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Varied levels of reactivity by different E-selectin/Fc constructs with cutaneous lymphocyte-associated antigen (CLA)⁺ CD4⁺ T cells

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SUMMARY

T cells utilize the vascular adhesion molecule E-selectin to enter inflamed skin. T lymphocytes identified by the mAb HECA-452 [cutaneous lymphocyte-associated antigen (CLA) T cells] are enriched in E-selectin ligand expressing cells. However, the proportion of CLA⁺ T cells reactive with an E-selectin/Fc chimeric construct, as determined by flow cytometry, can vary considerably between studies. One important variable in these studies has been the E-selectin/Fc chimeric used to assess ligand expression. We therefore compared the reactivity of mouse, rat, and human E-selectin/Fc from the same widely used commercial source with peripheral blood CLA⁺ CD4⁺ T cells, neutrophils, and the promyelocytic cell line HL-60 by flow cytometry and by shear flow assays. We observed that the binding activities of the different E-selectin/Fc chimeras were considerably different. Mouse E-selectin/Fc demonstrated the highest binding activity with human neutrophils and HL-60 cells by both assay approaches, whereas human E-selectin/Fc demonstrated the lowest binding activity. In addition, mouse E-selectin/Fc binding increased essentially in a linear manner with increasing expression of CLA by CD4⁺ T cells, whereas human and rat E-selectin/Fc binding occurred with only a subset of CLA⁺ CD4⁺ T cells. We conclude that there is substantial variability in the reactivity of different E-selectin/Fc constructs, thus caution should be used when assessing E-selectin ligand expression with these reagents. For instance, the discordance in expression of CLA and E-selectin ligands by T cells may in part be due to the E-selectin/Fc construct being used.

Keywords

Inflammation; T cells; adhesion molecules

INTRODUCTION

Effector/memory T cells display selectivity in their lymphoid and extralymphoid tissue trafficking. For instance, the vast majority of T cells in normal skin and at sites of cutaneous inflammation, express the cutaneous lymphocyte-associated antigen (CLA). CLA⁺ T cells are important for flexible immunosurveillance in the skin, but have also been implicated in many skin diseases [1].

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CLA⁺ T cells utilize the endothelial cell-expressed adhesion molecules E-selectin and P-selectin to initiate their accumulation along the microvasculature [2]. These selectins are Ca²⁺-dependent, selective lectins that recognize sialylated and fucosylated oligosaccharides [e.g. sialyl-Lewis x (sLe^X)] on discrete macromolecules [2]. CLA⁺ T cells are detected most notably by the mAb HECA-452, which binds glycans such as sLe^X [3–5]. CLA⁺ T cells can be stained by soluble recombinant E-selectin-immunoglobulin chimeric proteins, which are comprised of the extracellular region of E-selectin and the Fc region of an immunoglobulin (E-selectin/Fc) [6–9]. Of interest is that the proportion of CLA⁺ CD4⁺ T cells stained by E-selectin/Fc can vary a great deal between studies. For instance, some have reported a nearly precise correlation between E-selectin/Fc reactivity and HECA-452 staining [6,9], whereas others have shown E-selectin/Fc reactivity to occur with only a subset of CLA⁺ CD4⁺ T cells [7,8]. One important difference in these studies is the E-selectin/Fc construct being used to assess E-selectin ligand expression. Therefore, we compared the reactivity of mouse, rat, and human E-selectin/Fc from the same widely used commercial source with peripheral blood CLA⁺ CD4⁺ T cells, neutrophils, and the promyelocytic cell line HL-60 by flow cytometry and by shear flow assays. We report considerable variability in the binding activity by these constructs.

MATERIALS AND METHODS

Reagents

HECA-452 conjugated to biotin was purchased from BD Pharmingen (San Diego, CA). CD4-FITC was purchased from Ancell (Bayport, MN). Human, mouse, and rat E-selectin-human IgG Fc chimeric constructs and human and mouse P-selectin-human IgG Fc chimeric constructs were purchased from R&D systems (Minneapolis, MN). PE-conjugated F(ab')₂ goat anti-human IgG and allophycocyanin (APC)-conjugated streptavidin were purchased from Jackson ImmunoResearch (West Grove, PA). Mouse IgG and rat IgM isotype controls conjugated to FITC or biotin were purchased from Caltag (Burlingame, CA).

