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Heterogenous glycosylation of ICAM-3 and lack of interaction with Mac-1 and p150,95

Intercellular adhesion molecule (ICAM)-1, ICAM-2, and ICAM-3 have been identified as counter-receptors for the leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1). The other leukocyte integrins, Mac-1 and p150,95, also interact with ICAM-1. ICAM-1 and ICAM-3 are highly homologous, and an undefined ligand for Mac-1 is presnt on neutrophils where ICAM-3 is well expressed. In addition, glycosylation has been shown to affect the interaction of ICAM-1 with Mac-1. We therefore sought to characterize ICAM-3 heterogeneity and determine whether ICAM-3 was a ligand for either Mac-1 or p150,95. Despite extensive differences in N-linked glycosylation, ICAM-3 purified from lymphoid cells and from neutrophils supports adhesion of LFA-1-bearing cells equally well; however, neither supports adhesion of Mac-1 or p150,95-expressing chinese hamster ovary cell transfectants. Similarly, purified Mac-1 does not support adhesion of ICAM-2 or ICAM-3-expressing L cell transfectants. ICAM-3 on neutrophils does not participate in Mac-1-dependent homotypic aggregation. Thus, ICAM-3 is not a counter-receptor for either Mac-1 or p150,95.

1 Introduction

The leukocyte integrin family of adhesion molecules are critical for adhesive functions in the immune system [1, 2]. The three members of this family, lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) share a common β chain (CD18) that is noncovalently associated with homologous α (α_L, α_M, α_X) subunits. The three LFA-1 counter-receptors identified, intercellular adhesion molecule 1 (ICAM-1; CD54), ICAM-2 (CD102), and ICAM-3 (CD50), are all highly glycosylated type-I transmembrane proteins that belong to the immunoglobulin superfamily (IgSF). ICAM-1 [3, 3a] and ICAM-3 [4–6] contain five Ig-like domains, whereas ICAM-2 contains two Ig-like domains [7]. ICAM-1 has also been identified as a counter-receptor for other leukocyte integrins, namely Mac-1 [8–10], and possibly p150,95 [11]. The LFA-1 and Mac-1 binding sites on ICAM-1 are distinct, as they map to IgSF domains 1 and 3, respectively [10, 12]. Mac-1 interaction with ICAM-1 is weaker than the LFA-1/ICAM-1 interaction [9], and is affected by glycosylation, as removal of N-linked sugars from the third Ig-like domain of ICAM-1 increases adhesiveness to Mac-1 [10].

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ICAM-3 is a heavily glycosylated protein of M_r 124 000 that is well expressed on leukocytes, and absent on endothelium [13]. The high level of 52% amino acid identity between the extracellular domains of ICAM-1 and ICAM-3, which is comparable to the identity between ICAM-1 in the mouse and human, raised the possibility that ICAM-3, like ICAM-1, might bind to Mac-1. ICAM-1 and ICAM-3 are most highly conserved in domains 2 and the first half of domain 3. This region is 76% identical, whereas domain 3 is 52% identical overall. Since the LFA-1 binding site in the first Ig-like domain of ICAM-1 [12] and ICAM-3 [14] is functionally conserved, with only 38% sequence identity, conservation of the Mac-1 binding site in domain 3 is plausible. ICAM-3 is highly expressed on neutrophils, which are known to express an undefined ligand that binds to Mac-1 in chemoattractant-stimulated neutrophil aggregation [11, 15, 16]. In this report, we characterize ICAM-3 heterogeneity, and evaluate glycoforms of ICAM-3 on lymphoid cells and neutrophils as ligands for Mac-1 and p150,95.

2 Materials and methods

2.1 Monoclonal antibodies

mAb to ICAM-1, RR1/1 (IgG1) [17] and R 6.5 (IgG2a) [18]; ICAM-2, CBR-IC2/1 and CBR-IC2/2 (IgG2a) [19]; ICAM-3, CBR-IC3/1 (IgG1) [13] and CBR-IC3/2 (IgG2a) [20]; CD11a, TS1/22 and TS2/4 (IgG1) [21]; CD11b, OKM1 (IgG2a) [22] and LPM19c (IgG2a, a gift of Dr. K. Pulford, Oxford, GB) [23]; CD11c, CBRp150/4G1 (IgG2a) [24]; CD18, TS1/18 (IgG1) [21]; HLA, W6/32 [25]; and a myeloma protein, X63 (IgG1), have been described.

