Biochemical and Biophysical Research Communications 450 (2014) 1696–1701

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Plasminogen activator inhibitor-1 regulates infiltration of macrophages into melanoma via phosphorylation of FAK-Tyr^{925}

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ARTICLE INFO

Article history:
Received 12 July 2014
Available online 22 July 2014

Keywords:
Macrophage
PAI-1
FAK
Melanoma
Invasion
LRP1

ABSTRACT

Tumor-infiltrating macrophages are potential candidates for cancer immunotherapy. However, the detailed molecular mechanism underlying macrophage infiltration into tumors is poorly understood. Based on our previous finding that plasminogen activator inhibitor-1 (PAI-1) enhances vitronectin-dependent migration of macrophages, we investigated the potential role of PAI-1 in macrophage invasion into melanoma. Experimental evidence obtained from spheroid confrontation assay clearly showed that PAI-1 overexpression significantly enhanced the invasion of RAW 264.7 cells into B16F10 melanoma. We further demonstrated that PAI-1 induces phosphorylation of focal adhesion kinase (FAK) at Tyr^{925}, which, in turn, mediated the invasion of macrophages into the melanoma. This work further illustrates that low-density lipoprotein receptor related-protein 1 (LRP1) is essential for PAI-1-mediated FAK phosphorylation and macrophage invasion into melanoma. In conclusion, our study demonstrates a novel role of PAI-1 in macrophage invasion into melanoma and provides insights into the underlying molecular mechanism.

1. Introduction

Focal adhesion kinase (FAK) regulates the formation of focal adhesion complexes at focal contacts connecting the extracellular matrix (ECM)-associated integrins with the actin cytoskeleton. FAK acts as an adaptor protein that recruits various focal contact proteins or their regulators and mediates regulation of various intracellular signaling cascades [1]. Binding of integrin to ECM triggers the interaction of FAK with integrin-bound paxillin, leading to auto-phosphorylation of FAK at Tyr^{397}; this results in the recruitment of Src family kinases and activation of several downstream signaling pathways [2]. FAK is phosphorylated at tyrosine residues Tyr^{407}, Tyr^{576}, Tyr^{577}, Tyr^{861}, and Tyr^{925}. FAK phosphorylation at Tyr^{925} recruits growth factor receptor-bound protein-2 (GRB-2), which in turn activates the extracellular signal-regulated kinase-2 (ERK-2) cascade. Both of these events are proposed to release FAK from focal contacts, destabilizing them and allowing the formation new focal contacts at new sites [1]. Cells with high levels of phosphor-FAK-Tyr^{925}, exhibit increased cell protrusion and migration [3].

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) family, inhibits urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) by binding to their active forms on the cell surface, leading to attenuation of plasminogen activation [4]. In addition, PAI-1 interacts with ECM by binding to the somatomedin B domain of vitronectin, thereby hindering cell adhesion mediated by the uPA receptor (uPAR) and integrins [5,6]. Moreover, PAI-1 mediates internalization of the uPAR–uPA–PAI-1 complex and associated integrins via the interaction with low density lipoprotein (LDL) receptor-related protein 1 (LRP1) [7]. LRP1 is large endocytotic receptor that can bind to over 30 different ligands [8], a function that explains its involvement in numerous cellular processes, including cell migration and invasion [9–11].

Macrophages constitute the dominant population of immune cells that infiltrate tumors, including melanomas, resulting in promotion of angiogenesis and growth of the tumor [12]. However, macrophages also recently emerged as prominent candidates for cancer immunotherapy, as several studies have established that macrophages can acquire antitumor activity [13,14]. Thus, understanding the molecular mechanisms underlying tumor invasion by macrophages is of the utmost importance. Previously, we demonstrated that PAI-1 increases vitronectin-dependent migration of macrophages [15]. In this study, we investigated the role

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http://dx.doi.org/10.1016/j.bbrc.2014.07.070
0006-291X/

of PAI-1 in macrophage invasion of melanomas and the mechanisms underlying this process.

