Decreased expression of the plasminogen activator inhibitor type 1 is involved in degradation of extracellular matrix surrounding cervical cancer stem cells

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Abstract. The plasminogen activator (PA) system consists of plasminogen activator inhibitor type 1 (PAI-1), urokinase-type plasminogen activator and its receptor (uPA and uPAR). PAI-1 inhibits the activation of uPA (which converts plasminogen to plasmin), and is involved in cancer invasion and metastasis, by remodeling the extracellular matrix (ECM) through regulating plasmin. Cancer stem cells (CSCs) are a small subset of cells within tumors, and are thought to be involved in tumor recurrence and metastasis. Considering these facts, we investigated the relationship between PAI-1 and cervical CSCs. We used ALDH1 as a marker of cervical CSCs. First, we demonstrated that culturing ALDH1-high cells and ALDH-low cells on collagen IV-coted plates increased their expression of active PAI-1 (ELISA), and these increases were suggested to be at mRNA expression levels (RT-qPCR). Secondly, we demonstrated PAI-1 was indeed involved in the ECM maintenance. With gelatin zymography assays, we found that ALDH1-high cells and ALDH-low cells expressed pro-matrix metalloproteinase-2 (pro-MMP-2) irrespective of their coatings. With gelatinase/collagenase assay kit, we confirmed that collagenase activity was increased when ALDH1-low cells were exposed to TM5275, a small molecule inhibitor of PAI-1. Putting the data together, we hypothesized that cancer cells adhered to basal membrane secrete abundant PAI-1, on the other hand, cancer cells (especially CSCs rather than non-CSCs) distant from basal membrane secrete less PAI-1, which makes the ECM surrounding CSCs more susceptible to degradation. Our study could be an explanation of conflicting reports, where some researchers found negative impacts of PAI-1 expression on clinical outcomes and others not, by considering the concept of CSCs.

Introduction

The plasminogen activator (PA) system consists of plasminogen activator inhibitor type 1 (PAI-1), urokinase-type plasminogen activator and its receptor (uPA and uPAR) (1). PAI-1 inhibits the activation of uPA (which converts plasminogen to plasmin), and the PA system is involved in proteolysis (1). Dysregulation of the PA system is related to disorders such as thrombosis, atherosclerosis and type 2 diabetes (2-5). In addition, it has been reported that PAI-1 is involved in cancer invasion and metastasis, by remodeling the extracellular matrix (ECM) through regulating plasmin (6-12). Some studies reported that overexpression of PAI-1 correlated with poor prognosis in cancer patients, however, it is controversial (13-16).

Cancer stem cells (CSCs) are a small subset of cells within tumors and possess abilities similar to normal stem cells; the ability to self-renew and differentiate (17,18). This concept was first demonstrated in leukemia and increasing evidence supports this model in various types of solid tumors including cervical cancer (19-24). CSCs are thought to be involved in tumor recurrence and metastasis; failure to treat CSCs by surgery or chemotherapy would result in relapse (17). Considering these facts, investigating the impact of PAI-1 on CSCs could give insight into CSC features in terms of metastasis.

In this study, we investigated the significance of PAI-1 in CSCs and non-CSCs. In cervical cancer, ALDH1-positive cells, like other solid tumors, are known to be more tumorigenic than negative ones, and we used ALDH1 as a marker of cervical CSCs (25-34). First, using the cervical cancer cell line CaSki we confirmed that the expression of PAI-1 was significantly increased by changing coatings of culture plates as described (35), especially collagen IV-coating, confirmed by enzyme-linked immunosorbent assay (ELISA). These results

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were also obtained after sorting CaSki cells into ALDH1-high cells and ALDH1-low cells, and in addition, the expression levels themselves in the supernatants from ALDH1-high cells and ALDH1-low cells were different.

Secondly, we investigated the significance of PAI-1 in degradation of the ECM, by investigating gelatin zymography assays and collagenase activity assays. We found that matrix metalloproteinase-2 (MMP-2) was involved, and confirmed that the activity to degrade the ECM was increased by exposing ALDH1-low cells to TM5275 (a small molecule inhibitor of PAI-1). This result was suggestive that PAI-1 was indeed involved in maintaining the ECM, especially around non-CSCs. In conclusion, we investigated the significance of PAI-1 in CSCs and non-CSCs. The expression levels of PAI-1 were different from ALDH1-high cells to ALDH1-low cells, and also different depending on culture plates. Our study could be an explanation of conflicting reports, where some researchers found the impacts of PAI-1 expression on poor clinical outcomes and others not, by considering the concept of CSCs.

