ISSN : 0974 - 7435 Volume 8 Issue 3

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 8(3), 2013 [428-433]

Measuring site density of P-selectin on planar surface based on ^{125}I labeling

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ABSTRACT

Measuring the site density of P-selectin on planar substrate is the primary event in understanding the mechanism of P-selectin-induced cell adhesion on molecular level through flow chamber experiment. In order to overcome two major disadvantages of current measurement means: low signal noise ratio of fluorescence labels and the limitation of traditional gamma counter or scintillation counter, herein, a novel method based on combination of radioactively ^{125}I labels and Infinia Hawkeye 4 ECT is proposed to detect the low density of the P-selectin on planar surface. The results illustrate that site density of P-selectin is linear with their concentration, and glass plate shows better adsorption than polystyrene. For the adsorbed P-selectin on the plate at same concentration, the difference of radiation intensity between direct 125I-P-selectin and following bound by its antibody ^{125}I-9E1 suggests that the distribution of P-selectin head's orientation is random, with only about 10% P-selectins in their head up position. Comparing with the traditional method, this method is rapider, more efficient and can be widely applied to flow chamber experiment for further exploring cells rolling behavior under flows and obtaining a series of 2D molecular dynamic parameters.

KEYWORDS

P-selectin; site density; ^{125}I labeling; SPECT; Flow chamber.

INTRODUCTION

At sites of infection or noxious stimuli, circulating leukocytes adhere to endothelial cells and then migrate into tissues to destroy pathogens and repair tissue injury[1]. P-selectin, one of the three members in selectin family, binding with its ligand PSGL-1 (P-selectin glycoprotein ligand) induces the tethering and then rolling of neutrophils on the endothelial surface[2]. In general, parallel plate flow chamber (PPFC) is used to mimic the adhesion of circulating neutrophil and measure the 2D reaction kinetic parameters between adhesive molecules[3-5]. In the flow chamber experiment, cells are perfused through a planer substrate which is precoated with certain concentration of P-selectin. 2D reverse reaction rate can be calculated by measuring the bond lifetime through tethering experiment; however, the forward reaction rate is not only related to the binding affinity between receptor and ligand, but largely influenced by the concentration of receptor and ligand, cell con-

tact area, diffusion and etc**[5, 6]**. Thus, precisely measuring the site density of P-selectin on the planar substrate is the primary event in understanding the mechanism of selectin-induced cell adhesion on molecular level through flow chamber experiment.

The site density of PSGL-1 on cell surface can be measured by fluorescence or radiation labeling. It has been reported that the number of PSGL-1 molecules on neutrophil and HL-60 cell are 11, 0002, 000 and

18, 000 2, 000[7], and their site densities are 49 and 36 sites/μm2, respectively. However, P-selectins are used to coat on a planer glass coverslip before assembling into the flow chamber floor, and the site density of P-selectin is needed to be smaller than 10 sites/μm2 in the single bond tethering experiment[8]. The sensibility of fluorescence labeling is hard to fulfill this need; although the sensibility of 125I labeling is enough, the gamma counter used to measure the radiation of a single test tube is not suitable to measure the radiation on a planar.

INFINIA HAWKEYE 4 ECT is a type of multifunctional nuclear imaging equipment, it can complete 100% function of Single Photon Emission Computer Tomography (SPECT), and 90% function of Positron

Emission Tomography (PET) and computed tomography (CT), it carries out fusion positioning of ECT and CT imaging and radionuclide decay correction in whole energy range[9]. Moreover, it can measure multiple samples at a time, which saves a large amount of time. Therefore, after 125I labeling, SPECT was used to measure the site density of P-selectin on the planar. Using this method, the site densities of P-selectin physically adsorbed on glass and polystyrene (PS) were measured. It is found that the site density of P-selectin is linear with its adsorbent concentration, and the adsorbability of glass is slightly higher than PS. It has been proved that P-selectin molecules are randomly coated after physical adsorption, and only ~10% P-selectins have a head-up orientation that can easily bind with the cell surface PSGL-1.

