主論文の要旨

PAI-1 secreted from metastatic ovarian cancer cells triggers the tumor-promoting role of the mesothelium in a feedback loop to accelerate peritoneal dissemination

（腫瘍由来 PAI-1 が腹膜中皮との相互的フィードバックループを形成することによって卵巣癌の腹膜播種形成を促進する）

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Introduction

More than 70% of ovarian cancer patients are initially diagnosed at advanced FIGO stages, with peritoneal metastasis. Human peritoneal mesothelial cells (HPMCs) have played important roles in the microenvironment of ovarian cancer metastasis. As the front line for protection, HPMCs are a defensive barrier against cancer cells. However, we previously identified the cancer-associated mesothelial cells (CAMs), which acquired the tumor-promoting features. The transformation from HPMCs to CAMs and the underlying mechanism attracted our interest.

Plasminogen activator inhibitor-1 (PAI-1) was initially known as an inhibitor of fibrinolysis by inhibiting the proteolytic activity of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA). Aside from regulating the fibrinolytic system in cancer, PAI-1 independently and directly mediates cancer cell proliferation and migration. However, the role of PAI-1 in metastatic ovarian cancer remains unknown.

Here, we determined how HPMCs switched from their barrier function into CAMs and how CAMs promoted cancer metastasis. PAI-1 was a crucial regulator of ovarian cancer-mesothelium crosstalk. PAI-1 secreted from metastatic ovarian cancer cells triggered the CAM transition. In response, CAMs provided a pro-metastatic effect on the cancer cells. As a result, a feedback loop was gradually formed, making it easier for ovarian cancer cells to disseminate.

Methods

In this study, first, we performed cytokine antibody array and identified PAI-1 as the most abundant effectors inside conditioned media. Then, transwell migration and invasion assays were performed to confirm the function of PAI-1 *in vitro*.

To study the underneath mechanism, the luciferase reporter assay was performed and it was indicated that nuclear factor kappa B (NFκB) pathway was directly activated by PAI-1, at the transcriptional level. Then ChIP assay was conducted to further identify the downstream targets of PAI-1/NFκB regulatory axis. Meanwhile, Western blotting was performed in four independent samples of primary mesothelial cells, to analyze the activation of NFκB components. Lastly, BALB/C nude mice were used for the *in vivo* model of peritoneal metastatic ovarian cancer.

To understand the clinical significance of PAI-1, we analyzed the PAI-1 expression at both RNA level (from TCGA database) and protein level from tissue samples (from Nagoya University) by immunohistochemistry.

Results

1. PAI-1 is a key regulator to induce CAM formation both in vitro and in vivo.

From the cytokine antibody array, PAI-1 was the most abundantly released factor in the

supernatants from three ovarian cancer cell lines: ES2, SKOV3, and HEY cells. Therefore, recombinant PAI-1 was used to directly stimulate HMPCs. The wound-healing assay demonstrated that PAI-1 promoted the migratory ability of mesothelial cells in the same way that conditioned medium stimulated the migration of the cells, which indicated that PAI-1 might be the most likely candidate to induce CAM formation from HPMCs *in vitro* **(*Fig. 1A*)**.

To study the role of CAMs *in vivo*, we co-injected (by intraperitoneal injection) ovarian cancer cells and primary mesothelial cells. Under the *in vivo* imaging system (IVIS), the intraperitoneal tumor-bearing burden of each mouse was investigated. Due to the presence of mesothelial cells, peritoneal metastasis was severer than in mice injected solely with ovarian cancer cells. In dual injection groups, the metastasis-promoting effect was significantly repressed by the inhibition of PAI-1 secretion. Taken together, the malignant role of CAMs is markedly promoted by PAI-1, accelerating the severity of the metastasis **(*Fig. 1B*)**.

2. The nuclear NFκB pathway in CAMs is activated by PAI-1.

Recombinant PAI-1 was directly added to four independent cases of primary HPMCs. The activated NFκB components (p65, ser536, IKKs) were investigated. Although the level of ser536 was not efficiently elevated in case #2, both nuclear p65 and ser536 were significantly increased in the other three cases，further validating the NFκB activation in different primary samples. These results also indicated that PAI-1 was a key stimulator of nuclear NFκB activation in CAMs **(*Fig. 2A*)**.