Materials and methods

Cell culture. The cervical cancer cell lines CaSki and SiHa were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and sub-cultured by 0.25% trypsin/EDTA (Wako) detachment. The cells were grown in a humidified atmosphere at 37°C and 5% CO₂.

Coatings. Fibronectin solution, vitronectin solution and laminin solution were purchased from Wako (Cat# 063-05591, 220-02041 and 120-05751, respectively), and were used according to manufacturer's instructions. Collagen I and collagen IV-coated plates were obtained from Corning (Acton, MA, USA).

Reagents. Human PAI-1 peptide was purchased from Abcam (Cambridge, MA, USA). TM5275 was purchased from Axon Medchem (USA).

RNA extraction and RT-quantitative PCR (RT-qPCR). Total RNA was extracted with Tissue Total RNA kit (Favorgen Biotech Corp., Taiwan) according to manufacturer's protocols. First-strand cDNA was synthesized from 500 ng of total RNA using ReverTra Ace (Toyobo, Japan). qPCR was performed with SYBR Green PCR master mix (Roche) according to manufacturer's instructions. Denaturation was performed at 95°C for 2 min, followed by 35 cycles at 98°C for 10 sec, at 65°C for 10 sec and at 68°C for 10 sec. β -actin was used as a housekeeping gene and the results are presented as fold change relative to β -actin expression (2^{- $\Delta\Delta$ Ct}). Each experiment was performed in triplicate. The sequences of the primer pairs used in this study are shown in Table I.

Enzyme-linked immunosorbent assay (ELISA). Culture supernatants were assayed for active PAI-1 and active uPA by ELISA kits (Innovation Research Inc., USA, Cat# IHPAIKT and IHUPAKT) according to manufacturer's protocol. Cells

Table I. Primer sequences used in this study.

Gene name	Primer sequence	Size (bp)
PAI-1	GCACCACAGACGCGATCTT ACCTCTGAAAAGTCCACTTGC	112
uPA	GCCATCCCGGACTATACAGA AGGCCATTCTCTTCCTTGGT	417
uPAR	CTGGAGCTGGTGGAGAAAAG TGTTGCAGCATTTCAGGAAG	406
β-actin	CATGTACGTTGCTATCCAGGC CTCCTTAATGTCACGCACGAT	250

Gene names and primer sequences (5'-3') for RT-PCR and qPCR are shown. PAI-1, plasminogen activator inhibitor type 1; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

 $(2x10^5)$ were seeded into 6-well plates and were cultured for 24 h. After that, medium was switched to DMEM without FBS for 24 h, and then the supernatants were collected. Immediately after collection, these supernatants were centrifuged for 10 min at 300 x g to remove cell debris and were stored at -80°C. For detection of intracellular active PAI-1, cells were permeabilized with 1 ml of 0.5% Triton X-100 for 20 min. Plates were read on an ELISA reader, EPOCH (BioTek, Winooski, VT, USA).

Flow cytometry. The ALDH enzymatic activity of the cells was measured as described previously (34), using the Aldefluor kit (StemCell Technologies, Vancouver, BC, Canada). CaSki cells (5x10⁶ cells) were suspended in Aldefluor assay buffer containing ALDH substrate. The brightly fluorescent ALDH-positive cells were detected using MoFlo XDP (Beckman Coulter, Inc., Brea, CA, USA). As a negative control, cells were stained under identical conditions after treatment with the specific ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB). After sorting, 1x10⁵ cells were seeded into 6-well plates and were cultured for 24 h. Then medium was changed under each experimental condition. Experiment was repeated at least three times.

Gelatin zymograhpy assays. Gelatin zymography assays (Cosmobio, Japan, Cat# AK45) were performed as previously described (36). Collected supernatants (10 μ l) were mixed with 2X sample buffer and electrophoresed according to manufacturer's instructions. Subsequent enzymatic reactions were performed at 37°C for 40 h.