2.2 Cell culture

Peripheral blood lymphocytes (PBL) were obtained as described [19]. The T thymoma SKW3 cell line [19], the chinese hamster ovary (CHO) cell lines stably expressing ICAM-1 (domain 3–5 deleted, F185), Mac-1 or p150,95 [11], and the mouse L cells expressing human ICAM-1 [9]

and ICAM-3 [26] have been described. L cells expressing human ICAM-2 were generated as described [27], and maintained in identical selection media as ICAM-1- and ICAM-3-expressing L cells [9, 26].

2.3 Surface iodination

Surface labeling of cells with ^{125}I, preclearance of Triton X-100 (1%) lysates with bovine IgG-coupled-Sepharose, and immunoprecipitation with appropriate mAb-bound Sepharose, SDS-PAGE, and autoradiography were conducted as previously described [19].

Treatment of samples with N-glycanase (Genzyme Corp., Boston, MA) and O-glycanase (Genzyme) was carried out after washing the immunoprecipitates, and prior to elution in sample buffer. Antigen-complexed Sepharose beads were washed once and resuspended in N-glycanase buffer (100 mM NaH_2PO_4 pH 8.6, 10 mM DTT, 10 mM EDTA, 0.1% SDS, 1% Triton X-100) or O-glycanase-buffer (100 mM NaH_2PO_4 pH 6.0, 10 mM DTT, 10 mM EDTA, 1% Nonidet P-40). Samples were then treated for 18 h at 37 °C with either 2.5 U/ml of N-glycanase or 1 U/ml of O-glycanase. Sequential enzymatic digestions of immunoprecipitates were achieved through washing of digested immunoprecipitates with the second digestion buffer and incubation with the appropriate enzyme. Following digestion, beads were washed once in 50 mM Tris-HCl, pH 8.0, and subjected to SDS-PAGE and autoradiography as described [19].

2.4 Immunofluorescence flow cytometry

Indirect immunofluorescence and flow cytometry were performed as previously described [19].

2.5 Protein purification

Purification of ICAM-1, ICAM-3, Mac-1, and p150,95 was as previously described [9, 24, 28, 29], except for the addition of 5 mM diisopropylfluorophosphate (DFP) in the purification of ICAM-3 from neutrophils.

2.6 Adhesion assays

Binding analysis of transfected CHO cells to purified ICAM-1 and ICAM-3 was adapted from [11]. Briefly, purified ICAM-1 or ICAM-3 was diluted and adsorbed in 25-μl drops to 60-mm petri dishes. The number of ligand sites/μm² was determined using saturating amounts of ^{125}I-labeled mAb (ICAM-1: mAb RR1/1; ICAM-3: mAb CBR-IC3/1) and calculated assuming monovalent binding of the mAb [30]. After a 90-min incubation, non-specific binding sites were blocked with 0.5% heat-treated BSA. CHO cells transfectants, after removal from tissue culture plates with Hanks' balanced salts solution (HBSS), containing 10 mM Hepes, pH 7.3, 5 mM EDTA, or alternatively, SKW3 cells were washed twice and resuspended to 2×10^6 cells/ml in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, 0.5% heat-treated BSA. Cells (2×10^6 in 1 ml) were then added to petri plates containing 1 ml of 100 ng/ml

PMA in the same buffer, and allowed to adhere for 60 min at 37 °C. Unbound cells were removed by six washes with a transfer pipette. Bound cells were counted by visually scoring the number of cells in six microscopic fields (100 × magnification). This number was divided into the input number of cells which was determined in parallel, to obtain the percentage of cell binding.

Binding of ICAM-expressing L cells to purified Mac-1 was as described previously [10]. Briefly, purified Mac-1 was diluted and adsorbed in 25 μl drops to 60-mm petri dishes, and nonspecific binding sites were blocked as above. L cells were removed from tissue culture plates with trypsin-EDTA (Gibco BRL, Gaithersburg, MD), washed twice, and resuspended in 1 ml PBS containing 2 mM $MgCl_2$, 0.5% heat-treated BSA (2×10^6 cells/ml). Cells were then added (2×10^6 cells) to petri plates and the adhesion assay was carried out as described above.