2. Materials and methods

2.1. Reagents

Antibodies against myc, FAK, phosphorylated FAK-Tyr^{925}, GAPDH, phosphorylated paxillin, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA). Anti-PAI-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine serum albumin (BSA) and anti-FLAG antibody and anti-LRP1 (N-terminal) were purchased from Sigma–Aldrich (St. Louis, MO). Recombinant mouse LRP1-associated protein 1 (LRPAP, also known as RAP) was purchased from R&D Systems (Minneapolis, MN). Fibronectin and vitronectin were purchased from BD Biosciences (Bedford, MA).

2.2. Cell culture

The murine macrophage cell line, RAW 264.7, and melanoma cell line, B16F10, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin and streptomycin. Viability was assayed by Trypan blue dye exclusion, and was typically greater than 95%. RAW 264.7 cell cultures were maintained until passage 20, and then discarded.

2.3. Plasmid construction and transfection

Plasmids encoding wild-type and mutant PAI-1 were constructed and used as described previously [16]. Human FAK cDNA cloned into pCMV6-Entry vector (pCMV6-FAK) was purchased from OriGene Technologies (Rockville, MD). Wild-type FAK was mutated to replace Tyr^{925} with Phe using the Muta-Direct Site-Directed Mutagenesis Kit (Intron Biotechnology, Gyeonggi, Korea), according to the manufacturer's instructions and the following primers (mutated bases are underlined): FAK^{-Y925F} 5′-CGAATGAT AAGGTGTTCGAGAATGTGACGG-3′ (sense) and 5′-CCGTCACATTC TCGAACACCTTATCATTCG-3′ (antisense). Mutations were confirmed by sequencing. Cells were transfected using Fugene 6 Transfection Reagent (Promega, Madison, WI) and incubated for 24 h. Two micrograms of plasmid were used for a single transfection, and 1 μg of each plasmid was used for co-transfection of cells in 6-well culture plates.

2.4. Small interfering RNA (siRNA) transfection

RAW 264.7 cells were grown in 6-well plates and transfected with 80 nM PAI-1 siRNA1 (target sequence CCAACAAGAGCCAAUC ACATT) [17], PAI-1 siRNA2 (Santa Cruz, CA) or control siRNA (target

