Vol. 195, No. 1, 1993
August 31, 1993

Pages 478-483

NEUTROPHIL NCA-160 (CD66) IS THE MAJOR PROTEIN CARRIER OF SELECTIN BINDING CARBOHYDRATE GROUPS LEWIS^x AND SIALYL LEWIS^x

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Received July 7, 1993

SUMMARY: The neutrophil surface carbohydrate groups Lewis^x and sialyl Lewis^x have previously been shown to interact with the vascular selectins E- and P-selectin. However, the proteins expressing these carbohydrate groups have not been fully characterised. We show that these carbohydrate groups, and the structurally related Lewis^a group, are all carried predominantly on a sub-population of the carcinoembryonic antigen (CEA) related NCA-160, which is also recognised by CD66 antibodies. We demonstrate that the related NCA-160, NCA-90/95 and CD67 all undergo an increase in surface expression in response to fMLP stimulation, and that this increase is greater than that observed for the sialyl and non-sialyl Lewis^x carbohydrate groups. These data suggest that the expression of differently glycosylated forms of NCA is independently regulated.

The first steps in acute inflammation involve the low-level adherence of neutrophils to the endothelium, by an interaction known as "rolling" adhesion. This rolling adhesion has been shown to be mediated by the vascular selectins, E- and P-selectin, and to occur independently of the leucocyte integrins[1,2]. The vascular selectins recognise neutrophil glycoconjugates containing the carbohydrate groups sialyl Lewis^x and also the non-sialylated Lewis^x [3-7]. P-selectin interaction with sialylated Lewis^a has also been demonstrated[8]. Several different neutrophil glycoproteins have been implicated in the selectin mediated interaction of neutrophils with endothelial cells. Although we have previously demonstrated that most of the neutrophil Lewis^x is present on the CEA related glycoprotein, NCA-160, the identity of glycoproteins which carry the selectin binding carbohydrate groups has remained unclear.

Neutrophils carry several CEA-related glycoproteins, including NCA-160 (recognised by CD66 monoclonal antibodies) with a transmembrane spanning region, and the glycosyl-phosphatidyl-inositol-linked CD67 and NCA-90/95. All of the neutrophil NCAs are heavily glycosylated, with 59% of the molecular weight of the mature NCA-160 molecule being

0006-291X/93 $4.00

accounted for by glycosylation[9]. Several CEA family members have been demonstrated to be adhesion molecules, mediating homotypic and heterotypic adhesion events[9-13]. The neutrophil NCAs have also been shown to be involved in stimulation of aggregation and adhesion to plastic[14] with an increase in surface expression occurring on activation[15,16]. NCAs have also been implicated more directly in the signalling processes which lead to activation of the cell. The present study defines precisely the association between selectin binding carbohydrate groups and the neutrophil NCAs, and the changes in expression which take place on cellular activation.

MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Co. Poole, U.K., unless stated. Polyclonal anti-CEA immunoglobulin was purchased from Dako Ltd., High Wycombe, U.K., anti-Lewis^x antibody MC2 was produced as described previously[17], anti-Sialyl-Lewis^x antibody, SNH3, was a kind gift of Dr. S. Hakomori, Biomembrane Institute, Seattle, anti Lewis^a, LM112/161 and anti-CEA monoclonal IM1013.1 were obtained from the SAPU, Carluke, Scotland, YTH71.3, designated CD66, a rat monoclonal, was the kind gift of Dr. H. Waldmann, Cambridge, U.K., MHM23, designated CD18, was the kind gift of Dr. A. J. McMichael, Oxford, U.K., the CD67 antibody 80H3 was purchased from Serotec, Oxford, U.K.. Anti-immunoglobulin antibodies conjugates were purchased from Sigma.

