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A novel solution phase PAI-1/uPA-biotin complex assay for the measurement of active PAI-1 in plasma

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A B S T R A C T

We devised a new assay procedure to use biotinylated uPA to trace the active PAI-1 levels in the plasma. We show here that the potency of inhibitory monoclonal antibody 33B8 measured with the new assay is consistent with its *in vivo* efficacy in PAI-1 inactivation. We also found that among the three monoclonal antibodies tested, the traditional solid phase assay caused mechanism dependent significant right shift of IC_{50} values. As our new assay avoids the use of non-physiological large quantities of uPA, we conclude that it is a better measure of pharmacodynamic effects of anti-PAI-1 antibodies *in vivo*.

1. Introduction

Plasminogen activator inhibitor 1 (PAI-1) is the major physiological inhibitor of tissue-type and urokinase-type plasminogen activator (tPA and uPA), two proteases that are responsible for the conversion of inactive plasminogen into plasmin. PAI-1 is under tight and direct regulation by TGF-β, the master regulator of fibrosis. Large body of literature indicates that PAI-1 is implicated in fibrotic pathological processes in multiple organs such as kidney, lung, liver, skin and heart [1–4].

Deregulated wound healing process leads to fibrosis. It is caused by the hyperactivation of fibroblasts and manifested by the excessive accumulation of extracellular matrix. PAI-1/uPA/tPA axis plays a significant role for the turnover of extracellular matrix deposit. Under a normal physiological condition when PAI-1 level is low, the uPA/tPA activities are at high levels where excess extracellular matrix proteins are degraded to maintain a physiological homeostasis. In contrast, under pathological conditions, pro-fibrotic TGF-β drives high level expression of PAI-1 that inhibits uPA/tPA. This leads to the decrease of plasmin and plasmin-dependent proteolytic activities which dampens the degradation of extracellular matrix proteins [1,5,6]. Inhibiting PAI-1 therefore is an attractive therapeutic target for the treatment of multiple fibrotic conditions such as chronic kidney disease, idiopathic pulmonary fibrosis, cirrhosis, scleroderma and heart failure.

Because PAI-1 is an important therapeutic target, it is essential to develop a protocol that can accurately measure the active PAI-1 levels in plasma to reflect pharmacodynamic (PD) effects of PAI-1 inhibitor. There are three forms of PAI-1 in the body: active PAI-1, inactive latent and cleaved forms of PAI-1 [7]. Upon the addition of a PAI-1 small molecular inhibitor or an inhibitory antibody, the forth form, PAI-1/inhibitor complex is introduced in addition to these three physiological forms of PAI-1. In the literature, the main stream of plasma PAI-1 activity assay uses the principle that functionally active PAI-1 present in the plasma reacts with either uPA or tPA coated on a solid phase. In fact, multiple companies such as Molecular Innovations, Innovative Research have commercialized assay kits where tPA or uPA are coated and dried on a microtiter plates [8–11]. More recently, Cale et al. and Diaz et al. miniaturized solid phase assay by using uPA that was coupled to carboxylated beads. The miniaturized solid phase assay significantly reduce the plasma volume [12,13] and successfully used to demonstrate significant PAI-1 elevation in ApoE null mice and *in vivo* pharmacodynamic (PD) effect of polyphenolic inhibitors of PAI-1.

We are interested in testing the inhibitory effects of PAI-1 inhibitory monoclonal antibodies (mAbs) in whole plasma. We first studied 33B8, a PAI-1 mAb that was shown to exert *in vivo* effect in decreasing active PAI-1 and preventing the formation of fibrin deposit in the endotoxin-induced disseminated intravascular coagulation model [14]. To our surprise, the traditional solid phase assay revealed that 33B8 only inhibited plasma PAI-1 activity with an IC_{50} value of ~0.6 μM (Fig. 1) that is inconsistent with the reported *in vivo* efficacy. This prompted us to explore if there might be additional assay format which could be used to reflect PAI-1 PD effects.

Abbreviations: PAI-1, Plasminogen Activator Inhibitor 1; uPA, urokinase-type plasminogen activator; mAb, Monoclonal Antibody; pAb, Polyclonal Antibody; PE, Streptavidin-R-Phycoerythrin