2.7 Neutrophil aggregation assays

Neutrophil homotypic adhesion assays were carried out as described [31]. Following preincubation of neutrophils (10^7 cells/ml) with mAb (1:200 dilution W6/32 ascites, LPM19c ascites, ICAM-3 antiserum; 20 μg/ml purified mAb: TS1/18, CBR-IC3/1, CBR-IC3/2) for 25 min at room temperature, 0.5-ml aliquots were added to 24-well plates (Costar, Beverly, MA) and stimulated with either PMA (100 ng/ml) or N-formyl-L-met-L-leu-L-phe (fMLP) (10^{-7} M). After 15 min, aggregation was scored visually as previously described [17].
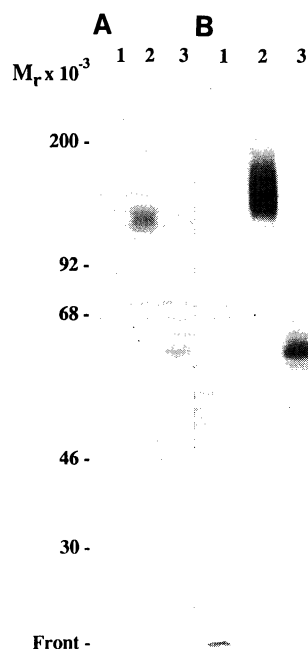

Figure 1. Glycosidase treatments of ICAM-3. ^{125}I-labeled (A) SKW3 or (B) neutrophil lysates were immunoprecipitated with control human Ig-Sepharose (lane 1) or CBR-IC3/1 mAb-Sepharose (lanes 2 and 3). Immunoprecipitates were either untreated (lane 2) or treated with N-glycanase (lane 3). Immunoprecipitates were subjected to reducing SDS-7.5% PAGE and autoradiography; molecular weight standards are shown to the left.

3 Results

3.1 Biochemical characterization of ICAM-3

ICAM-3 immunoprecipitated from PBL and SKW3 T lymphoma cells displays an M_r of 110000–130000 under reducing conditions (Fig. 1A, lane 2, and data not shown), whereas ICAM-3 from neutrophils migrates as a broad band of M_r 120000–160000 (Fig. 1B, lane 2). In contrast, LFA-1 is identical in M_r from lymphocytes and neutrophils (data not shown). N-glycanase cleaves ICAM-3 from neutrophils and SKW3 cells to a homogenous species of M_r 62000 (Fig. 1A and B, lane 3). Subsequent treatment of N-glycanase-treated immunoprecipitates with either O-glycanase or additional N-glycanase does not result in further reduction in M_r (data not shown).

3.2 ICAM/leukocyte integrin interactions

To investigate the possibility that ICAM-3 is a ligand for another leukocyte integrin, COS cells were transiently transfected with cDNA encoding LFA-1, Mac-1 or p150,95, and tested for binding to ICAM-1 or ICAM-3 purified from tonsils. Only the LFA-1-expressing COS cells bind to ICAM-3 (data not shown). In contrast, COS cells expressing both LFA-1 and Mac-1 bind to purified ICAM-1, as previously described [9]. A more sensitive appraisal of the ICAM-leukocyte integrin interaction was conducted using stably transfected CHO cells which express high levels of Mac-1, p150,95, or as a control, ICAM-1 [11]. CHO transfectants expressing Mac-1 and p150,95 do not bind to either ICAM-3 purified from a lymphoid source (SKW3 cells) or from neutrophils (Fig. 2). Both forms of ICAM-3 are functionally active, as they support adhesion of LFA-1-expressing SKW3 cells. CHO Mac-1 and CHO p150,95 cells bind significantly to purified ICAM-1 (Fig. 2).

Reciprocal experiments utilized transfected murine fibroblast L cells that express all three ICAM molecules at high, and comparable, levels (Fig. 3). These cells were tested for their ability to adhere to purified Mac-1 (Fig. 4). L-ICAM-1 cells bind strongly to purified Mac-1, as previously reported

Figure 3. Flow cytometry of L cell transfectants. L cells were labeled with either negative control X63 mAb, RR1/1 mAb to ICAM-1, CBR-IC2/1 mAb to ICAM-2, or CBR-IC3/1 mAb to ICAM-3, followed by FITC-conjugated anti-mouse Ig.

