DC-SIGN binds ICAM-3 isolated from peripheral human leukocytes through Lewis x residues

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Intercellular adhesion molecule-3 (ICAM-3) binds to the α1β2 integrin and mediates the contact between T cells and antigen-presenting cells. It has been suggested that dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN), a C-type lectin of macrophages and DCs, is an additional ligand of ICAM-3. So far, the glycan structure mediating the interaction of native ICAM-3 with DC-SIGN is undefined. Here, we demonstrate that native ICAM-3 from human peripheral leukocytes binds recombinant DC-SIGN, is recognized by monoclonal Lewis x antibodies, and specifically interacts with DC-SIGN on immature DCs. The presence of Lewis x residues on ICAM-3 was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. Investigations on different peripheral blood cell populations revealed that only ICAM-3 from granulocytes bound DC-SIGN. Cotransfection studies demonstrated that fucosyltransferase (FUT) IX and, to a significantly lesser extent, FUTs IV, but not FUTs III and VII, mediate the synthesis of Lewis x residues on ICAM-3. These findings indicate that FUT IX is the main FUT mediating the synthesis of Lewis x residues in ICAM-3 in cells of the myeloid lineage, and that these residues bind DC-SIGN. The results suggest that ICAM-3 assists in the interaction of granulocytes with DC-SIGN of DCs.

Key words: carcinoembryonic antigen-related cell adhesion molecule-1/dendritic cell-specific ICAM-3 grabbing nonintegrin/fucosyltransferases/intercellular adhesion molecule-3/Lewis x

Introduction

C-type lectins comprise a group of proteins, most of which are membrane bound. The prominent feature of C-type lectins is a conserved extracellular carbohydrate recognition domain (CRD) that requires Ca2+ for carbohydrate binding. Depending on the orientation of the N-terminus, type I and type II C-type lectins are distinguished (Drickamer, 1999). Macrophages and dendritic cells (DCs) are particularly rich in C-type lectins (Figdor et al., 2002). The macrophage mannose receptor (MMR) is a well-characterized example of a type I C-type lectin containing eight CRDs. DC-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) and DC-SIGN-related (DC-SIGNR) are examples of type II C-type lectins. It is well established that C-type lectins of macrophages and DCs bind glycans of infectious agents (Cambi and Figdor, 2003). For example, dectin-1, the β-glucan receptor, mediates the attachment and uptake of fungi, and the MMR binds the mannose-capped cell-wall component of Mycobacterium tuberculosis ManLAM (lipoarabinomannan).

ICAMs are transmembrane glycoproteins, which constitute a subfamily in the immunoglobulin superfamily. To date, five members of the ICAM family have been described in mammals. ICAM-1, -2, and -3 function in the immune system through the interaction with the α1β2 leukocyte integrin (Gahmberg, 1997; Gahmberg et al., 1997). It has been suggested that DC-SIGN is a ligand of ICAM-2 and ICAM-3 in addition to the α1β2 integrin (Geijtenbeek, Krooshoop et al., 2000; Geijtenbeek, Torensma et al., 2000). In fact, the designation DC-SIGN refers to the interaction with ICAM-3 (Geijtenbeek, Torensma et al., 2000). DC-SIGN is a type II lectin expressed by macrophages and DCs. It binds to a variety of pathogens. Mannose-containing glycoconjugates and fucose-containing Lewis blood group-related antigens have been identified as ligands (van Kooyk and Geijtenbeek, 2003).

Recently, the dual ligand-binding specificity of DC-SIGN was supported by screening of extensive glycan arrays (Guo et al., 2004). In addition to ICAM-2 and ICAM-3, Mac-1 (van Gisbergen, Sanchez-Hernandez et al., 2005), carcinoembryonic antigen (CEA), and CEA-related cell adhesion molecule 1 (CEACAM1) (van Gisbergen, Aarnoudse et al., 2005; Bogoevska et al., 2006) have been reported to bind DC-SIGN. In the case of CEACAM1 and Mac-1, Lewis x structures were identified in native preparations from human granulocytes as counter receptors for DC-SIGN (Lucka et al., 2005; van Gisbergen, Ludwig et al., 2005; van Gisbergen, Sanchez-Hernandez et al., 2005; Bogoevska et al., 2006). Importantly, DC-SIGN-binding glycan structures have not yet been

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Malto Tof MS. Detected ions could be interpreted as Na\(^+\) with an apparent molecular mass of 120–170 kDa, reflecting DC-SIGN.