The NFκB luciferase reporter assay was performed by co-transfection of HEK293T cells with the pTAL-NFκB vector (Firefly Luc) and the pRL-TK vector (Renilla Luc). The results demonstrated that the luciferase activity of NFκB was significantly increased by PAI-1, indicating that PAI-1 directly upregulated NFκB downstream cytokines, such as IL-8, CXCL5, TNFα and IL-6. After PAI-1 stimulation, IL-8 and CXCL5 mRNA expression was upregulated. However, TNFα and IL-6 mRNA expression was unaffected. Together with the results of ChIP qPCR, it was indicated that PAI-1 specifically activated the transcription of IL-8 and CXCL5 **(*Fig. 2B*)**.

3. PAI-1 predicts poor clinical outcomes for ovarian cancer patients.

The protein analysis by immunohistochemistry showed that all 67 patients positively expressed PAI-1. Kaplan-Meier analysis showed that upregulated PAI-1 predicted a poor survival rate among ovarian cancer patients ($P < 0.01$). For the first time, we showed that a high level of PAI-1 was significantly correlated with peritoneal dissemination ($P < 0.01$). From TCGA database, mRNA of PAI-1 was overexpressed in patients with late stage (III, IV) ovarian cancer ($P < 0.05$). A high level of PAI-1 also predicted a poor survival rate in

ovarian cancer patients ($P < 0.05$) **(*Fig. 3*)**.

Discussion

As the most superficial cell population in the peritoneal cavity, mesothelial cells are the first line of defense against cancer. However, constantly stimulated by cancer-secreted mediators, mesothelial cells transform into cancer promoters or CAMs. We characterized CAMs based on the supporting evidence: 1) mesothelial-mesenchymal transition (MMT) was induced and constantly maintained in CAMs, physically providing a convenient pathway for cancer cell penetration and residence; 2) CAMs secreted pro-tumoral factors, such as IL-8 and CXCL5, to accelerate tumor metastasis; 3) CAMs promoted metastasis *in vitro* and tumor progression *in vivo*. To these ends, CAMs are crucially responsible for the acceleration of peritoneal metastasis.

CAM formation forms a pro-metastatic feedback loop, accelerating the pace of the peritoneal dissemination of ovarian cancer. PAI-1 is a key activator. When we blocked PAI-1 in ovarian cancer cells, the release of IL-8 and CXCL5 in CAMs was suppressed, indicating that CAM formation was blocked, and the feedback loop was partially cut off by PAI-1 inhibition. This is further demonstrated in our *in vivo* model. To better emphasize the role of mesothelial cells in the microenvironment, we performed an intraperitoneal co-injection of primary human mesothelial cells with ovarian cancer cells. The presence of human mesothelial cells worsened peritoneal metastasis *in vivo*. However, the factor triggering CAM formation remains unclear. Here, we inhibited PAI-1 expression using shRNAs and found that the formation of CAMs was inhibited, and their pro-metastatic effect was significantly reduced. This suggests that PAI-1 is one of the essential triggering factors for CAM formation.

Clinically, PAI-1 is upregulated in ovarian cancer tissues. A high level of PAI-1 independently indicates a poor prognosis in ovarian cancer patients. Most importantly, PAI-1 is significantly correlated with peritoneal metastasis. For the first time, PAI-1 is described as a candidate for predicting tumor metastasis, and may be a promising diagnostic and prognostic marker.

Conclusion

This study illustrated the pro-metastasis feedback loop in the peritoneal microenvironment. Metastatic ovarian cancer cells activate the formation of CAMs by releasing PAI-1, which directly promotes NFκB activation to upregulate IL-8 and CXCL5 secretion from CAMs. IL-8 and CXCL5 further accelerate the progression of metastasis. Thus, targeting PAI-1 and blocking CAM formation in the loop could slow down the pace of ovarian cancer metastasis.