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ADAM17 cleaves CD16b (FcγRIIIb) in human neutrophils

Yue Wang [a,b], Jianming Wu [a], Robert Newton [c], Nooshin S. Bahaie [a], Chunmei Long [a], Bruce Walcheck [a,*]

[a] Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN 55108, USA
[b] Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China
[c] The Incyte Corporation, Wilmington, DE 19880, USA

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ABSTRACT

CD16b (FcγRIIIb) is exclusively expressed by human neutrophils and binds IgG in immune complexes. Cell surface CD16b undergoes efficient ectodomain shedding upon neutrophil activation and apoptosis. Indeed, soluble CD16b is present at high levels in the plasma of healthy individuals, which appears to be maintained by the daily turnover of apoptotic neutrophils. At this time, the principal protease responsible for CD16b shedding is not known. We show that CD16b plasma levels were significantly decreased in patients administered a selective inhibitor targeting the metalloproteases ADAM10 and ADAM17. Additional analysis with inhibitors selective for ADAM10 or ADAM17 revealed that only inhibition of ADAM17 significantly blocked the cleavage of CD16b following neutrophil activation and apoptosis. CD16b shedding by ADAM17 was further demonstrated using a unique ADAM17 function-blocking mAb and a cell-based ADAM17 reconstitution assay. Unlike human CD16, however, mouse CD16 did not undergo efficient ectodomain shedding upon neutrophil stimulation or apoptosis, indicating that this mechanism cannot be modeled in normal mice. Taken together, our findings are the first to directly demonstrate that ADAM17 cleaves CD16 in human leukocytes.

1. Introduction

The human IgG Fc receptor CD16 (FcγRIII) consists of two isoforms (CD16a/FcγRIIIa and CD16b/FcγRIIIb) that are encoded by two highly homologous genes [1]. However, CD16a is a membrane-spanning protein, whereas CD16b is linked to the plasma membrane via a GPI anchor [2,3]. CD16b is expressed only by neutrophils and it primarily recognizes IgG-containing immune complexes, providing an important link between innate and adaptive immunity. Immune complexes can also promote excessive neutrophil activation that results in the release of high quantities of cytolytic and pro-inflammatory factors leading to extensive tissue injury, as is the case for neutrophils infiltrating synovial tissues during rheumatoid arthritis [4].

The surface density of CD16b is rapidly modulated by a complex interplay between mobilization from intracellular stores and proteolytic release [5]. CD16b proteolysis occurs upon neutrophil activation and apoptosis [6–8], and the maintenance of soluble CD16b in the plasma of healthy individuals indicates that its cleavage is a physiological process [6]. Serine proteases and metalloproteases have been implicated in CD16b proteolysis [8–10]; however, the primary enzyme involved in generating plasma CD16b has yet to be defined. This represents a critical step for understanding how CD16b cleavage is regulated and for identifying potential therapeutic targets in inflammatory diseases.

Several members of the ADAM[1] (a disintegrin and metalloprotease) family of membrane-associated proteases facilitate ectodomain shedding of cell surface proteins [11]. The family member ADAM17 is a well described sheddase, which is expressed in resting neutrophils and its enzymatic activity is rapidly induced upon neutrophil activation and apoptosis [12–15]. Using highly selective small molecule inhibitors, a unique ADAM17 function blocking mAb, and a cell-based ADAM17 reconstitution assay, we provide the first direct evidence that ADAM17 is a sheddase of CD16b in neutrophils.

2. Materials and methods

2.1. Human subjects and animals

The indicated patients from study INCB7839-202 (ClinicalTrials.gov Identifier: NCT00864175) were orally administered the selective ADAM10 and ADAM17 inhibitor INCB7839 (Incyte Corporation, Wilmington, DE) at 300 mg twice daily for 28 days with trastuzumab and docetaxel, and plasma samples were collected. These procedures and peripheral blood collection from normal individuals were performed in accordance with protocols approved by the Institutional Review Board at the University of Minnesota and the Incyte Corporation. Bone marrow neutrophils were isolated from wild-type C57BL/6J mice and FcγRIIB-deficient mice (C57BL/6J) (Taconic, Germantown, NY) in

Abbreviations: ADAM, a disintegrin and metalloprotease

* Corresponding author at: University of Minnesota, 295j AS/VM Bldg., 1988 Fitch Avenue, St. Paul, MN 55108, USA. Tel.: +1 612 624 2282; fax: +1 612 625 0204.
E-mail address: walch003@umn.edu (B. Walcheck).