Immunoblotting and SDS-PAGE: Neutrophils were isolated by 6% dextran sedimentation of red cells followed by leukocyte separation over a discontinuous density gradient using lymphoprep (Nycomed) and Ficoll (*s.g.*1.119), centrifuged at 700*g* for 20 minutes. Membrane proteins were extracted with Triton X-100 as described previously[15]. Detergent extracted neutrophil membrane glycoproteins were resolved by SDS-PAGE on 10% acrylamide gels[18] under reducing conditions. Proteins were transferred to 0.45μm pore nitrocellulose membrane as described previously[19], except that transfer buffer contained 15% methanol. The membrane was blocked with 5% skimmed milk ("Marvel") in PBS for two hours followed by incubation with primary antibody for a further two hours. YTH71.3, MC2 and SNH3 were used as ascites, anti-CEA and CD67 antibodies as purified IgG all diluted 1:200 in milk/PBS, anti Lewis^a tissue culture supernatant was used undiluted. The membrane was washed with PBS 3 x 10 minutes followed by incubation with second antibody at 1:500 to 1:1000 dilution in milk/PBS. After washing the blot was developed using 5-bromo-3-chloro indolyl phosphate as substrate.

Activation of neutrophils: Isolated neutrophils were resuspended in HBSS/0.5% BSA at 10^6 cells ml^{-1} and either fixed at the start of the experiment or treated with fMLP prior to fixation for 15 minutes in 0.5% paraformaldehyde. Activation was carried out by addition of fMLP to a final concentration of 10^{-7}M, followed by an incubation for 30 minutes at 37°C. The cells were subsequently washed and labelled with primary antibody followed by a secondary antibody-FITC conjugate, both labelling steps being carried out in HBSS/BSA, which eliminated non-specific binding of FITC conjugates. Results were collected on a FACScan flow cytometer, collection and analysis being carried out using Consort 30 software.

Immunoprecipitation: 0.5ml membrane protein extracts (10^8 neutrophils/ml) were incubated with 15μl antibody at 4°C overnight, 200μl second antibody conjugated to agarose was added and the incubated for 1 hour. The effluent fraction (unbound proteins) was collected and the resin was washed with PBS/0.1% Triton X100, then 0.75M NaCl/20mM phosphate pH7.4/0.1% Triton X-100 and a further wash with PBS/0.1% Triton X-100. Bound proteins were eluted with 2 x 200μl 0.5M acetic acid and neutralised with 75 μl 2M Tris HCl pH 8.7.

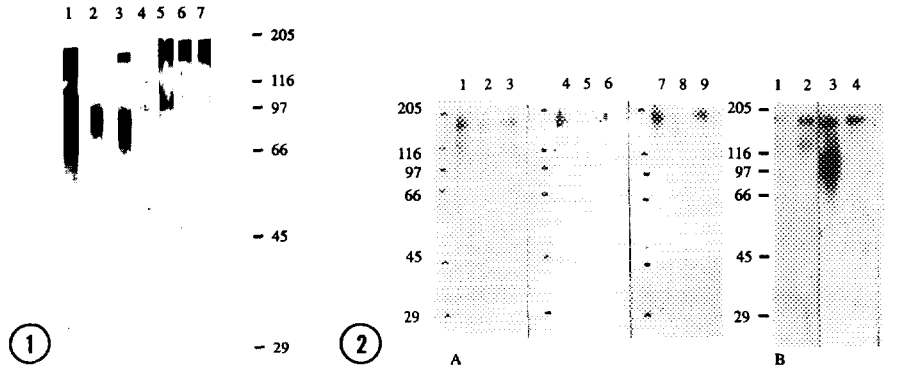

Figure 1. 10% non-gradient SDS-PAGE of neutrophil membrane extract. Immunoblotted with the following antibodies; anti-CEA polyclonal Ig (lane 1); CEA monoclonal IM1013.1 (lane 2); CD66, YTH 71.3 (lane 3); CD67, 80H3 (lane 4); Lewis^x, MC2 (lane 5); sialyl-Lewis^x, SNH3 (lane 6); Lewis^a, LM112/161 (lane 7).

Figure 2. **A;** Immunoblot of anti-CEA polyclonal immunoglobulin immunoprecipitates of neutrophil membrane proteins, resolved by 10% SDS-PAGE. Lanes 1, 4, 7, unfractionated material; lanes 2, 5, 8, unbound material after a second absorption with immunoglobulin; lanes 3, 6, 9, material eluted from the anti-CEA immunoglobulin. Lanes 1-3 are blotted with anti-Lewis^x, lanes 4-6 with anti-sialyl-Lewis^x and lanes 7-9 with anti-Lewis^a. **B;** Immunoblot of anti-Lewis^x precipitates of neutrophil membrane proteins, resolved by 10% SDS-PAGE. Lanes 1 & 3, material unbound after two absorptions with antibody; lanes 2 & 4 material eluted from anti-Lewis^x. Lanes 1 & 2 are immunoblotted with anti-Lewis^x; lanes 3 & 4 are immunoblotted with anti-CEA immunoglobulin.