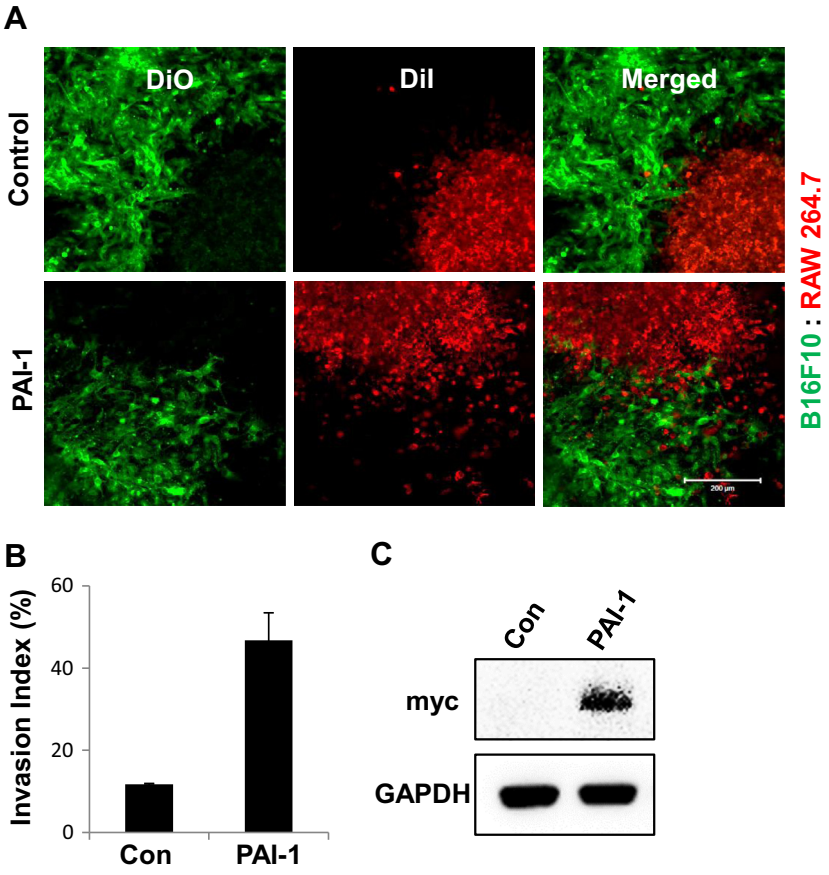

Fig. 1. Plasminogen activator inhibitor-1 (PAI-1) overexpression increases invasion of RAW 264.7 cells into melanoma. (A) B16F10 cells and RAW 264.7 cells transfected with control (con) plasmid or plasmid encoding PAI-1 were labeled with DiO (green) and DiI (red), respectively, and cultured on agarose-coated plates overnight, followed by seeding of spheroids on vitronectin-coated slides for 24 h. The cells were analyzed by confocal microscopy. Scale bars = 200 μm. (B) Invasion of RAW 264.7 cells, transfected with plasmid encoding PAI-1 or control (con) plasmid as shown in (A), was quantitated using Image J software and expressed as invasion index (%). (C) Western blotting analysis of RAW 264.7 cells transfected with plasmid encoding myc-tagged PAI-1 or control plasmid. GAPDH served as loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sequence ACCACGACGUACACAAACATT) [17] using the Fugene 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Cells were incubated for 48 h, resuspended, and seeded on BSA-, vitronectin-, or fibronectin-coated plates in serum free medium and cultured for 4 h. In other experiments RAW 264.7 cells were transfected with 100 nM of control siRNA, LRP1 siRNA1 (target sequence AAGCAGTTTGCCTGCAGAGAC) or LRP1 siRNA2 (target sequence AACTTCTTAAACTCATAGCTT) [18] and incubated for 24 h and re-transfected with various plasmids and subjected to western blotting or two-dimensional spheroid confrontation assay.

2.5. Western blotting

Cells transfected with plasmids or siRNA were washed and resuspended in serum-free medium followed by seeding on plates coated with BSA, vitronectin, or fibronectin for 4 h. Cells were washed with phosphate-buffered saline (PBS) and lysed in buffer containing 50 mM Tris (pH 7.8), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2% SDS, 1% NP-40, protease inhibitor cocktail (Roche, Indianapolis, IN), and phosphatase inhibitor cocktail (Calbiochem, San Diego, CA) for 30 min on ice. Equal amounts of protein lysates were resolved by denaturing electrophoresis in a 4–12% Tris–glycine polyacrylamide–SDS gel (Invitrogen, Carlsbad, CA), followed by transfer to a nitrocellulose membrane. The membrane was blocked with 5% skim milk, and the membrane was incubated with primary, and then secondary antibody. Protein bands were detected using the ECL Plus reagent (GE Healthcare, Sunnyvale, CA).

2.6. Two-dimensional (2D) spheroid confrontation assay

Two-dimensional spheroid confrontation assay was performed as described previously, with some modifications [19]. RAW 264.7 cells were labeled with the red fluorescent dye, DiI, and B16F10 cells were labeled with the green fluorescent dye, DiO, using Vybrant Multicolor Cell-labeling Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and cultured overnight in 1% agarose-coated 6-well plates allowing for spheroid formation. The spheroids were collected, randomly plated on vitronectin (10 μg/ml)-coated slides, and further incubated for 24 h, followed by fixation and mounting. Images of the junctions of differentially labeled spheroids were captured using a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

2.7. Invasion index and statistical analysis

To quantitate invasion of the red fluorescence-labeled RAW 264.7 cells into the green fluorescence-labeled B16F10 spheroids, images were analyzed using Image J software. The invasion index was calculated as the ratio of the overlapping area to the total green area expressed as percentage, as described previously [19]. The invasion index was expressed as mean ± standard deviation of at least three experiments.