Collagenase activity assays. Collagenase activity was detected with EnzChek Gelatinase/Collagenase Assay kit (Molecular Probes, USA, Cat# E-12055) according to manufacturer's instructions. The supernatant (100 μ l) was used, and incubated with DQ gelatin solution for 24 h at room temperature. The fluorescence intensity was measured in a fluorescence microplate reader, Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA, USA), set for excitation at 485 nm



Figure 1. Influence of types of coatings on PAI-1 expression. (A) Influence of types of coatings on active PAI-1 expression in the supernatants from CaSki and SiHa. Active PAI-1 levels in the supernatants from CaSki were significantly increased (ELISA). Experiments were performed in duplicate and repeated three times (n=3). The values shown represent the mean \pm SE. (B) Influence of collagen IV on PAI-1 mRNA expression of CaSki. Culturing CaSki cells on collagen IV-coted plates increased their expression levels of PAI-1 mRNA (RT-qPCR). Experiments were performed in triplicate and repeated three times (n=3). The values shown represent the mean \pm SE. ^{**} p<0.01; *p<0.05; n.s., not significant. n.d., not detected. (A) X-axis, non-coating, fibronectin, vitronectin, laminin, collagen IV. Y-axis, ng/ml/10⁶ cells. (B) X-axis, non-coating or collagen IV. Y-axis, relative gene expression.

and emission detection at 538 nm. The fluorescence intensity was corrected for background fluorescence by subtracting the value derived from the no-enzyme control. Clostridium collagenase (provided with the kit) was used as a positive control. Experiment was repeated three times.

Statistical analysis. ANOVA test was used for comparing the influence of the ECM. Student t-test was used to compare the means of expression levels of PAI-1. Wilcoxon rank-sum test was used to investigate the effects of TM5275. p-values <0.05 were considered statistically significant. JMP/SAS Institute software was used for statistical analysis.

Results

Expression levels of PAI-1 are significantly different depending on the coating. In advance, we confirmed the expression of PAI-1, uPA and uPAR of CaSki and SiHa by RT-PCR (data not shown). The concentration of active PAI-1 in the supernatants from CaSki and SiHa is shown in Fig. 1A. The expression levels of active PAI-1 in the supernatants from CaSki cells were significantly different depending on the coating, and we selected CaSki cells for further experiment. Besides, we decided to investigate the relationship between collagen IV and PAI-1, because i) We detected high levels of PAI-1 when culturing CaSki cells on collagen IV-coated plates as shown in Fig. 1A. ii) Few studies have investigated the relationship between PAI-1 and collagen IV, while most studies investigated the relationship between PAI-1 and fibronectin, vitronectin and laminin (1,8,11,35). iii) Collagen IV is known to be rich in basal membrane (where cervical cancer occurs) (37), and collagen IV could be important for CSCs. As shown in Fig. 1B, culturing CaSki cells on collagen IV-coated plates increased the expression of PAI-1 at mRNA levels.

Expression levels of PAI-1 are different from ALDH1-high cells to ALDH1-low cells. We performed the same procedures after sorting CaSki cells into ALDH1-high cells and ALDH1-low cells. As shown in Fig. 2A, culturing ALDH1-high cells and ALDH1-low cells on collagen IV-coated plates increased the expression levels of PAI-1 mRNA. The increase of ALDH1-high cells was statistically significant, and it was suggestive that ALDH1-high cells contributed to the increase of PAI-1 mRNA expression levels (Fig. 1A).

PAI-1 plays a role in maintaining the ECM when secreted (1), and we detected secreted active PAI-1 in the supernatants of cultured cells using ELISA. In addition, since the balance of uPA and PAI-1 is important rather than the concentration of PAI-1 itself (1,2,15), we investigated secreted active uPA as well (Fig. 2B). We found that the expression of PAI-1 is higher when cells were cultured on collagen IV-coated plates than when cultured on non-coating plates, and that the expression of PAI-1 of ALDH1-low cells was higher than that of ALDH1high cells.