The preparations bound DC-SIGN and DCs from peripheral granulocytes, but not from peripheral lymphocytes. (Bogoevska et al., 2006). In the present study, native ICAM-3 was identified by stepwise exoglycosidase digestion as having a complex pattern of putative N-glycan structures (Figure 2). In addition to hybrid/complex-type oligosaccharides, some high-mannose-type oligosaccharides were also found. In total, we identified 16 different oligosaccharides. Nine of these contained at least one fucose residue and four contained two or three fucose molecules, indicating that fucose is not only linked to the core structure of N-glycans, but also to the antennae, a characteristic feature of Lewis structures. To identify these enzymes, type I or type II antennae without fucose were digested by (A) sialidase (sia), (B) a mixture of \(\beta\)-(1-3,4)-fucosidase (\(+\) Fuc) prior to Western blot analysis. The mixture of ICAM-3 was pretreated by \(\alpha\)-(1-3,4) fucosidase (\(+\) Fuc) prior to Western blot analysis. LeX BSA conjugate served as a positive control. Equal loading of gels was confirmed by mAb 140.11 after stripping of blots (lower panel).

**Results**

DC-SIGN binds to ICAM-3 through Lewis x residues

The purified ICAM-3 preparation displayed a diffuse band with an apparent molecular mass of 120–170 kDa, reflecting heterogeneous glycosylation (Figure 1A). The broad band was also apparent in Western blots with a monoclonal ICAM-3 antibody (Figure 1B). A minor sharper band corresponding to a molecular weight of >180 kDa may represent aggregates. When the Lewis x-specific monoclonal antibody (mAb) L5 was applied, a comparable banding pattern was observed. The specificity of mAb L5 for Lewis x glycans has been established in previous studies (Streit et al., 1996; Lucka et al., 2005). Fucosidase III treatment, which leads to the release of \(\alpha\)-(1-3,4)-linked terminal fucose, but not fucose linked to the core structure of N-glycans, completely abolished binding of mAb L5, confirming the presence of fucose linked to the N-acetylgalactosamine residue, which is a key feature of the Lewis glycan epitope. When using recombinant Fc-DC-SIGN, the binding pattern was similar to the binding pattern of the Lewis x antibody. After treatment of the preparation with fucosidase III, no residual binding of Fc-DC-SIGN was observed. These findings indicate that native ICAM-3 from peripheral leukocytes binds DC-SIGN via Lewis x residues.

**Lewis x residues in the ICAM-3 preparation were identified by MALDI-TOF MS analysis after stepwise exoglycosidase digestion**

In order to undertake a detailed analysis of the glycan structure of ICAM-3, N-glycans were released from ICAM-3 tryptic peptides by \(\gamma\)-(N-acetyl-\(\beta\)-glucosaminyl)asparagine amidase (PNGase) F treatment. The mixture of ICAM-3 N-glycans was characterized after sialidase digestion with subsequent matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS). We obtained a complex pattern of putative N-glycan structures (Figure 2). In addition to hybrid/complex-type oligosaccharides, some high-mannose-type oligosaccharides were also found. In total, we identified 16 different oligosaccharides. Nine of these contained at least one fucose residue and four contained two or three fucose molecules, indicating that fucose is not only linked to the core N-acetyl glucosamine (GlcNAc), but also to the antennae, a characteristic feature of fucose within the Lewis epitope. Lewis structures were identified by stepwise exoglycosidases treatment as has been previously described in detail (Lucka et al., 2005). Since monoclonal antibodies against Sialyl Lewis x residues did not bind to the ICAM-3 preparation (not shown), PNGase F-released N-glycans could be treated with sialidase because potential Sialyl Lewis x residues were not destroyed. Next, a mixture of \(\beta\)-galactosidase and \(\beta\)-N-acetylgalactosaminidase was applied. \(\beta\)-Galactosidase cleaves \(\beta\)-(1-3,4,6)-linked galactose when no fucose is bound to the subterminal GlcNAc. \(\beta\)-N-acetylgalactosaminidase removes \(\beta\)-(1-2,3,4,6)-bound GlcNAc residues. Consequently, Lewis structures within the N-glycan antennae cannot be cleaved. By the combination of these enzymes, type I or type II antennae without fucose

**Fig. 1.** Analysis of native ICAM-3 purified from normal peripheral leukocytes. (A) Purity of immunaffinity purified human ICAM-3 demonstrated by SDS-PAGE followed by Coomassie staining. (B) Western blot analyses of the ICAM-3 preparation by mAb 140.11 (monoclonal ICAM-3 antibody), mAb L5 (monoclonal Lewis x antibody), and Fc-DC-SIGN (recombinant Fc-fusion protein of DC-SIGN). To demonstrate Lewis x-specific binding of mAb L5 and Fc-DC-SIGN, respectively, ICAM-3 was pretreated by \(\alpha\)-(1-3,4) fucosidase (\(+\) Fuc) prior to Western blot analysis. LeX BSA conjugate served as a positive control. Equal loading of gels was confirmed by mAb 140.11 after stripping of blots (lower panel).

