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Disulfide bond exchanges in integrins αIIbβ3 and αvβ3 are required for activation and post-ligation signaling during clot retraction☆

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ABSTRACT

Background: Integrin αIIbβ3 mediates platelet adhesion, aggregation and fibrin clot retraction. These processes require activation of αIIbβ3 and post-ligation signaling. Disulfide bond exchanges are involved in αIIbβ3 and αvβ3 activation.
Methods: In order to investigate the role of integrin activation and disulfide bond exchange during αIIbβ3- and αvβ3-mediated clot retraction, we co-expressed in baby hamster kidney cells wild-type (WT) human αIIb and WT or mutated human β3 that contain single or double cysteine substitutions disrupting C523-C544 or C560-C583 bonds. Flow cytometry was used to measure surface expression and activation state of the integrins. Time-course of fibrin clot retraction was examined.
Results: Cells expressed WT or mutated human αIIbβ3 as well as chimeric hamster/human αvβ3. The αIIbβ3 mutants were constitutively active and the thiol blocker dithiobisnitrobenzoic acid (DTNB) did not affect their activation state. WT cells retracted the clot and addition of αvβ3 inhibitors decreased the retraction rate. The active mutants and WT cells activated by anti-LIBS6 antibody retracted the clot faster than untreated WT cells, particularly in the presence of αvβ3 inhibitor. DTNB substantially inhibited clot retraction by WT or double C523S/C544S mutant expressing cells, but minimally affected single C523S, C544S or C560S mutants. Anti-LIBS6-enhanced clot retraction was significantly inhibited by DTNB when added prior to anti-LIBS6.
Conclusions: Both αIIbβ3 and αvβ3 contribute to clot retraction without prior activation of the integrins. Activation of αIIbβ3, but not of αvβ3 enhances clot retraction. Both αIIbβ3 activation and post-ligation signaling during clot retraction require disulfide bond exchange.

Introduction

Following vascular injury, the exposed subendothelial matrix activates platelets and initiates, along with coagulation mechanisms, the production of fibrin from fibrinogen [1]. Platelet activation induces inside-out signals that activate integrin αIIbβ3 resulting in ligand binding to its large globular head. Engagement of αIIbβ3 by fibrinogen or fibrin generates outside-in signals that mediate cytoskeleton rearrangement which is required for platelet spreading and fibrin clot retraction. Clot retraction enhances thrombus stability and wound healing by compressing the clot and drawing the edges of the wound together [2,3]. Integrin αIIbβ3 mediates clot retraction by transmitting contractile forces from the actin-myosin cytoskeleton to the fibrin polymers that are tethered outside the cells. Interestingly, several studies reported that αIIbβ3 expressed in nucleated cells such as Chinese Hamster Ovary (CHO) cells and M21 melanoma cell lines must be activated in order to mediate fibrin clot retraction and integrin activation by talin is required for platelet-mediated fibrin clot retraction [4,5]. Other studies indicated that αIIbβ3 expressed in mammalian cell lines was able to mediate fibrin clot retraction without prior activation of the integrin [6–9].

The vitronectin receptor, αvβ3, is another member of the β3 integrin subfamily. In contrast to αIIbβ3 which is restricted to platelets and megakaryocytes, αvβ3 is widely expressed in various cell lines including endothelial cells and platelets. αvβ3 was also shown to mediate fibrin clot retraction when expressed in mammalian cell lines [4,10–12].

Several studies report that activation of αIIbβ3 is induced by a cascade of multiple disulfide bond exchanges in the β3 subunit [13–15]. Although the precise disulfide bonds that participate in this disulfide bond shuffling have not been identified, they were shown to be located at a cysteine rich region of β3 comprising four epidermal growth factor (EGF)-like domains [13,14]. Each EGF-like domain contains 3–4

Abbreviations: BHK, baby hamster kidney; CHO, chinese hamster ovary; DMEM, dulbecco modified Eagle's medium; DTNB, dithiobisnitrobenzoic acid; PBS, phosphate-buffered saline; pCMBS, 4-(chloromercuri) benzenesulfonic acid; PDI, protein disulfide isomerase.