Of note, the patterns of secreted PAI-1 and PAI-1 mRNA expression levels were apparently different. In the context of maintaining the ECM, not only active PAI-1 but also many factors, such as non-active PAI-1, uPA, tPA and uPAR, and their combination are important (1,7,10,15,38-40). Considering these facts, the expression levels of PAI-1 mRNA do not have to accord with the concentration of secreted active PAI-1, however, we further detected intracellular active PAI-1 in order to obtain insights into the inconsistency. Of interest, the concentration of intracellular active PAI-1 was similar to the expression patterns of PAI-1 mRNA (Fig. 2A and C). These results can be interpreted as follows: The amount of active PAI-1 is consistent with the expression of PAI-1 mRNA, however, PAI-1 from ALDH1-high cells is more difficult to be secreted than PAI-1 from ALDH1-low cells [due to transportation system or membrane permiability, for instance (41)].



Figure 2. Influence of collagen IV on PAI-1 expression of ALDH1-high cells and ALDH1-low cells. (A) Influence of collagen IV on PAI-1 mRNA expression of ALDH1-high cells and ALDH1-high cells and ALDH1-high cells and ALDH1-high cells. Culturing ALDH1-high cells on collagen IV-coted plates significantly increased their expression levels of PAI-1 mRNA (RT-qPCR). Experiments were performed in triplicate and repeated four times (n=4). The values shown represent the mean ± SE. (B) Influence of collagen IV on active uPA and active PAI-1 levels in the supernatants from ALDH1-high cells and ALDH1-low cells. Culturing ALDH1-high cells and ALDH1-low cells on collagen IV-coted plates increased their expression of active PAI-1 (ELISA). The expression levels of PAI-1 in the supernatants from ALDH1-low cells cultured on collagen IV-coted plates were significantly higher than those from ALDH1-high cells cultured on both non-coating and collagen IV-coated plates. Experiments were performed in duplicate and repeated six times (n=6). The values shown represent the mean ± SE. (C) Influence of collagen IV on intracellular active PAI-1 levels (ALDH1-high cells and ALDH1-low cells. The patterns of intracellular active PAI-1 levels (ELISA) were similar to the patterns of PAI-1 mRNA expression levels (A). Experiments were performed in duplicate and repeated six times of repeated three times (n=3). The values shown represent the mean ± SE. (C) Influence of collagen IV coted plates. *p<0.05, n.s.; not significant. n.d.; not detected, N; non-coating, IV; collagen IV. (A) X-axis: ALDH1-high or ALDH1-high or ALDH1-low, N or IV and uPA or PAI-1. Y-axis: ng/ml/10⁶ cells. (C) X-axis, ALDH1-high or ALDH1-low and N or IV. Y-axis, ng/ml.



Figure 3. Results of gelatin zymography assays. Each supernatant expressed only pro-MMP-2. Representative data are shown. Experiments were performed three times. MMP, matrix metalloproteinase; N, non-coating; IV, collagen IV. X-axis, ALDH1-high or ALDH1-low and N or IV. Y-axis, MMP-2, pro-MMP-2 or pro-MMP-9.

ALDH1-high cells and ALDH1-low cells express pro-MMP-2. We then speculated on the impact of PAI-1 in maintenance of the ECM. With gelatin zymography assays, we investing the ability of each collected supernatant to degrade the general ECM, gelatin. As shown in Fig. 3, each supernatant expressed only pro-MMP2. This result was acceptable since pro-MMP-2 is the substrate of plasmin (1,42), although we could not find significant difference of pro-MMP-2 expression among these conditions.

Exposing ALDH1-low cells to TM5275, a small molecule inhibitor of PAI-1, increases collagenase activity of the supernatants. In order to quantify the activity of degrading the ECM, we proceeded to perform collagenase activity assays. After sorting, $1x10^5$ cells were seeded and cultured for 24 h. Then, medium was changed to DMEM with or without 10 nM TM5275 for 24 h, and the supernatants were collected (43). As shown in Fig. 4, collagenase activity was increased only when ALDH1-low cells were exposed to TM5275. The intensities ranged from the intensity of 0.02 U/ml of clostridium collagenase to that of 0.2 U/ml of clostridium collagenase.