EXPERIMENTAL

Labeling protein by IODOGEN

Human P-selectin/Fc chimera recombinant and mouse anti-human P-selectin mAb 9E1 were purchased from R&D system Inc., MN. In iodination reaction, dissolve 30 ~ 100 μg P-selectin (106 kDa) or 40 ~ 150 μg 9E1 (150 kDa) in 100 μl Tris iodination buffer (25 mM Tris-HCl, pH 7.5, 0.4 M NaCl). Wet the Pierce Pre-Coated Iodination Tube (Thermo Fisher Scientific Inc., IL) with 1 ml Tris iodination buffer and decant, add 100 μl high Tris buffer (0.125 M Tris-HCl, pH 6.8, 0.15 M NaCl) directly to the bottom of the tube, do not allow buffer to drain down the tube wall. Add 10 μl, 1.0 μCi , Na125I into the Iodination Tube and mix. Allow iodide to activate for 6 minutes at room temperature, swirl the tube every 30 seconds. Remove and add the activated iodide to the protein solution and mix, react for 6-9 minutes at room temperature, mix by gently flicking the tube every 30 seconds. Finally, add 50 μl scavenging buffer (10 mg tyrosine/ml in Tris iodination buffer), mix and incubate for 5 minutes with additional flicking at 1 and 4 minutes.

Purification of labeled protein

Before experiment, wash and equilibrate the 10 ml Sephadex G-25 column with 20 ml Tris buffer. Add the activated iodide into the column, and wash tube containing the iodinated protein with 0.5ml of Tris buffer and add this to the column to increase recovery of the iodinated protein. Collect 0.5 ~ 0.8 ml fraction in each polypropylene tube, and collect 25 tubes in total. The radiation intensity and the amount of protein are measured by SPECT and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., IL) respectively.

Measuring site density of P-selectin

We used both polystyrene (PS) and glass as adsorbent materials. Proteins are adsorbed into a Costar 48-well polystyrene plate, or glass coverslips fixed into a 6-well plate. 8 mm × 8 mm square hole is cut in each 2mm-thickness silica gel pad, and paste on the coverslip allowing the protein solution to adsorb into a certain area. Add 130 μl (48-well plate) or 102 μl (6-well plate) labeled or non-labeled P-selectin into the adsorbent area, incubate for 16 hrs at 4. Suck out the adsorbent solution, wash three times by Hank's balance solution (HBSS) containing 1% bovine serum albumin (BSA), and incubate for 1 hr at room temperature. Measure the radiation intensity by SPECT if P-selectin was labeled by 125I, otherwise such out the washing solution after incubation, and add 125I labeling P-selectin

FULL PAPER

antibody to incubate for 30 min at room temperature. Suck out solution and wash three times by the washing solution, then measure radiation intensity by SPECT. Take out the silica gel pad before measurement to avoid additional radiation intensity induced by silica adsorption.

Radioactive measurement of SPECT

Place samples on the detecting platform of GE Infinia Hawkeye 4 (Courtesy of GE Healthcare Inc.), and measure the radiation intensity of 125I. Detect 10 minutes for fractions collected from the Sephadex G-25 column, and detecting time for the site density samples can be adjusted by the actual radiation intensity, usually about 20 to 30 minutes. After detection, radiation quantity can be calculated by the software.

RESULTS

Labeling of P-selectin and P-selectin antibody

Human P-selectin or mouse anti-human P-selectin antibody 9E1 were labeled by Iodogen method and purified through Sephadex G-25 column, radiation intensity of collected fractions of P-selectin and P-selectin antibody are shown in Figure 1. 1A and 1B are the detected images shown on SPECT, 25 test tubes with collected fractions were measured in both pictures, and darkness of the image represents the intensity of radioactivity, the radiation counts of each tube are shown in 1C and 1D. One steep and another mild peak are both appeared in these two graphs. The first one is the labeled protein peak that appeared in tubes between number 3 to 6, and the second one is the peak of unbound 125I. There is a clear boundary between these two peaks, which implies a successful purification of the labeled protein. Furthermore, the first peak much higher than the second one meaning that most of 125I are labeled on the target protein, and only small fraction of 125I are remained unbound.