Figure 4. Adhesion of transfected L cells to purified Mac-1. L cells stably expressing ICAM-1, ICAM-2, ICAM-3, or as control, untransfected L cells, were tested for adhesion to spots of purified Mac-1. One of three representative experiments is shown, and error bars indicate 1 SD.

Table 1. Effect of ICAM-3 mAb on chemoattractant-stimulated neutrophil aggregation[a)]

Antibody treatment	PMA	fMLP
	Aggregation[b)]	
None	5+	5+
HLA mAb	5+	5+
ICAM-3 mAb	5+	5+
ICAM-1 mAb + ICAM-2 mAb	5+	5+
ICAM-1 mAb + ICAM-2 mAb + ICAM-3 mAb	5+	5+
ICAM-3 antiserum	5+	5+
ICAM-1 mAb + ICAM-2 mAb + ICAM-3 antiserum	5+	5+
CD18 mAb	0	0
CD11b mAb	0	0
CD18 mAb + ICAM-3 mAb	0	0
CD11b mAb + ICAM-3 mAb	0	0

a) Neutrophils pretreated with the indicated mAb to HLA (W6/32), ICAM-1 (R6.5), ICAM-2 (CBR-IC2/2), ICAM-3 (CBR-IC3/1 + CBR-IC3/2), CD11b (LPM19c), or CD18 (TS1/18), were stimulated with PMA (100 ng/ml) or fMLP (10^{-7} M) and after 15 min, aggregation was scored.

b) 0 indicates essentially no cells clustered; 1 indicates less than 10% of cells in clusters; 2 indicates between 10–50% of cells were in aggregates; 3 indicates 50–100% of cells were in aggregates; 4 indicates nearly 100% cells were in large clusters of aggregates; and 5 indicates that all cells were in very compact aggregates.
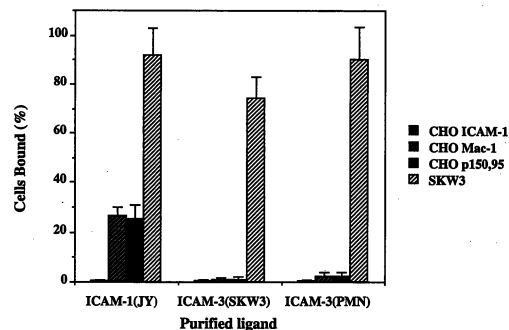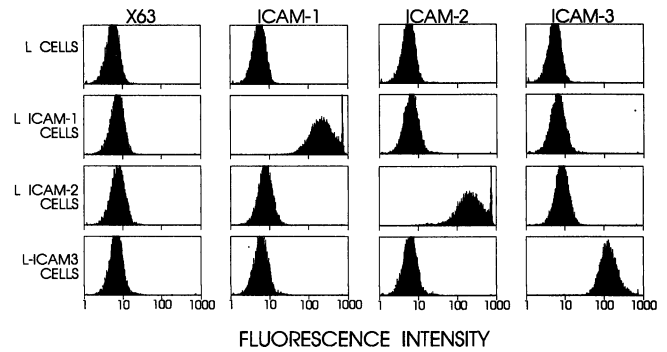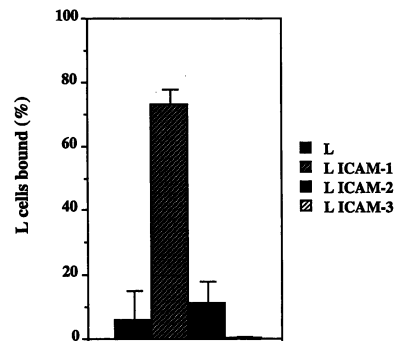

Figure 2. Adhesion of CHO cells to purified ICAM. SKW3 or CHO cells stably expressing ICAM-1, Mac-1, or p150,95 were tested for adhesion to spots of ICAM-3 purified from SKW3 cells or from PMN, or purified ICAM-1. The site density of ICAM-3 (SKW3), ICAM-3 (PMN) and ICAM-1 was 600 sites/μm^2, as determined by radioimmunoassay. One of three representative experiments is shown, and error bars indicate 1 SD.