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accordance with protocols approved by the Animal Care and Use Committee of the University of Minnesota.

2.2. Cell isolation and treatment

Human peripheral blood neutrophils and mouse bone marrow neutrophils were isolated as previously described [15,16]. NK cells were purified using a human NK cell isolation kit (Miltenyi Biotec, Auburn, CA), as per the manufacturer's instructions, resulting in >90% enrichment of $CD56^+CD3^-$ lymphocytes. Cell activation with PMA (10 ng/ml; Sigma, St. Louis, MO), formyl peptide receptor-like 1 agonist (1 μg/ml; Sigma), human TNFα (5 μg/ml; PeproTech Inc, Rocky Hill, NJ) or induction of apoptosis with anti-human Fas mAb CH-11 (500 ng/ml) was performed as previously described [15,17]. Mouse neutrophil activation was induced by formyl peptide receptor-like 1 agonist or LPS from *E. coli* O111:B4 (100 μg/ml; Sigma). Mouse neutrophil apoptosis was induced by mouse TNFα (20 ng/ml; PeproTech) and cycloheximide (35 μM), which reproducibly induces apoptosis [18–21]. Mouse TNFα was initially tittered down to a concentration that caused nominal neutrophil activation during the timeframe of the assay, as we have previously reported [17]. Some cells were pre-incubated for 30 min with the broad-spectrum metalloprotease inhibitor TAPI-I (Peptides International, Louisville, KY) at 50 μM, the selective ADAM17 specific inhibitors SP26 [22] (MERCK, Whitehouse Station, NJ) at 5 μM and BMS566394 referred to as inhibitor 32 in Ref. [23] (Bristol-Myers Squibb Company, Princeton, NJ) at 5 μM, the selective ADAM10 inhibitor GI254023X (kindly provided by Dr. Andreas Ludwig, Rhein-Westphalian Technical University, Aachen, Germany) at 0.5 μM, which is 10-fold selective for ADAM10 over ADAM17 in cellular assays [24], the anti-human ADAM17 function blocking mAb D1(A12) at 50 nM (kindly provided by Dr. Gillian Murphy, University of Cambridge, Cambridge, United Kingdom), or isotype-matched negative control antibody.

The EC2 fibroblast cell line derived from ADAM17-deficient mouse embryos has been previously described [14,25,26]. The two allelic forms of CD16b (NA1 and NA2) were amplified from human neutrophil cDNA, cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), and expressed in a stable manner in EC2 cells using described procedures [14,26]. The EC2 cells were then reconstituted with wild-type mouse ADAM17 using a bicistronic retroviral vector co-expressing eGFP, as previously described [14,26]. Apoptosis was induced by UV irradiation using a UV-C light source at a dosage of 60 mJ/cm^2, followed by incubation at 37 °C in 5% CO_2 for 2 h.

2.3. Flow cytometry

Flow cytometric analyses were performed on a FACSCanto instrument (BD Biosciences), as described [15,16]. Human CD16 was detected by the mAb 3G8 (Biolegend). The mAb 196001 (R&D Systems, Minneapolis, MN) detects mouse CD16 but not FcγRIV, and the mAb 2.4G2 (Santa Cruz Biotech, Santa Cruz, CA) detects mouse FcγRIIB, CD16, and FcγRIV [27]. Mouse L-selectin was detected with Mel-14 (eBioscience, San Diego, CA). Externalized phosphatidylinositol on apoptotic cells was detected by fluorochrome-conjugated annexin-V, as per the manufacturer's instructions (BD Biosciences, San Jose, CA).