Discussion

In the present study, we investigated the significance of PAI-1 in cervical CSCs and non-CSCs, and its impact on the ECM maintenance. PAI-1 inhibits the activation of uPA (which converts plasminogen to plasmin), and it is involved in cancer invasion and metastasis, by remodeling the ECM through regulating plasmin (1,6-12). The studies investigating CSCs are now increasing in various types of solid tumors including cervical cancer, and CSCs are thought to be involved in tumor recurrence and metastasis (17,18). Putting these facts together,



Figure 4. Effects of TM5275, a small molecule inhibitor of PAI-1, on collagenase activity. Collagenase activity was significantly increased only when ALDH1-low cells were exposed to 10 nM TM5275 (Wilcoxon rank-sum test). These intensities were ranged from the intensity of 0.02 U/ml of clostridium collagenase to that of 0.2 U/ml of clostridium collagenase (positive control). Experiments were performed in duplicate and repeated three times (n=3). The values shown represent the mean \pm SE. *p<0.05; n.s., not significant; N, non-coating; IV, collagen IV. X-axis, ALDH1-high or ALDH1-low, N or IV and TM5275 - or +, Y-axis, arbitrary units.

we aimed to investigate the relationship between PAI-1 and cervical CSCs.

First, using the cervical cancer cell line CaSki we confirmed that the expression levels of PAI-1 were significantly increased when the cells were cultured on collagen IV-coated plates compared to when cultured on non-coated plates, employing ELISA and RT-qPCR. When we use collagen IV as the representative of the ECM, we can put a focus on proteolytic activity of PAI-1 unlike using fibronectin and vitrinectin (nonproteolytic, or direct interaction between PAI-1 and the ECM is known) (1). At the same time, we have to consider the protein downstream of uPA and plasmin such as MMP-2, because the substrate of uPA itself is not collagen IV but fibronectin (44). This is why we performed gelatin zymography assays. With gelatin zymography assays, we found the involvement of pro-MMP-2.

Secondly, we investigated the significance of PAI-1 in maintenance of the ECM, by investigating collagenase activity. We confirmed that the activity to degrade the ECM was increased by exposing ALDH1-low cells to TM5275 (a small molecule inhibitor of PAI-1). On the contrary, adding recombinant PAI-1 (the final concentration of 1 μ g/ml), to the supernatants from ALDH1-high cells cultured on noncoating plates, reduced its fluorescence intensity by 7% (data not shown). These results were suggestive that PAI-1 was indeed involved in maintaining the ECM in CSCs and non-CSCs.

The limitation of our experiment is that we could not assess to what extent PAI-1 was involved in maintaining the ECM. The question remains from that we could not show a clear inverse correlation between concentration of PAI-1 and fluorescence intensity (Figs. 2B and 3). This is due to the complexity of the PA system, and considering only PAI-1 would not be enough



Figure 5. Hypothetic schema from the data we obtained. Cancer cells adhered to basal membrane secrete abundant PAI-1, whereas, cancer cells distant from basal membrane secrete less PAI-1. The concentration of active PAI-1 around CSCs is lower than that around non-CSCs, and the ECM surrounding CSCs is more susceptible to degradation.

in the context of degradation of the ECM (10). However, we may say that we shed light on the necessity to consider the concept of CSCs when investigating the PA system.

ALDH1-low cells expressed PAI-1 more highly than ALDH1-high cells (Fig. 2A). PAI-1 is known to be related to senescence, and it is more highly expressed in 'old cells' than in 'young cells', and 'more passaged cell line' than 'less passaged cell line' (45-47). Considering the direction of differentiation from ALDH1-high cells to ALDH1-low cells, it is reasonable to think that ALDH1-low cells express PAI-1 more highly than ALDH1-high cells.

Although the increase of PAI-1 expression in the supernatants from SiHa cells when cultured on laminin-coated plates was not statistically significant (Fig. 1A), it is suggestive that PAI-1 is important for the interaction between cancer cells and basal membrane, considering that laminin is a major component of basal membrane of uterine cervix as well as collagen IV (37).

In conclusion, we investigated the significance of PAI-1 in CSCs and non-CSCs. The expression levels of PAI-1 of ALDH1-high and ALDH1-low cells were both increased when cultured on collagen IV-coated plates, however, the basic expression levels of PAI-1 of ALDH1-high were lower than those in ALDH1-low cells. This result was suggestive that the ECM surrounding CSCs (especially distant from basal membrane) is more susceptible to degrade due to its low expression levels of PAI-1 (hypothetic schema from the data we obtained is shown in Fig. 5). Although verification to other kinds of CSC markers, and other types of cancers are needed, our study could be an explanation of conflicting reports, where some researchers found negative impacts of PAI-1 expression on clinical outcomes and others not, by considering the concept of CSCs.

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