dependent cells.

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