Cell isolation and culture

Peripheral blood was collected from normal donors in sodium heparin in accordance with an approved protocol by the Institutional Review Board: Human Subjects Committee at the University of Minnesota. Neutrophils (polymorphonuclear leukocytes) and mononuclear cells were isolated and cell viabilities assessed by exclusion of the vital dye trypan blue, as described previously [5,10]. HL-60 cells (ATCC, Manassas, VA) were maintained per the manufacturer's instructions.

Cell staining for flow cytometric analyses

To assess E-selectin ligands on human lymphocytes, neutrophils and HL-60 cells, the cells were incubated with 10 – 30 µg/ml of E-selectin/Fc at 4°C with gentle shaking for 30 min in the presence or absence of EDTA. Cells were then treated with PE-conjugated F(ab')₂ goat anti-human IgG. The lymphocytes were also sequentially treated with HECA-452-biotin, streptavidin-APC, and CD4-FITC. All cell staining steps were performed at 4°C with washing (PBS) between each step. Isotype-matched negative control mAbs were used to evaluate the levels of background staining. Cells were fixed in 1% paraformaldehyde and 5,000 – 50,000 cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA).

Hydrodynamic shear flow adhesion assay

Adhesion of HL-60 cells to E-selectin/Fc was assayed in a parallel plate flow chamber (Glycotech, Rockville, MD), as described previously [10].

RESULTS AND DISCUSSION

Different E-selectin/Fc constructs vary in their reactivity with CLA⁺ CD4⁺ T cells

We examined the reactivity of three different E-selectin-human IgG Fc chimeric constructs (mouse, rat, and human E-selectin/Fc) from the same commercial source with resting peripheral blood CLA⁺ CD4⁺ T cells by flow cytometry. These E-selectin/Fc constructs have been extensively used for leukocyte staining and typically at concentrations $\leq 10 \mu\text{g/ml}$ [7,8,10–12]. Representative profiles of CD4⁺ T cells dual stained with mouse, rat, or human E-selectin/Fc (10 $\mu\text{g/ml}$) and HECA-452 are shown in Fig. 1. We observed that mouse E-selectin/Fc staining increased linearly with increasing expression of CLA, whereas a smaller proportion of CLA⁺ CD4⁺ T cells demonstrated reactivity with both rat and human E-selectin/Fc (Fig. 1). The latter construct being the least reactive. When concentrations of rat or human E-selectin/Fc were increased up to 30 $\mu\text{g/ml}$, we observed only a marginal increase in their reactivity with CLA⁺ CD4⁺ T cells (Fig. 1). Staining by all three E-selectin/Fc constructs was diminished in the presence of EDTA (Fig. 1), demonstrating Ca²⁺-dependent binding.

The binding activity by different E-selectin/Fc constructs is consistent in flow cytometry and shear flow assays

The results above indicate considerable variability in the binding activities of the different E-selectin/Fc constructs tested with CLA⁺ CD4⁺ T cells. However, this is difficult to assess by staining lymphocytes, since these cells are heterogeneous in their expression of E-selectin ligands and they display diffuse staining by HECA-452, suggesting varied expression levels of sLe^X. Therefore, we examined the reactivity of mouse, human, and rat E-selectin/Fc with human peripheral blood neutrophils. These cells are quite homogeneous in their expression of E-selectin ligands, as well as P-selectin ligands, and are uniformly stained by HECA-452 [5]. We found that mouse E-selectin/Fc (10 $\mu\text{g/ml}$) stained these cells the brightest and in a uniform manner (Fig. 2A). Rat and human E-selectin staining levels were considerably lower even when used at 30 $\mu\text{g/ml}$, with human E-selectin/Fc demonstrating the lowest level of reactivity (Fig. 2A). In contrast to the different levels of staining by mouse and human E-selectin/Fc, mouse and human P-selectin/Fc stained neutrophils in an equivalent manner (Fig. 2B), suggesting that the species differences of the E-selectin constructs is not the cause of their dissimilar binding activities.