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disulfide bonds, of which one bond is unique for integrins and is not conserved in other EGF domains [16–19]. We showed that disruptions of the unique disulfide bonds in the EGF domains of the β3 subunit resulted in constitutively active αIIbβ3 and αvβ3 expressed in Baby Hamster Kidney (BHK) cells and that these bonds play a primary functional role in αIIbβ3 and αvβ3 activation involving disulfide bond exchange [20,21]. We also showed that disulfide bond exchange is necessary for αIIbβ3 post-ligation signaling events including sustained fibrinogen binding to the integrin and adhesion to immobilized fibrinogen [22,23].

In the current study, we investigated the role of integrin activation and disulfide bond exchange in αIIbβ3-mediated fibrin-clot retraction by using BHK cells expressing WT αIIbβ3 or αIIbβ3 harboring cysteine substitutions in the β3 subunit. Since transfected BHK cells also express chimeric hamster/human αvβ3 receptors, we also investigated the relative contribution of αvβ3 to clot retraction. We found that disulfide bond exchanges are involved in both receptor activation and post-ligation signaling during β3 integrin-mediated clot retraction.

Materials and Methods

Reagents and Antibodies

Dulbecco modified Eagle's medium (DMEM), L-glutamine and fetal calf serum were purchased from Biological Industries (Beit-Haemek, Israel), lipofectamine reagent and G418 were from Gibco BRL (Paisley, UK), hygromycin was from Roche Diagnosis Gmbh (Mannheim, Germany), human fibrinogen, ε-amino-n-caproic acid and the membrane impermeant free thiols blockers, dithiobisnitrobenzoic acid (DTNB) and 4-(chloromercuri) benzenesulfonic acid (pCMBS) were from Sigma (St Louis, MO) and Toronto Research Chemicals (Toronto, Canada), respectively. Bovine thrombin was purchased from Instrumentation Laboratory, (MA, USA). FITC-conjugated monoclonal antibody P2 against αIIbß3 was from Immunotech (Marseille, France). The monoclonal antibody LM609 against αvß3 was from Millipore (Temecula, California) and the FITC-conjugated monoclonal antibody 23C6 against αvß3 was from eBioscience (San Diego, CA). FITC-conjugated fibrinogen-mimetic murine monoclonal antibody PAC-1 was obtained from BD Biosciences (San Jose, CA). The αvß3 inhibitor RO0655233-001 was from Roche Applied Science (Basel, Switzerland). The activating monoclonal antibody anti-LIBS6 was kindly provided by Dr. Mark Ginsberg (Department of Medicine, University of California, San Diego, La Jolla, CA).

Construction of Expression Vectors for Wild Type (WT) and Mutant cDNAs

cDNAs of human αIIb and ß3 in pCDNA3 vector were kindly provided by Dr. Peter Newman from the Blood Center of Wisconsin, Milwaukee, WI. cDNA of αIIb was subcloned into the PvuII site of pCEP4 mammalian expression vector carrying the hygromycin resistance gene as a selection marker (Invitrogen, San Diego, CA) as previously described [24]. Substitutions of selected cysteine residues by serine or other residues were created in the pCDNA3/ß3 vector using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using two overlapping oligonucleotide primers containing single base pair substitution (the primers are available upon request). Correct incorporation of the mutations into the pCDNA3/ß3 vectors was verified by DNA sequencing. For creation of double mutants containing two cysteine substitutions together, we first introduced one mutation into normal pCDNA3/ß3 vector and then used the mutant pCDNA3/ß3 clone as a template for introducing the second mutation.