P-selectin adsorbed on glass and polystyrene

After purification, the amount of 125I labeled protein was quantified by Pierce BCA Protein Assay Kit, and radiation counts of single protein molecule were calculated. In our experiment, we collected 5 ml 125I labeled P-selectin, the total radiation counts were 63.61 per second, and the amount of P-selectin was measured to be 1.003 μg/ml. According to the M.W. of 106 kDa for the P-selectin/Fc chimera, the radiation counts of a single 125I labeled P-selectin are calculated to be 2.23 ×10-12 per second.

Gradient diluted the 125I labeling P-selectin in a series of concentrations (0, 8.3, 33.3, 83.3, 125 ng/ml), and added onto the 8 mm× 8 mm glass surface, incubated 16 hrs at 4. After washing three times by 1% BSA in HBSS, radiations were detected on SPECT. The radiation image detected for 20 min by SPECT is shown in Figure 2A, the adsorbability of 125I-P-selectin was increased as the concentration increased, showing a linear relationship (as in 2C). In the flow chamber

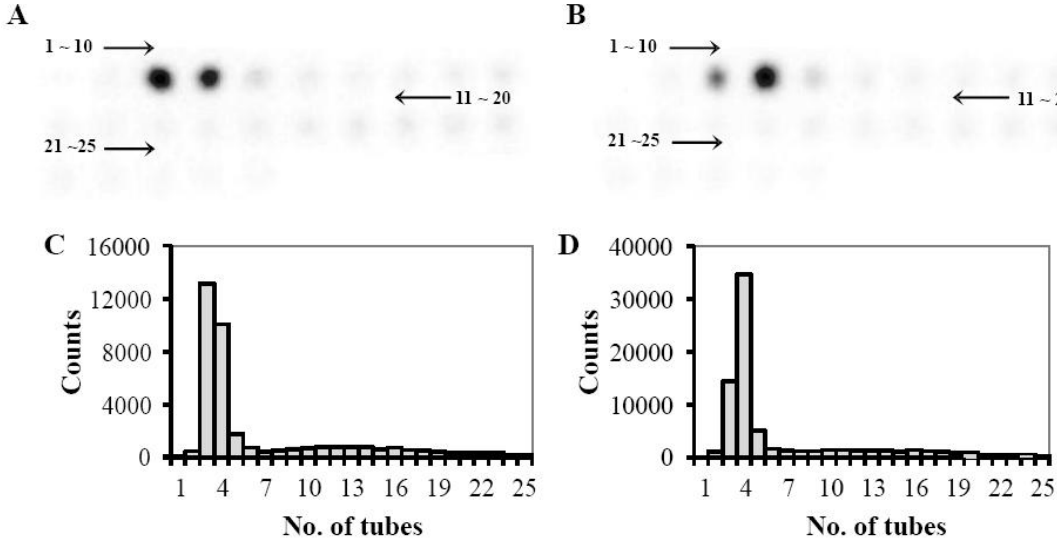

Figure 1 : ^{125}I-labeled P-selectin or P-selectin antibody purified by Sephadex G-25

experiment, the concentrations of P-selectin for HL-60 cells to tether or rolling on are 30ng/ml and 500ng/ml respectively. According to the relationship between P-selectin concentration and the site density (shown in 2E), the site density of P-selectin for HL-60 cells to tether on is 99 sites/μm2, and the site density to support rolling is 1662 sites/μm2.

measuring by the two-factor analysis of variance ($F > F0.01$).

Measure P-selectin site density by labeling P-selectin antibody

It is known that P-selectin bind with its ligand PSGL-1 by interaction in its lectin domain which is located on its head piece in N-terminal[10]. Therefore, in the flow chamber experiment, the actual site density of effective P-selectin is needed in calculating the kinetic parameters of molecular reaction. However, P-selectin is randomly adsorbed after physical adsorption, only a small number of P-selectins are adsorbed in a right