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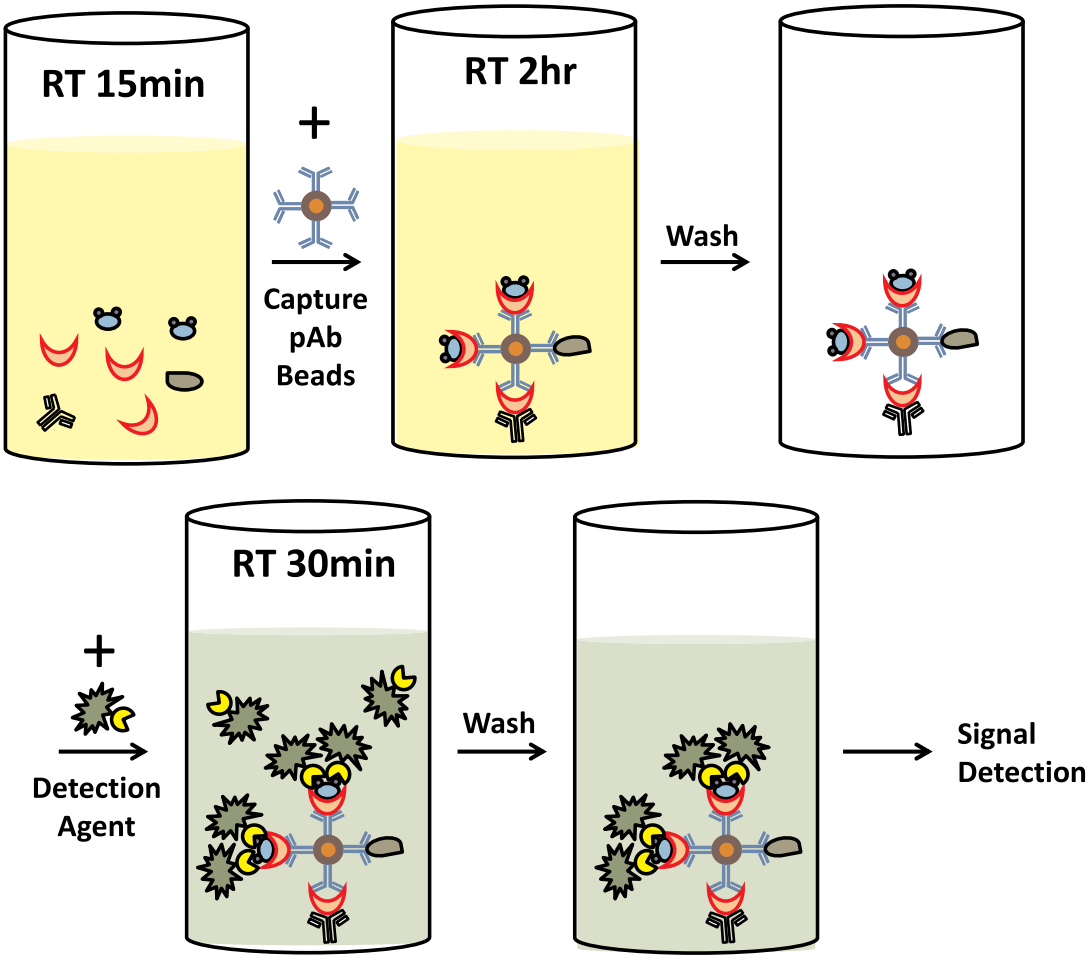

Symbol						
Name	Anti-Hu PAI-1 Capture pAb Coated Magnetic Microsphere	Anti-Hu PAI-1 Inhibitory mAb (testing mAb)	Active HuPAI-1	Biotinylated Human uPA	Streptavidin -PE	Inactive HuPAI-1

Fig. 1. Schematic diagram of solution phase PAI-1/uPA complex assay for the measurement of active human PAI-1.
Human uPA-Biotin was added to human plasma and was incubated in the presence of inhibitory anti-human PAI-1 mAb. The reaction mixtures were then incubated with microspheres coated with polyclonal anti-human PAI-1 capture antibody. All forms of PAI-1, including active PAI-1, latent PAI-1 and inactive PAI-1 that was inhibited by anti-human PAI-1 mAb were all captured. The excess uPA-biotin was washed off. The specific fluorescence signal of streptavidin-R-Phycoerythrin (PE) bound to the uPA-Biotin that was associated with human PAI-1 was detected to reflect the level of active PAI-1. The degree of inhibition by the anti -PAI-1 mAb in human plasma is calculated according to the decrease of the fluorescent signal. The background color of yellow in the vessel denotes reaction that contains materials derived from plasma which could interfere with the signal detection; the background color of green in the vessel denotes signal detection buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In the current study, we devised a "solution phase" PAI-1/uPA complex assay that reflects more faithfully the 33B8 *in vivo* PAI-1 inhibitory activity. We also reported the results of comparison of solution phase assay with the traditional solid phase assay for 31C9 and 33H1F7, two additional anti-PAI-1 mAbs with distinct mechanisms of inhibition.

2. Materials

Recombinant wild type human PAI-1 expressed in *E. coli* was prepared in-house. Other assay reagents were obtained from commercial sources: Glycosylated active form human PAI-1 (Molecular Innovations, GLYHPAI-A); Recombinant human HMW uPA (Molecular Innovations, UPA-HTC-INS); Biotinylated rabbit anti-human PAI-1 polyclonal antibody (Molecular Innovations, ASHPAI-GF-BIO); Biotin labeled human uPA (Molecular Innovations, UPA-HTC-BIO); Anti-human PAI-1 antibody coated MagPlex microspheres (EMD Millipore, HTPAI1-MAG); Inhibitory mouse monoclonal antibody to human PAI-1 clone 33B8 (Molecular Innovations, MA-33B8); Mouse monoclonal antibody to human PAI-1 clone 31C9 (Molecular Innovations, MA-31C9); Inhibitory mouse monoclonal antibody to human PAI-1 clone 33H1F7 (Molecular Innovations, MA-33H1F7); Region code # 76 MagPlex microspheres (Luminex, MC10076-01); xMAP Antibody Coupling Kit (Luminex, 40–50016); Streptavidin-R-Phycoerythrin (PE, Life Technologies, S866); Magpix Drive Fluid (Luminex, MPXDF); Human platelet poor plasma (3.2% Sodium Citrate as anti-coagulant, Bioreclamation,