RESULTS

Expression of NCAs and Lewis carbohydrates on neutrophils

Neutrophil membrane glycoproteins, extracted with Triton X-100 and resolved on SDS-PAGE were shown to contain a number of NCAs which give broad bands of approximately 160-190, 95-105 and 80-100 kDa (figure 1). Polyclonal antibody raised against CEA recognised two broad protein bands corresponding to NCA species of 160-190 and 80-100 kDa (figure 1, lane 1), a protein with identical mobility to the smaller of these two proteins was recognised by the anti-CEA monoclonal IM10 13.1 (figure 1, lane 2), and by several other monoclonal anti-CEAs (not shown). The CD66 monoclonal antibody YTH71.3 showed the same reactivity as the polyclonal anti-CEA antibody (figure 1, lane 3). The CD67 antibody 80H3 recognised a glycoprotein of around 100kDa, distinct from the lower molecular weight NCA recognised by a CD66 antibody (figure 1, lane 4). This combination of antibodies distinguishes between several NCAs, namely CD66, CD67 and the NCA-90. Clearly CD66 terminology requires updating in view of the diversity of the proteins recognised by these monoclonals. The broad bands exhibited by each of the NCAs on PAGE whilst other glycoproteins in the same preparation gave very tight bands was consistent with their heavy, heterogeneous, glycosylation.

Lewis carbohydrates are expressed predominantly on NCA-160

Anti-Lewis carbohydrate group antibodies recognised neutrophil membrane glycoproteins of the molecular weights 160-210kDa and around 100 kDa. All of the antibodies tested recognised proteins of identical mobility (figure 1, lanes 5-7), although the anti-Lewis^x antibody MC2 recognised the lower molecular weight protein more strongly than either anti-sialyl Lewis^x or anti-Lewis^a. We were however unable to demonstrate any reactivity on these glycoproteins or indeed, any neutrophil expression at all, of the related carbohydrate group sialyl-Lewis^a, using histochemistry, flow cytometry or immunoblotting (the antibody used stained various positive control tissues strongly) (not shown). Lewis^a expression did not depend on the Lewis phenotype of the individual, identical neutrophil expression being observed in three individuals who were Lewis^a, Lewis^b or Lewis^{a-b-} phenotype.

Immunoprecipitation of neutrophil membrane NCAs with anti-CEA polyclonal antibodies followed by probing with anti-carbohydrate antibodies demonstrated that the neutrophil NCA of 160-190kDa (NCA160) is the major carrier of the Lewis carbohydrate groups (Fig. 2A). Two successive absorptions of neutrophil NCA with the anti-CEA antibodies removed virtually all of the Lewis^x, sialyl-Lewis^x and Lewis^a reactive material on glycoproteins of this size, whilst failing to absorb the lower molecular weight protein which expressed these groups. In contrast, when solubilised neutrophil membrane proteins were immunoprecipitated twice using an antibody which recognises the Lewis^x carbohydrate group, immunoblotting with both MC2 and anti-CEA antibodies, the absorption clearly failed to remove all of the NCA-160 in spite of the