**Fig. 2.** MALDI-TOF mass spectrometry of intact and exoglycosidase-treated N-glycans released from ICAM-3. Mass spectrometric analysis was performed by MALDI-TOF MS. Detected ions could be interpreted as Na\(^+\)-adducts (M + Na\(^+\)) of the glycans. Desialylated N-glycans in (B), (C), and (D) correspond to the data presented in Table I. Indicated mass values in (A) correspond to the data presented in (E). Mass peaks corresponding to the K\(^+\)-adduct are not indicated. N-glycans were stepwise digested by (A) sialidase (sia), (B) a mixture of \(\beta\)-N-acetylgalactosaminidase and \(\beta\)-galactosidase (hex/gal), (C) \(\alpha\)-(1-3,4)-specific fucosidase (fucIII), and (D) a \(\beta\)-(1-4)-specific galactosidase (gal). (E) Mass spectrometric data of desialylated N-glycans released from ICAM-3 and putative corresponding oligosaccharide composition. Detected ions could be interpreted as Na\(^+\)-adducts (M + Na\(^+\)) of the glycans. The likely carbohydrate composition was determined by the GlycoMod tool of the Expasy Molecular Biology Server (nomenclature according to the GlycoMod tool). Detected masses that could not be clearly interpreted are not listed.
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bound to the subterminal GlcNAc will be removed completely. The use of \( \alpha(1-3,4) \)-specific fucosidase (FucIII) then leads to the release of fucose that is bound to the antennae, but not of fucose that is bound to the core structure. Finally, we used the \( \beta \) -galactosidase with \( \beta(1-4) \) specificity to demonstrate that the released fucose residues were part of Lewis x and not of Lewis a structures. The data are summarized in Table I and the corresponding positive ion mass spectromograms are shown in Figure 2. From these results, it is concluded that at least three structures were sensitive to FucIII treatment.

**Lewis x groups mediate the binding of ICAM-3 to DCs**

To investigate the interaction of Lewis x-carrying ICAM-3 and DC-SIGN of DCs, isolated ICAM-3 was covalently coupled with fluorescent microbeads and incubated with immature DCs. Lewis x–bovine serum albumin (BSA) coupled beads were used as a positive control and fluorescent beads coated with BSA served to determine background binding. Monocytes were isolated from peripheral human blood and differentiated with interleukin-4 and granulocyte macrophage colony-stimulating factor to immature DCs showing high levels of DC-SIGN expression (data not shown). Bound beads were visualized and quantified by fluorescence microscopy. Strong binding of ICAM-3-coated beads was observed compared to beads pre-treated by \( \alpha(1-3,4) \) fucosidase (Fuc) and lacking Lewis x residues (Figure 3). A strong decrease in binding was observed when Lewis x groups on ICAM-3 were blocked by mAb L5, or when DCs were preincubated with DC-SIGN blocking antibody DC28, demonstrating that the interaction between DC-SIGN and ICAM-3 is mediated by Lewis x residues present in the native ICAM-3.

**DC-SIGN binds only to ICAM-3 from granulocytes**

The ICAM-3 preparation used in this study was isolated fromuffy coats of healthy donors. In order to verify which of the populations of peripheral blood leukocytes contained ICAM-3 and bound the Lewis x antibody and recombinant Fc-DC-SIGN, respectively, monocytes, T lymphocytes, B lymphocytes, and granulocytes were isolated by gradient centrifugation followed by cell-type specific magnetic cell sorting. ICAM-3 was immunoprecipitated using ICAM-3-specific mAb 3.1. As shown in Figure 4, ICAM-3 from granulocytes was recognized by the monoclonal Lewis x antibody L5 as well as by recombinant Fc-DC-SIGN. In contrast, ICAM-3 immunoprecipitated from monocytes and T or B lymphocytes, respectively, did not bind the monoclonal Lewis x antibody L5. Similarly, recombinant Fc-DC-SIGN was not bound. Comparable results were obtained with cellular extracts of the whole fraction of unselected mononuclear cells isolated by density centrifugation (data not shown). In addition, no significant binding of the mannose-specific *Galanthus nivalis* agglutinin (GNA) was observed. These findings indicate that among peripheral blood leukocytes, only granulocyte ICAM-3 carries Lewis x residues and binds DC-SIGN.

**Recombinant ICAM-3 binds Lewis x antibody L5 and DC-SIGN after cotransfection with fucosyltransferase IX**

HEK293 cells were cotransfected with ICAM-3 cDNA and cDNAs encoding different fucosyltransferases (FUTs). As shown in Figure 5, binding of the Lewis x-specific mAb L5 to ICAM-3 was observed in the presence of FUT IX. A band with a corresponding electrophoretic mobility was detected by Fc-DC-SIGN. With FUT IV, some binding of the monoclonal Lewis x antibody and a faint binding of Fc-DC-SIGN were present. No Lewis x residues were detectable in ICAM-3 coexpressed with FUT III or FUT VII. In all the HEK293 transfectants, binding of DC-SIGN to a band of higher electrophoretic mobility was observed in each of the cotransfectants; no band was detected in untransfected HEK293 cells (data not shown). In the loading control using an antibody against the myc-tagged ICAM-3, it is apparent that this is the main form of expressed recombinant ICAM-3. Since a band of identical electrophoretic mobility binds the high-mannose-specific GNA, the lower molecular weight form of ICAM-3 expressed in HEK293 cells probably binds DC-SIGN via high-mannose residues. These results indicate that FUT IX and, to a lesser extent, FUT IV mediate the synthesis of Lewis x residues in ICAM-3, and that these residues bind DC-SIGN. In addition, a recombinant form of ICAM-3 devoid of Lewis x residues interacts with Fc-DC-SIGN, probably via high-mannose residues.