2.4. SDS-PAGE and immunoblotting

Western blotting was performed as previously described [14,15]. Human CD16 was detected by the mAb DJ130c (Santa Cruz Biotech, Santa Cruz, CA), mouse and human caspase-3 was detected by antibody #9662 (Cell Signaling, Beverly, MA), and mouse GAPDH was detected by antibody G9545 (Sigma).

2.5. Cytometric bead assay

A well established, commercially available human CD16 ELISA is not currently available. We developed a quantitative immunosorbent assay using cytometric functional beads A8 and A5 (BD Biosciences) conjugated with the anti-CD16 mAb 3G8 and an IgG1 isotype-matched negative control antibody, respectively, as per the manufacturer's instructions. A multiplexed quantitative cytometric bead assay was performed by flow cytometry, as previously described with some modifications [15]. Briefly, a suspension of A8 and A5 beads was incubated with supernatants from treated neutrophils or with human plasma diluted by 2-fold serial dilutions, followed by PE-conjugated anti-human CD16 mAb DJ130c (10 μg/ml). DJ130c detects an epitope distinct from 3G8 [28]. Soluble CD16 concentrations were determined from a standard curve obtained from serial dilutions of recombinant human CD16b containing BSA (R&D Systems).

3. Results and discussion

3.1. Effect of an ADAM inhibitor on plasma CD16 levels

INCB3619 is a potent and selective inhibitor that targets both ADAM10 and ADAM17 when compared with a panel of matrix metalloproteases and ADAM family members [29,30]. The second-generation inhibitor INCB7839, which has a specificity profile identical to INCB3619 [31], has been examined in clinical trials in HER2-positive metastatic breast cancer patients, and found to cause a marked reduction in plasma levels of the ADAM product, soluble HER2 [32]. Using clinical samples from those studies, we assessed the plasma levels of soluble CD16 pre- and 28 days post-treatment with INCB7839. As shown in Fig. 1, CD16 plasma levels were significantly reduced in patients following INCB7839 treatment, with the highest level of reduction being 67%. These data suggest that human plasma CD16 levels are regulated by ADAM10 and/or ADAM17 activity.

3.2. Effects of selective ADAM10 and ADAM17 inhibitors on CD16 shedding

We next examined the effects of other hydroxamate-based metalloproteinase inhibitors on CD16 shedding, which differ in their selectivity for ADAM10 and ADAM17. GI254023X is an ADAM10 inhibitor that blocks ADAM10, but not ADAM17, in cells at a concentration ranging from 0.2 to 1 μM [33,34]. Using GI254023X within this concentration range, we found the inhibitor had little to no effect on the down-regulation of CD16b surface expression upon neutrophil treatment with PMA, a cell activator that induces robust CD16b shedding [8,9,35,36]. SP26 is a highly potent inhibitor as well, but with a selectivity more than 10,000-fold greater for ADAM17 than ADAM10 [22]. SP26 selectivity has been assessed in cellular assays and *in vivo* [33]. SP26 markedly attenuated CD16b down-regulation from PMA-activated neutrophils (Fig. 2A). BMS566394 is another highly selective ADAM17 inhibitor, again with a potency orders of

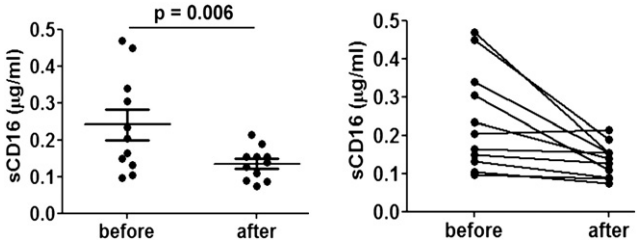

Fig. 1. Role of ADAM proteases in the homeostatic maintenance of CD16 plasma levels. Plasma levels of CD16 from 11 individuals before and after treatment with INCB7839 were quantified by ELISA. Shown in the left plot is the mean ± SD. In the right plot, patient CD16 plasma levels were compared before and after treatment.