3. Results

3.1. PAI-1 induces invasion of macrophages into melanoma

Tumor-infiltrating macrophages are extensively studied for various types of cancer immunotherapies. However, the mechanism underlying the infiltration process is not well understood. Based on our previous study showing that PAI-1 induces vitronectin-dependent migration of macrophages [15], we sought to investigate the role of PAI-1 in macrophage invasion into tumors. To determine this role, we overexpressed PAI-1 in RAW 264.7 cells and performed 2D confrontation assay with B16F10 cells after labeling the cells with DiI and DiO, respectively. The cells were seeded on agarose-coated plates overnight to allow formation of spheres. The differentially-labeled spheres were seeded on vitronectin-coated slides, and after 24 h, spheres were analyzed by confocal microscopy. Our data clearly demonstrated that RAW 264.7 cells overexpressing PAI-1 showed significant invasion into the melanoma spheres, compared with control cells (Fig. 1A and B). Western blotting showed PAI-1 overexpression in the transfected RAW 264.7 cells (Fig. 1C). These data indicate that PAI-1 is involved in macrophage invasion into melanoma.

3.2. PAI-1 induces phosphorylation of FAK at Tyr^{925} in RAW 264.7 cells

FAK plays an important role in cell movement [1]. FAK phosphorylation at Tyr^{925} causes the dissociation of focal contacts, which in turn drives cell motility [3]. In order to assess the effect of PAI-1 on phosphorylation of FAK at Tyr^{925}, we overexpressed PAI-1 in RAW 264.7 cells and seeded the cells on plates coated with BSA, vitronectin, or fibronectin. The western blot data revealed elevated levels of FAK phosphorylated at Tyr^{925} in cells overexpressing PAI-1 and cultured on vitronectin (Fig. 2A). However, cells grown on BSA or fibronectin displayed no change in phosphorylation.

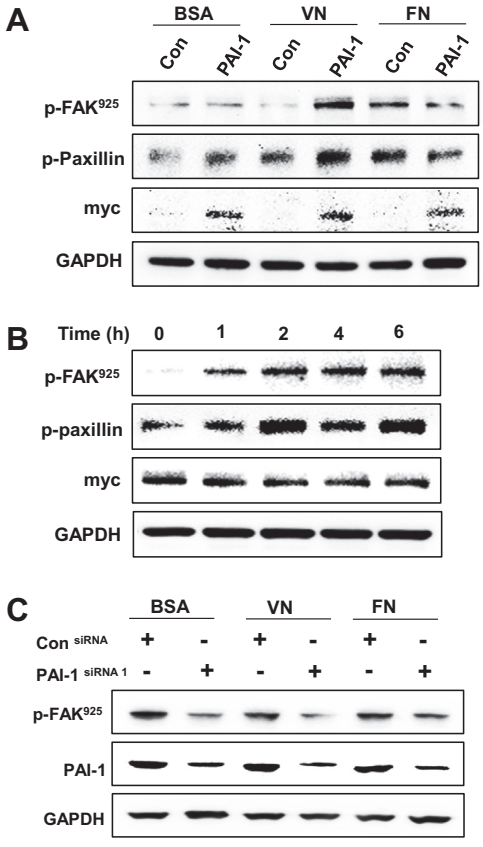

Fig. 2. PAI-1 overexpression induces focal adhesion kinase (FAK) phosphorylation at Tyr^{925} (p-FAK^{925}) in RAW 264.7 cells. (A) RAW 264.7 cells were transfected with control (con) plasmid or plasmid encoding myc-tagged PAI-1 and incubated for 24 h, followed by seeding on BSA-, vitronectin (VN)-, or fibronectin (FN)-coated plates for 4 h. Cells were lysed and analyzed by western blotting. p-paxillin = phosphorylated paxillin. (B) RAW 264.7 cells were transfected as shown in (A), seeded on VN-coated plates for the indicated time periods, lysed, and analyzed by western blotting. (C) RAW 264.7 cells were transfected with control (con) siRNA or 80 nM siRNA targeting PAI-1, incubated for 48 h, seeded on BSA-, VN- or FN- coated plates for 4 h, and analyzed by western blotting.