**Discussion**

Human macrophages and DCs express a number of C-type lectins. One of these lectins was first described through the

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**Table I.** Mass spectrometric data of desialylated N-glycans released from ICAM-3 after treatment with different glycosidases and putative corresponding oligosaccharide composition

<table>
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<tr>
<th>Ions m/z composition*</th>
<th>( \beta )-Galactosidase/hexosaminidase</th>
<th>( \alpha(1-3,4) ) Fucosidase</th>
<th>( \beta(1-4) ) Galactosidase</th>
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<td>2174.483</td>
<td>1850.226</td>
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</table>

*Mass spectrometric analysis was performed by MALDI-TOF MS. Detected ions could be interpreted as Na\(^+\)–adducts (M + Na\(^+\)) of the glycans. The likely carbohydrate composition was determined by the GlycoMod tool of the Expasy Molecular Biology Server (nomenclature according to the GlycoMod tool). Detected masses that could not be clearly interpreted are not listed. This table corresponds to Figure 2B, C and D.*
CD4-independent binding of human immunodeficiency virus (Curtis et al., 1992). Later, it was postulated that the lectin mediates the interaction of immature DCs with ICAM-3 on resting T cells. Based on this postulate, the lectin was designated DC-SIGN (Geijtenbeek, Torensma et al., 2000). As first revealed by the binding of pathogens, DC-SIGN exhibits a dual binding specificity for mannose- and fucose-containing ligands. Thus, Mycobacterium tuberculosis, the yeast Candida albicans, and Leishmania parasites are bound via mannose-containing glycans, whereas Helicobacter pylori and Schistosoma eggs bind via fucosylated ligands (Appelmelk et al., 2003; Cambi and Figdor, 2003). The dual-binding specificity of DC-SIGN was further substantiated by the use of glycan arrays. It was shown that DC-SIGN binds to N-linked high-mannose oligosaccharides and to glycans containing a terminal fucose such as Lewis x and Lewis a structures (Guo et al., 2004). From cocrystals of the CRD of DC-SIGN with high-mannose structures or Lewis x glycans, the dual specificity of DC-SIGN has been explained at an atomic level (Guo et al., 2004; Feinberg et al., 2005).

Previously, we identified Lewis x residues on native CEACAM1 isolated from human granulocytes by MALDI-TOF MS analysis (Lucka et al., 2005) and showed that the Lewis x glycans of CEACAM1 bind to DC-SIGN (Bogoevska et al., 2006). Besides CEACAM1, Mac-1 has been identified as a granulocyte glycoprotein which binds DC-SIGN via Lewis x residues (van Gisbergen, Sanchez-Hernandez et al., 2005). These results prompted us to ask which glycan structures may be responsible for the interaction of ICAM-3 with DC-SIGN. Since glycans of recombinant proteins do not reflect the in vivo situation, ICAM-3 was purified from a native

**Fig. 3.** Binding of microbeads coated with ICAM-3 to DC-SIGN on immature DCs. Immature dendritic cells were incubated with fluorescent microbeads to which ICAM-3 purified from peripheral leukocytes was covalently attached. Subsequent to washing, bead binding was visualized by fluorescence microscopy; dendritic cells are shown by phase contrast microscopy (scale bar represents 80 μm). (A) Attachment of beads coated by ICAM-3 was strongly reduced when DC-SIGN was blocked by preincubation of DCs by the blocking DC-SIGN antibody DC28 or after removal of Lewis x residues from ICAM-3 by α(1-3,4) fucosidase (+Fuc) or blocking of Lewis x residues on ICAM-3 by Lewis x-specific mAb L5. (B) Absolute numbers of microbeads bound to dendritic cells per microscopic field were quantified from digital images. Binding experiments were performed in triplicate and compared to untreated beads highly significant decrease in binding is marked by asterisks ($P < 0.01$).