The late myeloblast cell line HL-60 also expresses E- and P-selectin ligands in a uniform manner [13]. We found that the levels of reactivity by mouse, human, and rat E-selectin/Fc with these cells were similar to neutrophils (Fig. 3A). Discrepancies in the binding activity of selectin/Fc constructs in a soluble form vs. an immobilized form have been reported [14]. Therefore, we further compared the binding activities of mouse and human E-selectin/Fc (the most dissimilar by flow cytometry) using an *in vitro* shear flow assay that allows for cellular analyses under hydrodynamic conditions simulating the microvasculature. The shear resistance of HL-60 cells was assessed on mouse or human E-selectin/Fc over a range of shear stresses (1 – 16 dynes/cm²). Upon incremental increases in shear stress, we noted that significantly greater numbers of HL-60 cells remained attached to mouse E-selectin/Fc compared to human E-selectin/Fc (Fig. 3B). Thus together, the above findings indicate distinct binding activities by the different E-selectin/Fc constructs, with mouse E-selectin/Fc demonstrating the highest level of reactivity.

In summary, we performed a direct comparison of three different E-selectin/Fc constructs from the same commercial source, which have been extensively used to assess E-selectin ligand expression by leukocytes [7,8,10–12]. For human neutrophils, HL-60 cells, and CLA⁺ CD4⁺ T cells, our findings demonstrate that the binding activities of the E-selectin chimeras tested to be mouse E-selectin/Fc > rat E-selectin/Fc > human E-selectin/Fc. CLA⁺ CD4⁺ T

lymphocytes are well recognized as being enriched in E-selectin binding cells. However, Takahashi et al. have reported that CLA expression by CD4⁺ T cells does not necessarily correspond with E-selectin binding because, in part, a considerable proportion of peripheral blood CLA⁺ CD4⁺ T cells were not found to be reactive with rat E-selectin/Fc [7]. We demonstrate a substantial discrepancy in the staining of peripheral blood CLA⁺ CD4⁺ T cells by mouse and rat E-selectin/Fc. Mouse E-selectin/Fc binding increased essentially in a linear manner with increasing expression of CLA, whereas rat E-selectin/Fc binding occurred by only a subset of CLA expressers. Hence, we conclude that the discordance in expression of CLA and E-selectin ligands by T cells may in part be due to the E-selectin/Fc construct being used.

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Figure 1. CLA⁺ CD4⁺ T cells demonstrate different levels of reactivity with human, rat, and mouse E-selectin/Fc

Resting peripheral blood lymphocytes were gated on CD4⁺ T cells and dual analyzed for their reactivity with various concentrations of human, rat or mouse E-selectin/Fc in the presence or absence of EDTA and HECA-452 staining, as described in the Materials and Methods. The first dot plot (upper left) indicates non-specific antibody labeling (GAH, goat anti-human; rIgM, rat IgM; APC, allophycocyanin). For all dot plots, the indicated Ab or chimera reactivities on the x and y axes represent Log 10 fluorescence. The numbers in each quadrant of the dot plots indicate percentage of cells. Data are representative of three independent experiments using T cells isolated from separate donors.

Figure 2. Neutrophils demonstrate different levels of reactivity with E-selectin/Fc chimeras, but not P-selectin/Fc chimeras

Resting peripheral blood neutrophils were analyzed for their reactivity with human, rat, and mouse E-selectin/Fc (A) or human and mouse P-selectin/Fc (B) in the presence or absence of EDTA. Neutrophils were identified by their characteristic forward and side light scatter characteristics. For all histograms, the indicated chimera reactivities on the x axes represent Log 10 fluorescence. Data are representative of three independent experiments using neutrophils isolated from separate donors.

Figure 3. Human and mouse E-selectin/Fc binding activities are different by flow cytometry and shear flow assays

(A) HL-60 cells were analyzed for their reactivity with human and mouse E-selectin/Fc in the presence or absence of EDTA. For all histograms, the indicated chimera reactivities on the x axes represent Log 10 fluorescence. (B) HL-60 cells were perfused into a flow chamber containing adsorbed human or mouse E-selectin/Fc (2 µg/mL), as indicated. Following a 1.5 min static period, the shear stress was incrementally increased. Gravity sedimented cells were enumerated prior to shear flow induction (shear stress = 0) and tethered cells were enumerated for each shear stress (100X magnification). Data are expressed as percent of cells prior to shear flow induction. Inclusion of 1mM EDTA completely blocked HL-60 cell tethering (data not shown). HL-60 cells were examined three times per experiment and data are indicated as mean $\pm$ SD (*, $p < 0.05$ vs. human E-selectin/Fc). Data are representative of three independent experiments using neutrophils isolated from separate donors.