Phosphorylation of paxillin was also increased in PAI-1 overexpressing cells grown on vitronectin (Fig. 2A). Furthermore, western blot analysis of these cells after periods of up to 6 h of culture on vitronectin showed persistent levels of FAK-Tyr^{925} and paxillin phosphorylation (Fig. 2B). To further confirm the role of PAI-1 in FAK-Tyr^{925} phosphorylation, we carried out siRNA-mediated knockdown of PAI-1 in RAW 264.7 cells using two different siRNAs, and examined the level of FAK-Tyr^{925} phosphorylation. Our results demonstrated that the basal level of FAK-Tyr^{925} phosphorylation was reduced in PAI-1 knockdown cells (Fig. 2C and Supplementary Fig. 1). Taken together, these data suggest that PAI-1 induces the phosphorylation of FAK-Tyr^{925} in macrophages grown on vitronectin.

3.3. Role of FAK-Tyr^{925} phosphorylation on PAI-1-mediated invasion of macrophages into melanoma

Because PAI-1 was able to induce FAK-Tyr^{925} phosphorylation and macrophage invasion into melanoma, we sought to establish the role of Tyr^{925} phosphorylation in invasion. We replaced Tyr^{925} in wild-type FAK (FAK-WT) with phenylalanine, generating nonphosphorylatable FAK^{-Y925F}. RAW 264.7 cells were co-transfected with pCMV6-FAK (encoding FLAG-tagged FAK) and pcDNA3.1-PAI-1 (encoding myc-tagged PAI-1) and incubated for 24 h, followed by seeding and culture on vitronectin-coated plates for 4 h. Western blotting results revealed that co-expression of FAK-WT and PAI-1 increased the level of phosphorylated FAK-Tyr^{925} in the cells (Fig. 3A). However, co-expression of FAK^{-Y925F} resulted in drastic reduction of PAI-1-induced FAK-Tyr^{925} phosphorylation (Fig. 3A). These cotransfected RAW 264.7 cells were then subjected to 2D spheroid confrontation assay with B16F10 melanoma cells. Results of confocal microscopy clearly demonstrate the inhibitory effect of FAK^{-Y925F} expression on PAI-1-induced invasion of macrophages into melanoma (Fig. 3B and C). PAI-1 overexpression resulted in invasion of RAW264.7 cells into B16F10 cells; however, with expression of FAK^{-Y925F}, invasion was suppressed, possibly via a dominant negative effect (Fig. 3B and C). Collectively, these results indicate that PAI-1 induced invasion of macrophages into melanoma via induction of FAK-Tyr^{925} phosphorylation.

3.4. LRP1 is essential for PAI-1-mediated macrophage invasion into melanoma

LRP1 plays an important role in PAI-1-mediated migration in various cell types [9–11]. To illustrate the role of LRP1 in PAI-1-induced FAK-Tyr^{925} phosphorylation, we pre-treated cells overexpressing PAI-1 with the LRP1 inhibitor RAP before seeding on vitronectin. The western blot data revealed that, in the presence of LRP1, PAI-1 overexpression was unable to induce phosphorylation of FAK at Tyr^{925} (Fig. 4A). Moreover, PAI-1-induced paxillin phosphorylation was also hindered by RAP (Fig. 4A). Furthermore, siRNA mediated knockdown of LRP1 also resulted in inhibition of PAI-1-induced FAK phosphorylation at Tyr^{925} (Supplementary Fig. 2A). To further confirm the involvement of LRP1 in FAK-Tyr^{925} phosphorylation, we transfected RAW 264.7 cells with various PAI-1 mutants and incubated for 24 h, followed by seeding on vitronectin-coated plates and further incubation for 4 h. PAI-1 encoded by the construct PAI-1$^{\Delta LRP1}$ contains the R99E mutation and has low binding affinity for LRP1, whereas PAI-1 encoded by PAI-1$^{\Delta VN}$ contains the Q123K mutation and has impaired binding affinity for vitronectin. Likewise, PAI-1, encoded by PAI-1$^{\Delta uPA}$, contains the T365R and A358R mutations and has greatly reduced binding affinity for uPA [20]. Western blotting data revealed that overexpression of PAI-1$^{\Delta LRP1}$ did not induce phosphorylation of FAK at Tyr^{925} (Fig. 4B). A similar effect was observed with cells overexpressing PAI$^{\Delta uPA}$ (Fig. 4B). In fact, both PAI-1$^{\Delta LRP1}$ and PAI-1$^{\Delta uPA}$ slightly reduced FAK-Tyr^{925} phosphorylation unlike