**Fig. 4.** Representative Western blot analysis of ICAM-3 immunoprecipitated from purified populations of peripheral blood leukocytes. M, monocytes, T, T lymphocytes, B, B lymphocytes, G, granulocytes. mAb L5, monoclonal Lewis x antibody; Fc-DC-SIGN, recombinant fusion protein. GNA, Galanthus nivalis agglutinin; poly ICAM-3, polyclonal ICAM-3 antiserum. Antigen was immunoprecipitated using ICAM-3-specific mAb 3.1, and antibody N-19 was applied for detection. Western blot analyses were performed side-by-side under identical conditions. To exclude variations between donors, analyses were performed in three independent experiments with cell preparations of three different donors. Comparable results were obtained for all the three healthy donors.
preparation of peripheral leukocytes. Here, we provide conclusive evidence that ICAM-3 contains Lewis x residues, and that these residues interact with DC-SIGN. (1) In Western blots, ICAM-3 bound the Lewis x-specific mAb L5. (2) DC-SIGN binds ICAM-3. The binding of both DC-SIGN and Lewis x antibody was abolished by pretreatment of the ICAM-3 preparation with fucosidase III. (3) Beads coated with ICAM-3 bound to cultured DCs, and the binding was inhibited by a mAb to DC-SIGN. Pretreatment of the ICAM-3-coated beads with either fucosidase III or Lewis x-specific mAb drastically decreased the binding. (4) In the ICAM-3 preparation, the presence of Lewis x residues was confirmed by MALDI-TOF MS analysis after stepwise exoglycosidase digestion of the ICAM-3 N-glycans.

The cellular synthesis of Lewis x groups is guided by α(1,3)-FUTs. The main FUTs responsible for the synthesis of the Lewis x structures are FUT IV and FUT IX (de Vries et al., 2001). Our previous studies indicate that FUT IX is the main FUT mediating the synthesis of Lewis x residues of CEACAM1 (Bogoevska et al., 2006). When ICAM-3 was coexpressed with different FUTs, the most intense binding of the Lewis x-specific mAb was observed with FUT IX. The activity of FUT IV toward ICAM-3 was significantly lower, whereas no Lewis x groups were identified in cotransfectants with FUT III or FUT VII. Thus, ICAM-3 is a substrate of FUT IX similar to CEACAM1. Our unpublished results indicate that in addition to CEACAM1 and ICAM-3, PECAM-1 is also fucosylated by FUT IX. In contrast, other studied members of the immunoglobulin superfamily such as the receptors for platelet-derived growth factor or fibroblast growth factor are not. Thus, it appears that FUT IX fucosylates a distinct class of adhesion molecules of the immunoglobulin superfamily.

It is unclear which glycan structures on ICAM-3 are bound by DC-SIGN. This may be ascribed to the fact that the ICAM-3 preparations shown to interact with DC-SIGN were generated from transfected cell cultures in vitro. This applies to the first description of the interaction of ICAM-3 and DC-SIGN (Geijtenbeek, Torensma et al., 2000) as well as to a more recent study in which ICAM-3 was expressed in COS cells (Jimenez et al., 2005). In the latter study, high-mannose residues were identified as glycans interacting with DC-SIGN, though it was noted that only a fraction of the molecules bore an endo-H-sensitive high-mannose type of glycans. In a previous study, ICAM-3 from buffy coats was found to contain about 6% of high-mannose-type oligosaccharides (Funatsu et al., 2001). The presence of high-mannose structures was confirmed by the MALDI-TOF analysis as reported here. It has been speculated that these high-mannose residues might be responsible for the binding of DC-SIGN with ICAM-3 (Funatsu et al., 2001; Geijtenbeek et al., 2004). However, the results presented here do not support the notion that high-mannose-type glycans are the major glycan structures in native ICAM-3, which bind to DC-SIGN. After treatment of the preparation with fucosidase III, no residual binding of DC-SIGN could be observed in Western blots, though high amounts of ICAM-3 were loaded onto the gel. Similarly, pretreatment of ICAM-3-coated beads by a monoclonal Lewis x antibody or fucosidase III reduced the adhesion to cultured DCs to near background levels. As binding of DC-SIGN to high mannose is dependent on the number and/or the linkage of mannose residues (Guo et al., 2004), the mannose structures detected in native ICAM-3 may not be such that DC-SIGN binds efficiently. In this context, it should be noted that high-mannose oligosaccharides were identified on β2 integrins (Asada et al.,

Fig. 5. Western blots of recombinant ICAM-3 preparations produced by HEK293 cells coexpressed with different FUTs. HEK293 cells were cotransfected by ICAM-3 cDNA in combination with different FUT cDNAs. mAb L5, monoclonal Lewis x antibody; Fc-DC-SIGN, recombinant fusion protein. GNA, Galanthus nivalis agglutinin; mAb MYC, monoclonal antibody directed against the MYC tag of recombinant ICAM-3; mAb HA, monoclonal antibody directed against the hemagglutinin tag of FUTs. Lewis x-carrying ICAM-3 is marked by black arrowheads and the fraction of ICAM-3 positive for GNA- and Fc-DC-SIGN-binding is marked by gray arrowheads.
Nevertheless, the glycoprotein may assist to make contact with Lewis x residues in comparison with CEACAM1. ICAM-3 is a minor Lewis x expressing component. In Western blots, CEACAM1 is the major glycoprotein carried by unfractionated extracts from granulocyte membranes (van Gisbergen, Sanchez-Hernandez et al., 1991); however, DC-SIGN binding by Mac-1 was attributed to Lewis x residues (van Gisbergen, Sanchez-Hernandez et al., 2005).