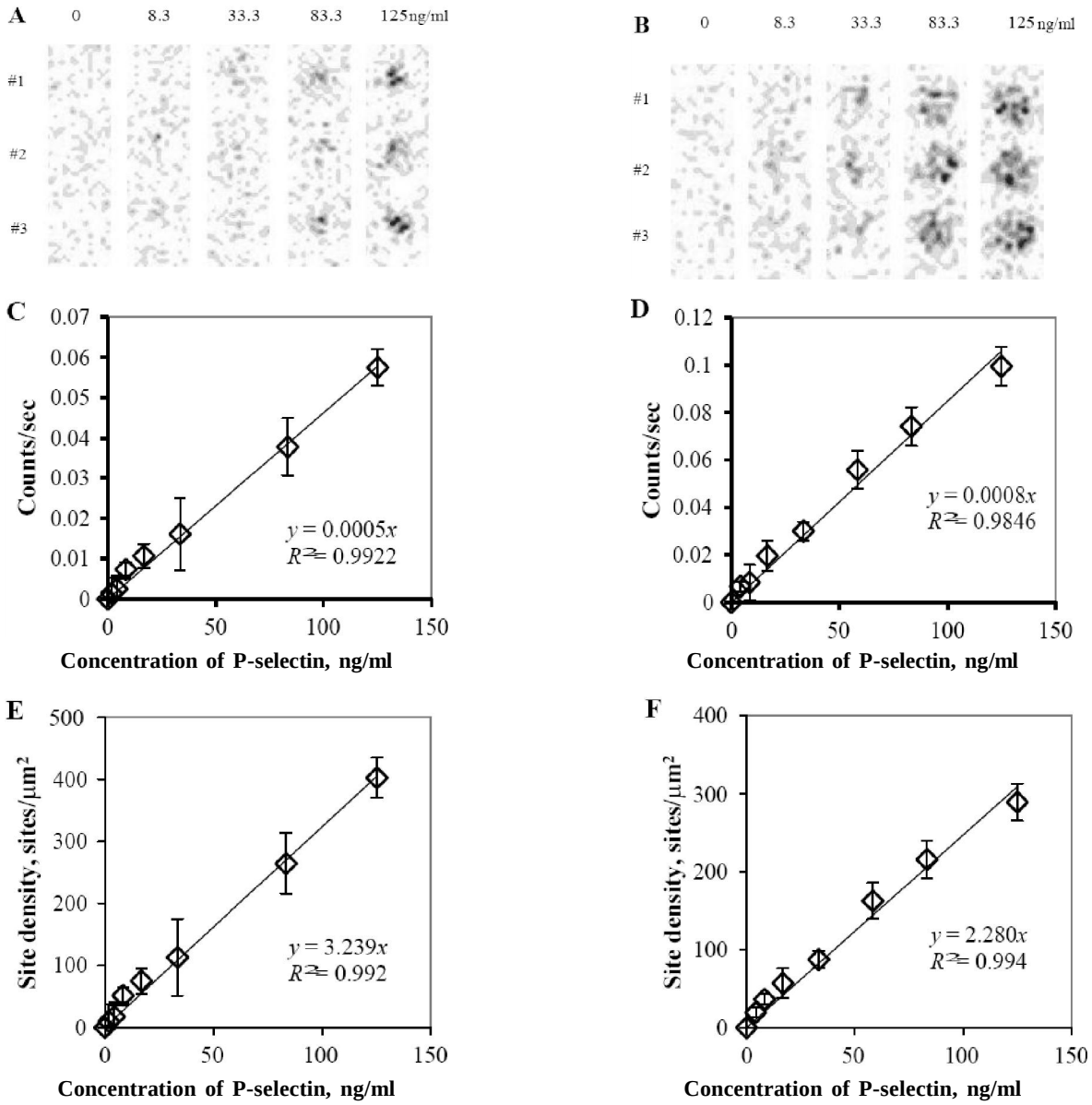

Figure 2 : Site density of P-selectin adsorbed on planar glass or PS surface

Other than glass material, we also measured the site densities of P-selectin adsorbed on polystyrene, as shown in 2B, 2D and 2F. The site densities of HL-60 cells tether to or rolling on were 69 sites/μm2 and 1140 sites/μm2, respectively, which were lower than the value measured on glass, and the difference is distinct when

FULL PAPER

orientation, in other words is in its head-up position, with its binding site exposed. The above results of P-selectin site densities were measured by P-selectin labeling, thus all kinds of orientations are included in the above results. In order to measure the actual site density of effective P-selectin, monoclonal antibody for the extracellular region of P-selectin was labeled, then radioactivity was detected after binding.