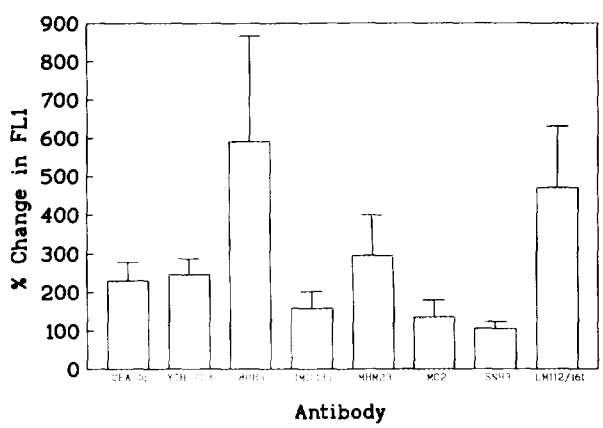

Figure 3. Change in surface expression of NCAs and carbohydrate groups on activation with 10^{-7}M fMLP. Cells were activated for 30 minutes at 37°C and analysed on a Becton Dickinson FACScan. Results are expressed as percentage of fluorescence due to same staining procedure of non-activated cells, background due to non-specific labelling with fluoresceinated antibody has been deducted. The antibodies used recognise the following antigens: anti-CEA polyclonal Ig (recognises all neutrophil NCAs, including CD66, CD67 and NCA-90); YTH 71.3 (CD66); 80H3 (CD67); CEA monoclonal IM1013.1 (NCA-90); MC2 (Lewis^x); SNH3 (sialyl-Lewis^x); LM112/161 (Lewis^a). The mean of seven different experiments performed with blood from different donors on different days is shown ±standard deviation.

fact that virtually all of the Lewis^x reactive material in glycoproteins of this mass had been removed (Fig. 2B). It is apparent that Lewis^x is expressed only on a subpopulation of NCA-160.

Expression of NCAs and Lewis carbohydrate groups are increased on fMLP activated cells

Flow cytometry was used to assay the change in surface expression of neutrophil NCAs. In Figure 3 data from 7 experiments showed the independent increase in surface expression of CD67 and NCA-90/95 as recognised by anti-CD67 and anti-CEA monoclonal antibodies, and also the increase in CD66, glycoproteins recognised both by anti-CD66 monoclonal and anti-CEA polyclonal antibodies. Of the NCAs, CD67 showed by far the greatest increase in expression, although both CD66 antibodies and anti-CEA polyclonal antibodies demonstrated a more than two-fold increase. The integrin β_2 chain, recognised by CD18 antibody, demonstrated a threefold increase in expression. Surface expression of the Lewis^a carbohydrate was also significantly upregulated on activation. Interestingly, the increase in expression of Lewis^x and its sialylated derivative was not as great, with both showing only a small increase in fluorescence-detected expression.

DISCUSSION

The data presented in this paper show that the major carrier of Lewis^x and sialyl-Lewis^x on neutrophils is the carcinoembryonic antigen related NCA-160, recognised by CD66 monoclonal antibodies. We have shown for the first time that a large number of anti-CEA monoclonal antibodies specifically recognise an NCA with approximate Mr= 90-95kDa. NCA-160, NCA-90/95 and CD67 on neutrophils are upregulated on activation with fMLP. All of the NCAs are apparently heavily but variably glycosylated. NCA-160 but not the other NCAs express the fucosylated structures, Lewis^x, sialyl-Lewis^x and Lewis^a.

Several factors support the hypothesis that neutrophil NCA may be involved in neutrophil adhesion (reviewed in ref.18). Importantly, all CEA family members for which a function has been demonstrated have been shown to be involved in cell-cell interaction, and neutrophil NCAs in particular have been shown to mediate adhesion[13]. It is also well known that other neutrophil adhesion molecules which mediate adherence to the endothelium are upregulated on activation, as are the neutrophil NCAs[15,16]. NCA-160 is clearly the major carrier of the Lewis carbohydrate groups. Although Lewis^x, sialyl-Lewis^x and Lewis^a expression was also increased on treatment of the neutrophils with fMLP, the increase in surface expression of the NCAs is markedly different to that of the carbohydrate groups. The data suggest that it is the Lewis^a-glycosylated population of NCA that is upregulated on activation of the cell, whilst the Lewis^x carrying NCA is constitutively expressed.

The present data provide further circumstantial evidence that NCA-160 is one target molecule of the vascular selectins. It is known that the P-selectin ligand is a glycoprotein[6], and that sialyl-Lewis^x is an important component of the ligand[4]. We have shown previously that

monovalent fragments of anti-CEA antibody, which recognise the same neutrophil glycoproteins as CD66, elicit an aggregation response in neutrophils[14]. The homotypic adhesion of neutrophils is integrin mediated[21] and as the selectins are thought to elicit activation of the integrins[22-24], it is likely that the selectin receptor is able to transduce some level of "activation" signal. NCA therefore fulfils two important requirements for the vascular selectin ligand, in that it is a glycoprotein which carries the carbohydrate groups involved in selectin recognition, and it is able to signal to the cell to increase integrin mediated adhesion.

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