Recombinant CEACAM1 preparations expressed in HEK293 cells exhibit an intense binding of DC-SIGN in the absence of Lewis x residues (Lucka et al., 2005). Recombinant ICAM-3 from HEK293 cells, too, bound DC-SIGN in the absence of Lewis x residues, although binding was lower than that of CEACAM1. According to Jimenez et al., (2005), among the N-terminal domains of ICAMs expressed in COS cells, only the two N-terminal domains of ICAM-2 and ICAM-3, but not of ICAM-1, bound DC-SIGN. Notably, the introduction of an additional N-linked glycan at different positions in the first domain of ICAM-1 resulted in binding of DC-SIGN. From these and related reports, it can be concluded that the expression of high-mannose-type residues in recombinant proteins depends upon the structural features of the proteins. In our previous studies on CEACAM6, we noted that the presence of mannosetype residues on the recombinant glycoproteins significantly depends on the cell line expressing the protein (Sauter et al., 1993). Similarly, CEACAM1 expressed in Chinese hamster ovary cells did not bind DC-SIGN in the absence of Lewis x groups, whereas CEACAM1 from HEK cells did. The recombinant glycoproteins, which bind DC-SIGN in the absence of Lewis x groups, bound the GNA. Since this lectin interacts with high-mannose-type oligosaccharides (Sharon and Lis, 2003), the DC-SIGN binding recombinant glycoproteins, which are devoid of Lewis x residues, most probably bind DC-SIGN via high-mannose-type oligosaccharides. Collectively, the presence of DC-SIGN binding high-mannose-type residues on recombinant proteins depends both on the particular protein as well as on the cell line and culture conditions.

In order to establish which of the peripheral blood cell populations expressed Lewis x-containing ICAM-3 glycoproteins and bound DC-SIGN, ICAM-3 was immunoprecipitated from purified populations of peripheral granulocytes, monocytes, as well as T and B lymphocytes. ICAM-3 was identified in each of the populations using an ICAM-3-specific antiserum. Interestingly, however, only ICAM-3 from granulocytes expressed Lewis x residues and bound DC-SIGN. This result appears to contradict published results, which postulate that DC-SIGN is bound by ICAM-3 expressed by T cells (Geijtenbeek, Torensma et al., 2000). However, as discussed before, this contradiction may be resolved by the fact that the glycosylation of native glycoproteins and of recombinant glycoproteins obtained from cultured cells can differ significantly.

After CEACAM1 and Mac-1 (van Gisbergen, Aarnoudse et al., 2005; Bogoevska et al., 2006), ICAM-3 is the third membrane glycoprotein of human granulocytes found to express Lewis x residues. When Lewis x antibodies are bound to unfractionated extracts from granulocyte membranes in Western blots, CEACAM1 is the major glycoprotein carrying Lewis x residues. The fact that Lewis x groups of ICAM-3 are detectable only after immunoprecipitation indicates that ICAM-3 is a minor Lewis x expressing component. In addition, MALDI-TOF analysis revealed a lower number of Lewis x residues in comparison with CEACAM1. Nevertheless, the glycoprotein may assist to make contact between granulocytes and DCs. As suggested previously, such contacts may regulate DC maturation and direct T-cell polarization to the type of pathogen (van Gisbergen, Geijtenbeek et al., 2005).

Our results indicate that ICAM-3 from human granulocytes contains Lewis x residues, and that these residues mediate the binding to DC-SIGN of DCs and possibly macrophages. The results do not support the view that ICAM-3 is a major ligand of DC-SIGN in resting T lymphocytes. Since it has been shown that the interaction of T cells with immature DCs can be blocked by monoclonal DC-SIGN antibodies, molecules other than ICAM-3 may mediate the binding of resting T lymphocytes to DC-SIGN of DCs.