Antibody 9E1 was labeled by 125I using Iodogen method, the amount of purified labeling protein was quantified by Pierce BCA Protein Assay Kit, and radiation counts were detected in SPECT. In our experiment, we collected 125I labeling 9E1 in a volume of 6.2 ml, the total radiation counts were 138.53 per second, and the concentration of 9E1 was measured to be 16.625 μg/ml. According to the M.W. of ~ 150 kDa for IgG, the radiation counts of a single 125I labeled 9E1 are calculated to be 2.07 ×10-12 per second. P-selectin was diluted in a series of concentrations (0, 8.3, 33.3, 83.3, 125 ng/ml), and was added onto the 8 mm×8 mm glass surface, incubated 16 hrs at 4. After washing three times by 1% BSA in HBSS, 125I labeled 9E1 was added to bind with P-selectin, incubated for 30 min at room temperature. Radioactivity was detected in SPECT after washing another three times by 1% BSA in HBSS. Figure 3 shows the radioactive image detected by SPECT, it is suggested that binding of 125I-9E1 increased as the P-selectin concentration increased, radiation counts and site density were both in linear with the P-selectin concentration (as shown in 3B and 3C). Site densities for HL-60 cells to tether (30ng/ml) or rolling (500ng/ml) on P-selectin are 9 and 148 sites/μm2 respectively. These two values are much lower than the site densities detected by direct labeling of P-selectin (99 sites/μm2 and 1662 sites/μm2). Lee D. *et. al.*[11] used a CRD sequence mAb and fluorescence labeling to detect the site density of P-selectin adsorbed by different methods. It was found that the site density results were in relevant with the molecule orientations. Therefore, it is suggested in our results that P-selectin has a random orientation after physical adsorption, and only about 10% are in its