[9]. No significant binding to purified Mac-1 is seen with ICAM-2- or ICAM-3-expressing L cells, thus confirming that neither ICAM-2 nor ICAM-3 is a counter-receptor for Mac-1.

Lastly, to confirm that ICAM-3 is not the undefined Mac-1 ligand on neutrophils responsible for the chemoattractant-induced homotypic aggregation of neutrophils, different mAb were tested for inhibition of fMLP- and PMA-stimulated neutrophil aggregation (Table 1). While Mac-1 and CD18 mAb completely inhibit aggregation, antiserum and mAb to ICAM-3 have no effect on neutrophil aggregation (Table 1). Both the ICAM-3 antiserum, and the combinations of ICAM-3 mAb, CBR-IC3/1 and CBR-IC3/2, were previously found to be capable of complete inhibition of ICAM-3/LFA-1-dependent aggregation of lymphoid cell lines [20]. These results confirm that ICAM-3 is not a Mac-1 ligand, and further indicate that an undefined Mac-1 ligand is responsible for the Mac-1-dependent neutrophil aggregation.

4 Discussion

In this study, we extend the biochemical characterization of ICAM-3 by showing that unlike ICAM-1, ICAM-3 interacts solely with LFA-1. ICAM-3 from lymphoid cells migrates as a band of M_r 124 000 on reducing SDS-PAGE [13], while neutrophil ICAM-3 migrates as a very broad band of M_r 120 000–160 000. N-glycanase treatment of ICAM-3 from neutrophils and lymphoid cells produces a sharp band of M_r 62 000, showing that the observed differences are due solely to the size of N-linked carbohydrates. The size of the deglycosylated polypeptide is in good agreement with that of M_r 56 980 predicted by analysis of cDNA sequences [4–6]. ICAM-3 is more heavily glycosylated and heterogeneous than ICAM-1, which displays M_r heterogeneity in different cell types with an M_r of 97 000 on fibroblasts, 114 000 on the myelomonocytic cell line U937, and 90 000 on the B lymphoblastoid cell line JY [32]. ICAM-3 has an unusually high frequency of N-linked sites for a cell surface protein: about 1 for every 30 amino acid residues. In the first Ig-like domain, ICAM-3 contains five potential N-linked glycosylation sites, whereas ICAM-1 and ICAM-2 contain none. The large contribution of carbohydrate, especially apparent in neutrophils, to the molecular mass of ICAM-3, may have profound effects on its function as an adhesion molecule. However, purified ICAM-3 isolated from both neutrophils and lymphoid cells bind equally well to LFA-1-bearing cells, suggesting that the differences in carbohydrate processing does not correlate with differences in ICAM-3 avidity for LFA-1.

Neutrophil homotypic aggregation is Mac-1-dependent, but the ligand responsible is unknown [11, 15, 16, 33]. L-selectin interaction with a carbohydrate ligand on neutrophils also contributes to neutrophil homotypic aggregation [16, 34], and to the rolling of neutrophils on adherent neutrophils [35]. Given the abundant expression of ICAM-3, and little or no ICAM-1 or ICAM-2 on neutrophils [13], and the high degree of homology between the third Ig-like domain of ICAM-3 and the corresponding Mac-1 binding-domain in ICAM-1 (52% amino acid identity), we tested whether ICAM-3 serves as a counter-receptor for the other leukocyte integrins, Mac-1 and p150,95. Although ICAM-1

interacts with all three leukocyte integrins, ICAM-2 and ICAM-3 interact solely with LFA-1. In support of these findings, Mac-1-dependent aggregation of neutrophils is not ICAM-3 dependent. The significant Mac-1/ICAM-1 binding [8–11], p150,95/ICAM-1 binding [11, 24] and the inability of ICAM-2 to interact with Mac-1 [9], confirm previous reports.

In summary, the results presented here show that ICAM-3 is a leukocyte-specific molecule that demonstrates considerable heterogeneity among cell types. This heterogeneity does not affect its ability to interact with LFA-1, or with the related leukocyte integrins, Mac-1 and p150,95. The glycoforms of ICAM-3 found on lymphoid cells and neutrophils clearly do not serve as counter-receptors to either Mac-1 or p150,95. A ligand for Mac-1 on neutrophils remains to be characterized.

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