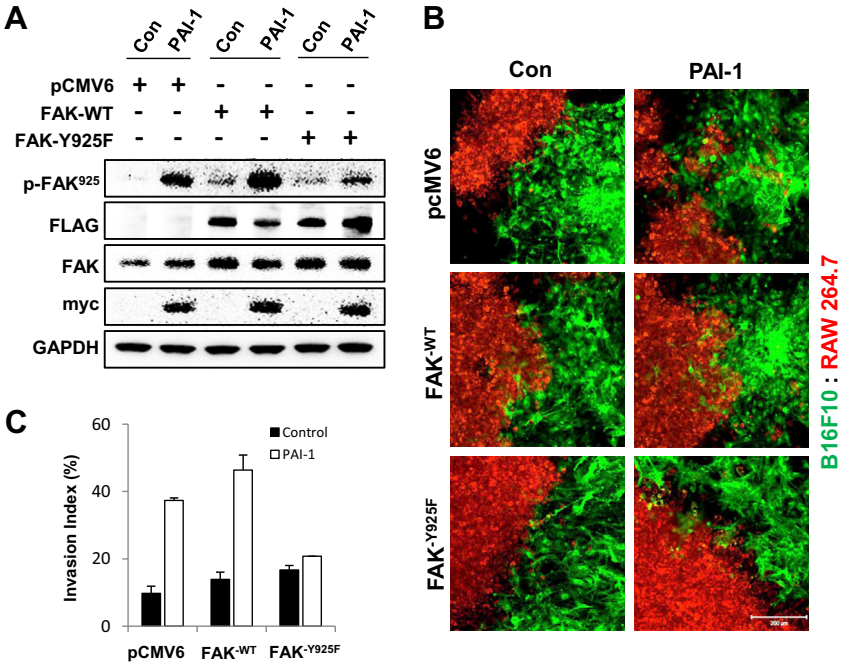

Fig. 3. PAI-1 mediates macrophage invasion into melanoma via FAK-Tyr^{925} phosphorylation. (A) RAW 264.7 cells were transfected with control plasmid or plasmid encoding myc-tagged PAI-1, alone or together with plasmid encoding FLAG-tagged wild-type FAK (FAK-WT) mutated FAK (FAK^{-Y925F}) and incubated for 24 h, followed by seeding on VN-coated plates for 4 h. Cells were lysed and subjected to analysis by western blotting. (B) B16F10 cells and RAW 264.7 cells transfected with the indicated plasmids were labeled with DiO (green) and DiI (red) respectively and cultured on agarose-coated plates overnight, followed by seeding of the spheroids into VN-coated slides for 24 h. The cells were then analyzed by confocal microscopy. Scale bars = 200 μm. (C) Invasion of RAW 264.7 cells shown in (B) was quantitated using Image J software and expressed as invasion index (%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