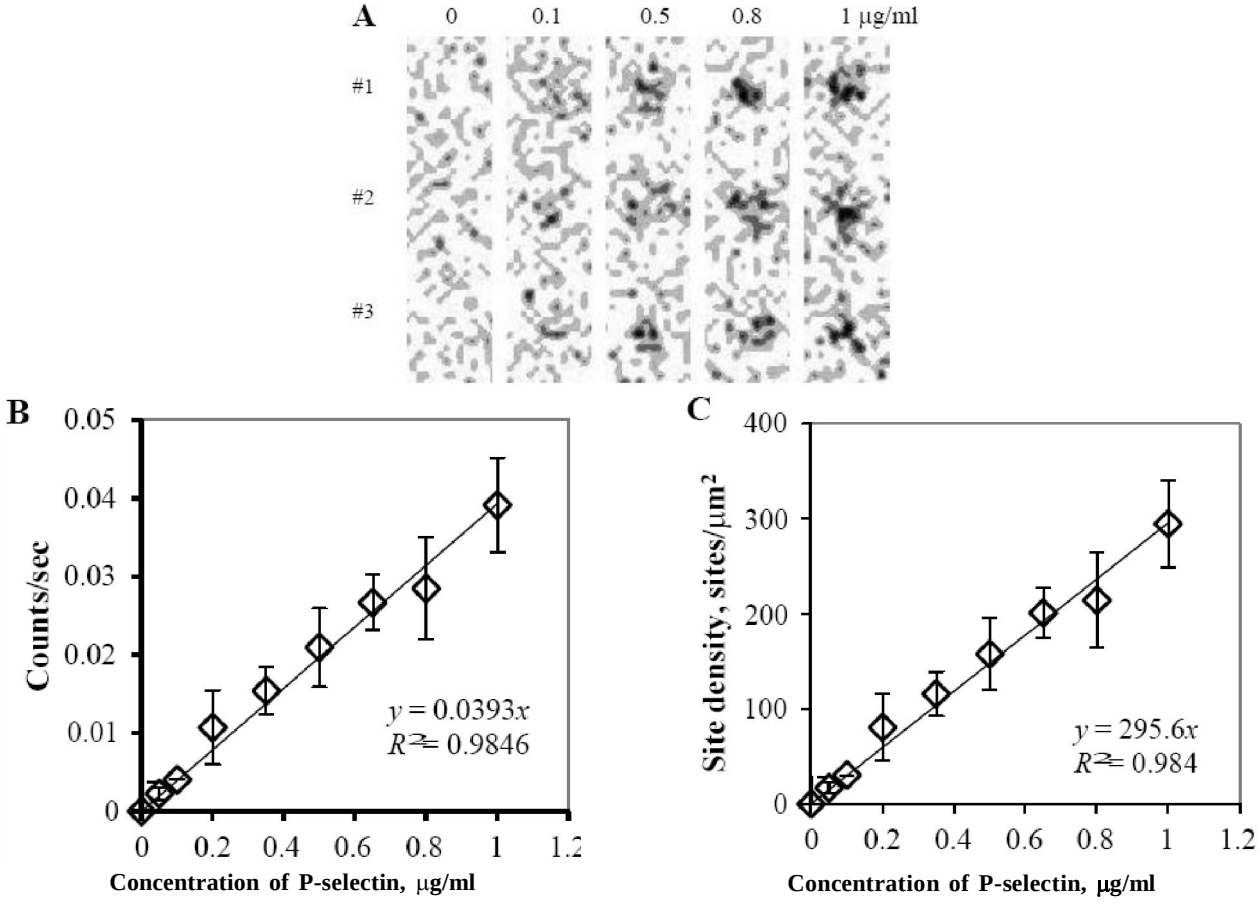

Figure 3 : Measuring the site density of P-selectin on planner glass by ^{125}I-labeled antibody

head-up position, with its binding site exposed.

DISCUSSION

In compare with the traditional gamma counter that can only detect radioactivity of solutions inside test tubes, SPECT not only can detect radioactivity on planar, but also can deal with multiple samples at a time. As shown in the above results, site density of P-selectin is linear with the adsorbing concentration, and glass has a higher adsorbability than polystyrene. It has been suggested in other paper that P-selectin is randomly oriented on planar after physically absorption[11]. We labeled P-selectin or its antibody 9E1 with 125I, and site density detected by 125I-P-selectin is about 10 times higher than 125I-9E1, which suggested that after physically absorption, only about 10% P-selectins are in its head up position, with binding site exposed.

In flow chamber experiment, it is useful to obtain the site density of protein on the flow chamber floor to calculate some of the molecular kinetic parameters. For example, when measuring forward reaction rate *kon* between PSGL-1 on HL-60 cell and P-selectin on the planar by flow chamber experiment, the site density we measured above must be used. According to equation[12], *kon* can be calculated. Here, *Pad* is probability for a cell to tether before it flows a distance, which approximates to the ratio of tether rate and length of the view; *mr* is the site density of P-selectin on the planar floor, which is 9 sites/μm2 when measuring by the above method; *ml* is the site density of PSGL-1 on HL-60 cell, it is reported to be 36 sites/ μm2[7]; *Ac*0*tc*0 are cell contact area, contact time and collision frequency, which can be obtained by the side view flow chamber[13]; *V* is the near wall flowing velocity of HL-60 cell.

In summary, the new method using 125I labeling and SPECT detection to measure the site density of protein adsorbed on a planar is feasible, as shown in the above results. Compared with the traditional method by using gamma counter, this method works more efficiently, and redeems the defect that planar cannot be detected. The results measured by using this new method can be combined with the flow chamber experiment to obtain a series of 2D molecular dynamic parameters, which may provide some help in understanding of the mechanisms of cell rolling adhesion related disease, such as inflammation, thrombus, and etc.

ACKNOWLEDGEMENT

This work was supported by National Natural Science Foundation of China (NSFC) grant 11272125 (YF) and China Postdoctoral Science Foundation (CPSF) grant 2013M542180 (YL).

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