Materials and methods

Antibodies, reagents, and expression vectors

For detection of ICAM-3, mouse mAb 3.1, goat polyclonal Ab N-19 (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA), and mAb 140.11 (Dr R. Vilella, Barcelona University Hospital, Spain) were used. The DC-SIGN blocking mAb DC28 was purchased from R&D Systems Inc. (Minneapolis, MN). Lewis x residues were detected by the rat monoclonal IgM antibody L5 (a kind gift of M. Schachner, ZMNH, Hamburg, Germany). Digoxigenin-labeled GNA and the horseradish peroxidase (HRP)-labeled anti-digoxigenin mAb were purchased from Roche (Mannheim, Germany). Lewis x–BSA conjugate and Fuc were obtained from Calbiochem (Darmstadt, Germany). α1-acid glycoprotein was purchased from Roche (Schwalbach, Germany) and served as a control for all glycosidase treatments. The following enzymes were used for the specific release of oligo- or monosaccharides. Recombinant PNGaseF asparagine amidase from Flavobacterium meningosepticum (EC 3.5.1.52) was purchased from Roche (Mannheim, Germany). Recombinant sialidase from Arthrobacter ureafaciens (exo-α-sialidase, EC 3.2.1.18), β-N-acetylgalatosidase from jack bean (EC 3.2.1.52), β-galactosidase from bovine testes (EC 3.2.1.23), α(1-4)-specific galactosidase from jack bean (EC 3.2.1.23), and Fuc from Xanthomonas manihotis (α-fucosidase III, EC 3.2.1.51) were obtained from PROzyme/Glyko (Novaton, CA) and Calbiochem. All other reagents were obtained from Merck (Darmstadt, Germany). Soluble Fc-DC-SIGN consisting of the extracellular portion of human DC-SIGN fused at the N-terminus to a human IgG1-Fc fragment was generated as described previously (Bogoevska et al., 2006). DNA-encoding human FUTs III, IV, VII, IX and full-length ICAM-3 were amplified from human cDNA by polymerase chain reaction, cloned in the EF-1α promoter carrying mammalian expression vector pEF-BOS expression vector, and HEK293 cells were transiently transfected (Ebrahimbnejad et al., 2004).

Purification of ICAM-3

ICAM-3 was purified from human blood buffy coat cell lysates by affinity chromatography, essentially as previously described (Funatsu et al., 2001). Human blood buffy coats were from Finnish Red Cross Blood Transfusion Service, Helsinki, Finland. The purity of the ICAM-3 preparation was analyzed by Coomassie staining after separation of proteins.
by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Western blot analyses, fucosidase treatment, and immunoprecipitation**

Proteins were separated by 4–12% NuPage gradient gels (Invitrogen, Karlsruhe, Germany) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocking overnight at 4 °C in 1% blocking reagent (Roche), membranes were incubated with primary antibodies (1 µg/mL) in TBST (Tris-buffered saline with Tween: 150 mM NaCl, 10 mM Tris (pH 8.0), 0.05% Tween 20) for 1 h at room temperature (RT). Subsequent to washing, the membranes were incubated with an appropriate HRP-conjugated, secondary antibody at a dilution of 1:25 000 for 1 h at RT. Signals were detected by chemiluminescence (ECL reagent, Amersham, Germany). Digestion of ICAM-3 with Fuc (25 µU/mL) was performed overnight at 37 °C in 50 mM sodium phosphate buffer (pH 5.0). The DC-SIGN overlay assay was performed as previously described (Bogoevska et al., 2006). For the analysis of different blood cells, mononuclear cells and granulocytes were isolated from fresh buffy coats of normal human blood donors by density centrifugation (Ficoll–Paque, d = 1.077 g/cm³; Pharmacia, Freiburg, Germany). To remove contaminating erythrocytes from the granulocyte preparation, the granulocyte fraction was resuspended in erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 8.0), incubated for 15 min at RT, and washed 3 times in ice-cold phosphate-buffered saline (PBS). Peripheral blood monocytes, B and T lymphocytes were isolated from the mononuclear cell fraction applying human anti-CD14, anti-CD19 or anti-CD3 magnetic microbeads following the protocol of the supplier (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). The homogeneity and purity of cell populations was controlled by fluorescence activated cell sorting analysis (data not shown). For immunoprecipitation, whole cellular lysates (1 mg per reaction) were preclared by protein G Plus agarose (Santa Cruz, CA) and incubated at 4 °C overnight with 5 µg of the ICAM-3-specific mAb 3.1 bound to protein G Plus agarose (20 µL). After washing, beads were boiled in 1× Laemmli buffer, and proteins were separated by SDS-PAGE and precipitated ICAM-3 was detected by Ab N-19.

**Trypsin digestion and release of N-linked glycans from purified ICAM-3**

Trypsin (sequencing grade) was purchased from Serva (Heidelberg, Germany) and trifluoroacetic acid was obtained from Sigma (Taufkirchen, Germany). To improve the release of N-linked glycans by subsequent enzymatic digestion, 100 µg of native ICAM-3 was predigested with trypsin according to standard procedures (Nuck, 2002). The protein to enzyme ratio was 1:50 (w/w). Digestion at 37 °C was carried out overnight in a buffer containing 50 mM N-methyl-2,2-iminodithiolan (pH 8.0) and stopped by heat inactivation. For the release of N-glycans, tryptic peptides derived from 100 µg ICAM-3 were digested with 5 µL PNGaseF in 500 µL of the above-described N-methyl-2,2-iminodithiolan buffer for 18 h at 37 °C. Peptides were separated by cation-exchange chromatography (AG-50WX12; Bio-Rad, Munich, Germany). Released oligosaccharides were further purified on a reverse phase cartridge (RP18).