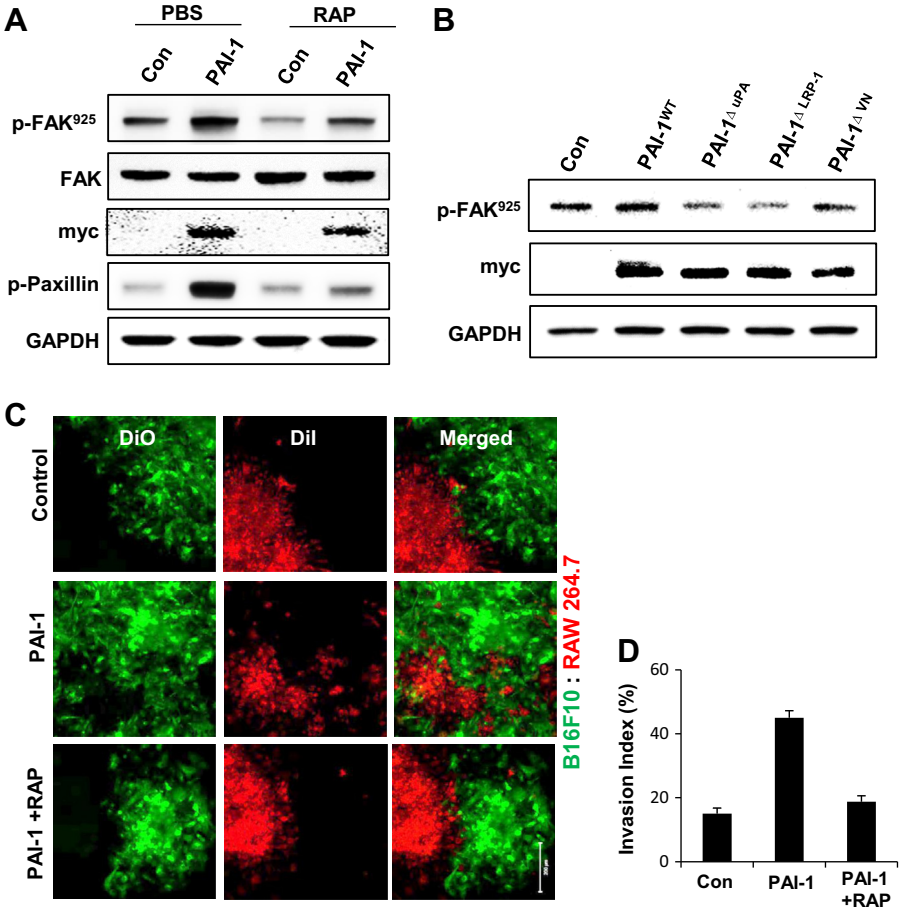

Fig. 4. Role of LRP1 in PAI-1-mediated macrophage invasion into melanoma. (A) RAW 264.7 cells, transfected with plasmid encoding myc-tagged PAI-1 or control plasmid, were pretreated with 50 μM RAP for 30 min prior to culture on VN-coated plates for 4 h. Cells were lysed and analyzed by western blotting. (B) RAW 264.7 cells were transfected with plasmids encoding wild-type (WT) and the indicated mutant forms of PAI-1, were incubated for 24 h, followed by seeding into VN-coated plates. After 4 h, cells were lysed and subjected to western blot analysis. (C) B16F10 cells and RAW 264.7 cells transfected with control plasmid or plasmid encoding PAI-1 were labeled with DiO (green) and DiI (red), respectively, and cultured on agarose-coated plates overnight, followed by seeding of the spheroids into VN-coated slides and culture in the presence or absence of 50 μM RAP for 24 h. The cells were analyzed by confocal microscopy. Scale bars = 200 μm. (D) Invasion of RAW 264.7 cells shown in (C) was quantitated using Image J software and expressed as invasion index (%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PAI-$1^{\Delta VN}$ (Fig. 4B). Next, we investigated the role of LRP1 in PAI-1-induced invasion of macrophages into the melanoma. The differentially labeled B16F10 and PAI-1 overexpressing RAW 264.7 spheroids were seeded on vitronectin-coated slides in the presence of 50 μM RAP. Results of confocal microscopy showed that PAI-1-induced invasion of macrophages into melanoma was blocked by RAP (Fig. 4C and D). Moreover, siRNA mediated knockdown of LRP1 in RAW 264.7 cells also inhibited the invasion process (Supplementary Fig. 2B and C). Taken together, these data indicate that LRP1 plays an important role in PAI-1-induced macrophage invasion into melanoma.