Co-Transfection of αIIb and ß3 cDNAs

Baby Hamster Kidney (BHK) cells were grown in DMEM supplemented by 2 mg/ml L-glutamine and 5% fetal calf serum. The cells were co-transfected with 1 μg of normal or mutated forms of pCDNA3/ß3 and 1 μg of normal pCEP4/αIIb using lipofectamine reagent. The transfected cells were grown in a selection medium containing 0.7 mg/ml G418 and 0.5 mg/ml hygromycin. Mock cells were produced by transfecting BHK cells with 1 μg of pCEP4 and 1 μg of pcDNA3 and were selected in the same medium.

Flow Cytometry Analysis of Transfected BHK Cells

Transfected BHK cells were harvested with phosphate-buffered saline (PBS) supplemented with 1 mM EDTA, pelleted and incubated in DMEM for 30 min at room temperature. Then, cells were pelleted again, resuspended in PBS supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (5×10^5 cells/100 μl) and incubated for 30 min at room temperature with either 20 μl FITC-conjugated P2 antibody, 5 μl FITC-conjugated 23C6 antibody or 20 μl FITC-conjugated PAC-1 antibody. To measure PAC-1 binding after activation of αIIbß3 by activating antibodies, 1 μl anti-LIBS6 was added to the cells suspended in PBS supplemented with 0.25 mM $MnCl_2$ (5×10^5 cells/100 μl) prior to the addition of FITC-conjugated PAC-1. The cells were then diluted to 5×10^5 cells/600 μl and analyzed for surface fluorescence by flow cytometery (Becton Dickinson, NJ). To measure ligand binding after blocking free thiols, we repeated these experiments in the presence of 2.5 mM DTNB. Baseline for nonspecific binding of the antibodies was measured in mock cells. PAC-1 binding to αIIbß3 was expressed as percent of αIIbβ3 expression level measured by P2 binding. The effects of anti-LIBS6 and DTNB were compared by two tailed paired *t*-test.

Fibrin Clot Retraction

Transfected BHK cells were harvested with PBS/1 mM EDTA, pelleted and incubated in DMEM for 30 min at room temperature. 3.5×10^6 cells were mixed with 250 μl DMEM supplemented with 25 mM Hepes, 5 mM ε-amino-n-caproic acid, 15 mM $CaCl_2$ and 0.5 units of bovine thrombin. The cells were placed in glass aggregometry cuvettes and 100 μg fibrinogen was added. The clots were allowed to retract at 37 °C and photographed after 1, 3, 5 and 18 hours. All experiments were performed in the presence or absence of 5 μM of the αvß3 inhibitor RO0655233-001 and/or 2.5 mM DTNB that were added 5 min prior to the addition of thrombin. In some experiments, 3 μl of anti-LIBS6 antibody was added to WT cells either with or without DTNB added 5 min before or after anti-LIBS6. Some experiments were also performed in the presence or absence of the anti-αvß3 blocking antibody LM609 at 1:50 dilution or in the presence or absence of 100 μM pCMBS. The images were processed by Adobe Photoshop CS software program to produce black and white images and were analyzed by the imageJ program to measure the percent of the retracted clot (black) from the whole clotting medium (black and white). At least 3 experiments for each WT and mutant were conducted. The differences between the mutated and WT cells and the effects of the αvß3 inhibitor and DTNB in each time point were analyzed by two tailed paired *t*-test. Since the initial retraction curves appeared to be linear until 3 to 5 hours, we also assessed the differences in the initial rate of clot retraction by the slope of the first 5 hours of the retraction using a linear regression analysis function of the GraphPad Prism 5 software program.

Results

Effect of Free Sulfhydryl Inhibitor (DTNB) on the Activation State of WT and Mutated αIIbß3

We previously showed that disruptions of the unique β3 EGF-3 disulfide bond C523-C544 by substituting either Cys523 or Cys544 for Serine (C523S or C544S, respectively) resulted in constitutively active αIIbβ3 and αvβ3. The double mutant C523S/C544S, in which