**Sequential exoglycosidase digestions**

Digestion of purified ICAM-3 derived N-glycan mixture led to specific fragments, which could be determined with subsequent mass spectrometry. All digestions were carried out with oligosaccharides derived from 100 µg ICAM-3 peptides according to the manufacturer’s protocol in the provided incubation buffer at 37 °C. After each enzymatic treatment, appropriate aliquots were taken and analyzed by MALDI-TOF MS as described in the Mass spectrometry section. Prior to the next digestion, oligosaccharides were desalted by anion/cation exchange chromatography. For specificities of the used enzymes see Kobata and Ginsburg (1969), Dwek et al., (1993), and Prime et al., (1996). As a control, all steps of sequential exoglycosidase digestions and subsequent mass spectrometry were done in parallel also with the oligosaccharides released from α1-acid glycoprotein, as described previously (Lucka et al., 2005).

Recombinant sialidase with the specificity for α(2-3,6,8,9)-linked sialic acid was used to remove all bound sialic acid residues. Oligosaccharides were incubated with 100 µL in a final volume of 100 µL for 48 h, with a fresh enzyme aliquot added after 24 h. A mixture of β-galactosidase with β(1-3,4,6) cleavage specificity from bovine testes and β-N-acetylhexosaminidase with the cleavage specificity of β(1-2,3,4,6) was used to remove galactose and GlcNAc residues from antennae that do not carry a fucose residue. Desialylated ICAM-3 oligosaccharides were incubated with 0.15 U of β-N-acetylhexosaminidase and 1.5 U of β-galactosidase in a final volume of 100 µL for 18 h. α-Fucosidase III with cleavage specificity for α(1-3,4)-linked fucose was used to remove terminally linked fucose residues; 1.5 µL was incubated in a final volume of 30 µL for 3 h. β-Galactosidase led to the release of β(1,4)-linked galactose residues, which carried an α(1-3)-linked fucose residue prior to the fucosidase treatment; 8 µL was used in 100 µL of the provided incubation buffer for 16 h.

**Mass spectrometry**

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was carried out on a Bruker Biflex instrument equipped with a 337-nm nitrogen laser (Bruker, Bremen, Germany) as described previously (Gohlke et al., 1996; Kannicht and Flechner, 2002). Measurement was done using the positive ion mode. An amount of 0.5 µL of each digestion mixture was used in 0.5-µL arabinosazon matrix solution.

The GlycoMod tool (http://www.expasy.org/tools/glycomod/) of the Expasy Molecular Biology Server was used to verify all calculations of oligosaccharide compositions. Experimental masses were calculated as monoisotopic Na⁺—adducts of derivatized N-glycans from PNGaseF release.

**Fluorescent bead binding assay**

Native ICAM-3 from human leukocytes was covalently coupled in the presence of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide to carboxylate-modified FluoSpheres® (diameter 1 µm) at 4 °C overnight as recommended by the supplier (Invitrogen). Beads were washed in PBS, briefly sonicated
to disperse particles, and kept in 1% BSA/PBS at 4°C until further use. Immature DCs (1 × 10^5), generated from monocytes of normal human blood donors as previously described (Bogoevska et al., 2006), were cultivated on glass chamber slides (Lab Tek®, surface area 1.8 cm^2; Nunc, Wiesbaden, Germany) and incubated with 1 × 10^7 ligand-coupled beads for 1 h at 37°C. After incubation, DCs were washed 5 times in prewarmed PBS and fixed in 4% paraformaldehyde/PBS. Bound beads were visualized by fluorescence microscopy (Leitz DMRB fluorescence microscope, Leitz, Wetzlar, Germany) and photographically documented with a digital camera. The number of beads per microscopic field was determined from digital images applying the cell-counting tool of the NIH image software package (version 1.63; NIH, Bethesda, MR); for statistical significance, Student’s t test was performed. For blocking experiments, immature DCs were preincubated by DC-SIGN mAb DC28 and an irrelevant myc antibody or ICAM-3-coated beads were preincubated with Lewis x-specific mAb L5 for 30 min at 37°C at a final concentration of 20 μg/mL. Defucosylation of ICAM-3-coated beads was performed under conditions as described.

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Conflict of interest statement
None declared.

Abbreviations
BSA, bovine serum albumin; CEA, carcinoembryonic antigen; CEACAM, CEA-related cell adhesion molecule; CRD, carbohydrate recognition domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin; DC-SIGNR, DC-SIGN-related; Fuc, α(1-3,4) fucoside; FUT, fucosyltransferase; GlcNAc, N-acetylglucosamine; GNA, Galanthus nivalis agglutinin; HRP, horseradish peroxidase; ICAM, intercellular adhesion molecule; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; MMR, macrophage mannose receptor; PBS, phosphate-buffered saline; PNGase, N^4-(N-acetyl-β-glucosaminyl)asparagine amidase; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween.

References