4. Discussion

Macrophage infiltration into melanoma promotes the growth of the melanoma [12]. In contrast, recent studies have demonstrated the antitumor function of these macrophages [13,14], further highlighting the importance of mechanistic studies of the infiltration process. Our previous study showed that PAI-1 increases vitronectin-dependent migration of macrophages [15]. In the current study, we investigated the role of PAI-1 in invasion of macrophages into melanoma and the mechanisms underlying the process.

The roles identified for PAI-1 in cell migration are contradictory, depending upon cell type and ECM composition [4]. Human lung cancer cells require PAI-1 for effective cell migration and invasion [21], whereas migration of ovarian cancer cells is reduced by PAI-1 [22], and invasion of prostate carcinoma cell line is not affected by PAI-1 [23]. Furthermore, we have previously shown that PAI-1 induces the migration of macrophages through vitronectin, but not fibronectin [15]. In the current study, we demonstrated that PAI-1 overexpression drives the invasion of macrophages into melanoma in the presence of vitronectin. PAI-1 expression level was found to be significantly higher in melanoma than in normal tissue [24]. The role of elevated PAI-1 in melanoma is still not well understood in terms of its inability to affect invasion and metastasis of the melanoma [25]. Our data support a novel function of PAI-1 in melanoma, because its overexpression in macrophages significantly increased macrophage invasion into melanoma via phosphorylation of FAK-Tyr^{925}.

Phosphorylation of FAK-Tyr^{925} is known to destabilize focal adhesions, allowing formation of new focal contacts, which drives cell motility. In fact, cells expressing constitutive phosphor-FAK-Tyr^{925} showed enhanced cell protrusion and migration [3]. PAI-1 is known to play an important role in the migration of cells, including macrophages [15]. However, the function of PAI-1 in

FAK-Tyr^{925} phosphorylation is not yet known. In this study, we demonstrated that phosphorylation of FAK-Tyr^{925} is a PAI-1/vitronectin-dependent event, because overexpression of PAI-1 increased the level of phosphorylated FAK-Tyr^{925}, and siRNA-mediated knockdown of PAI-1 also reduced the basal level of FAK-Tyr^{925} phosphorylation, in RAW 264.7 cells grown on vitronectin. Phosphorylation of FAK Tyr^{925} and paxillin Tyr^{118} are related to destabilization of focal contacts [3]. In agreement with this, we have also shown persistent, elevated levels of phospho-FAK-Tyr^{925} and phospho-paxillin in PAI-1-overexpressing RAW 264.7 cells. LRP1 interacts with the uPAR–uPA–PAI-1 complex via PAI-1 and inactivates associated integrins, followed by internalization of the whole complex and recycling of the receptors back to the surface [7]. Our results showed that PAI-1-mediated phosphorylation of FAK Tyr^{925} is dependent on interaction of PAI-1 with uPA and LRP1, suggesting the involvement of internalization of the uPAR–uPA–PAI-1 complex and associated integrins via LRP1. It is plausible that the alteration of surface integrins in this event might be responsible for FAK-Tyr^{925} phosphorylation; however, an in-depth mechanistic study is still needed.

Antimacrophage infiltration approaches have successfully reduced tumor size in mouse models using chemokine CCL5 antagonists [26] and various natural products and pharmacological drugs [27]. The current study has presented PAI-1 as a potential therapeutic target for reducing macrophage infiltration into tumors. Other therapeutic approaches convert the tumor-promoting macrophages into tumor-suppressing macrophages by activation of a proinflammatory program that leads to the destruction of tumor cells [14,28]. This approach requires enhanced infiltration of macrophages into the tumor tissue. PAI-1 is also a potentially important candidate in such therapeutic studies.

In summary, our study demonstrated the novel role of PAI-1 in macrophage invasion into melanoma via phosphorylation of the Tyr^{925} residue of FAK. Furthermore, this study also highlighted the therapeutic potential of PAI-1 in cancer immunotherapy as a regulator of macrophage infiltration into melanomas.

Acknowledgments

This research was supported by a Grant from the National Research Foundation (2013M3A9A9050126) funded by the Ministry of Science, ICT & Future Planning in the Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.070.

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