C-type lectins of natural killer cells: carbohydrate ligands and role in tumour cell lysis

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Introduction

Natural killer (NK) cells constitute a distinct lymphocyte subset defined by the absence of B- or T-cell surface receptors, and characterized by their natural cytotoxicity against certain tumour or virally infected cells [1]. As physical contact is required for this spontaneous cytotoxicity, the molecular basis for NK cell reactivity should involve effector (NK) cell membrane receptors engaging their specific ligands at the target (tumour) cell surface. Type I1 integral membrane proteins that contain extracellular domains related to the carbohydrate-recognition domain (CRD) of C-type animal lectins have recently been identified among candidates for NK cell receptor functions [2,3]. Here I summarize our recent investigations aimed at identifying the ligands for these receptors. These include high-affinity oligosaccharide ligands of the type represented on the tumour cell surface that are recognized by rat NKR-P1 or similar receptors at the NK cell surface and are intimately linked to natural killing. Unpublished work is also presented, pointing to the possible involvement of one of these molecules, rat NKR-P1, in adhesive reactions of NK cells.

NK cell receptors related to Ca\(^{2+}\)-dependent animal lectins

In recent years, extensive experimental efforts of immunologists have been concentrated on the identification of NK cell receptors, employing both hybridoma technology (production of monoclonal antibodies against purified NK cell populations) and molecular genetic approaches (such as subtractive hybridization) [3-6]. Both strategies have ultimately converged in the identification of a group of type II disulphide-linked dimeric transmembrane proteins related to Ca\(^{2+}\)-dependent animal lectins [3]. These molecules, the protein products of polymorphic gene families residing in the natural killer gene complex in the distal region of mouse chromosome 6 or the syntenic human chromosome 12q13-22 [2,3], have been assigned to a separate group (group V, Figure 1a) in the evolutionary tree of this lectin superfamily [7]. They are notable among C-type lectins by possessing, within their intracellular tails, peptide sequences that seem to enable them to transduce signals to the NK cell upon cross-linking with antibodies or engage-

Abbreviations used: NK, natural killer; CRD, carbohydrate-recognition domain; IL-2, interleukin-2.
ment of their ligands.

Immunological studies suggest the existence of two functionally distinct classes among these molecules. Proteins of the first group, represented here by human CD69 and rat NKR-P1 antigens (Figure 1a), have been shown to transmit positive signals in the NK cell resulting in production of inositol phosphates, an increase in the concentration of intracellular calcium and granule exocytosis [4,8,9]; these molecules will be referred to here as ‘activation’ receptors. There is an apparently reciprocal group of proteins, such as mouse Ly-49 antigens, that transmit negative signals causing a global inactivation of the NK cell cytolytic pathways [10]; these are referred to as ‘inhibitory’ receptors. A detailed knowledge of the ligands with which these molecules interact should improve our understanding of the molecular mechanisms responsible for the delicate balance between the positive and negative signals elicited by these molecules, which are thought to regulate many biological activities of NK cells. In this regard, the work of Karlhofer et al. [11] has indicated that MHC class I glycoproteins H-2D\(^\text{b}\), and possibly H-2D\(^\text{d}\), may be ligands for the inhibitory receptors. However, no ligands could be identified for the activation receptors [10]. Moreover, because of the considerable evolutionary distance of group V proteins from other members of the C-type lectin family, it was not clear if they would bind calcium or carbohydrates, which are the established ligands for many other C-type lectins [7]. In order to address these issues, we have embarked on production of soluble forms of group V proteins suitable for ligand identification studies.

Production of soluble dimeric forms of NK cell activation receptors

The lectin-type proteins, rat NKR-P1 and human CD69, are transmembrane, disulphide-linked glycoproteins on NK cells (Figure 1b), each having at least one potential N-glycosylation site in their extracellular parts. In our experiments with NKR-P1 we established that N-glycosylation is not required for the ligand-binding activity of this protein [12]. Therefore, our strategy for production of soluble forms of these antigens has been based on recombinant expression of the entire extracellular portions of the molecules (see Figure 1b) in bacteria, as fusion proteins with maltose-binding protein [12,13]. Affinity-purified fusion proteins were cleaved with Factor X\(a\) protease, and receptor portions obtained in good yields by gel filtration under denaturing conditions [12,13]. These purified receptors refolded efficiently in the presence of calcium, resulting in proteins immunochemically reactive with the respective conformation-sensitive monoclonal antibodies. Both dimeric and monomeric forms of these proteins were recovered in varying proportions in the refolded preparations (Figures 1d and 1f). The isolated forms of these proteins, used in most of our ligand identification and functional studies, were disulphide-linked dimers (Figures 1e and 1g), thus resembling the natural forms of these proteins in NK cell membrane [4,14].

NK cell receptors that are carbohydrate-binding proteins

Carbohydrate-binding activity of soluble NKR-P1 was initially tested by neoglycoprotein overlays (Figure 2), which revealed its ability to bind N-acetyl-\(\alpha\)-hexosamines, and, to a lesser extent, \(\alpha\)-fucose. When the carbohydrate-binding activities of soluble dimeric NKR-P1 and CD69 proteins were tested by quantitative inhibition assays, the hierarchy of their affinities towards individual monosaccharides was Gal\(\text{NAc}\) or Glc\(\text{NAc}\) > Fuc > Man > Gal > Glc [12,13]. The affinities of both proteins for simple sugars seem to be higher than those observed for most other C-type lectins investigated [13], e.g. binding of soluble dimeric NKR-P1 to Glc\(\text{NAc}\)\(_2\)-BSA was inhibited by D-Gal\(\text{NAc}\) at a concentration of \(3 \times 10^{-4}\) M. Recently, evidence has been provided that two of the inhibitory receptors, Ly-49A and Ly-49C, are also carbohydrate-binding proteins [15,16].

NK cell lectins bind carbohydrates independently of external calcium

Carbohydrate binding by soluble NKR-P1 was further investigated by binding experiments with BSA-linked monosaccharides performed in buffer lacking calcium and containing 10 mM EGTA. Surprisingly, this treatment did not abolish binding (Figure 2c). The specificity of the interaction was subsequently confirmed in an overlay performed with unlabelled protein followed by specific immunochemical detection of bound protein (Figure 2d). The explanation for this apparent calcium independence of NK cell lectins is their unusually high affinity for calcium, such that calcium ions bound in the CRD of these proteins do not easily dissociate even in the presence of the chelating agent [12]. Similar independence of carbohydrate binding on
Rat NKR-P1 and human CD69, two type II leucocyte proteins related to C-type animal lectins; structure of their genes, the corresponding protein products and an outline of strategy used to produce and characterize their soluble dimeric forms.

(a) Possible evolutionary relationships among genes that determine the structures of the C-type lectins (adapted from [21]). Association of exons coding CRD with those for transmembrane domains leads to formation of type II receptors. Further association with the exons coding different cytoplasmic tails results in the occurrence of either endocytic receptors (group II) or signalling receptors (group V, [7]). CD69 is an example of an activation receptor transmitting positive signals (+) in cells of many haematopoietic lineages. NK-cell-specific proteins, that have evolved by integration of an additional exon between those coding transmembrane domain and CRD, have been shown to transmit either activating (+) or inhibiting (−) signals [10, 19] and are referred to here as activation and inhibitory receptors, respectively. There is a third group of NK-cell-specific antigens, including NKG-2 and CD94, the modes of signalling of which are not yet known. (b) and (c) Schematic representation of two activation receptors, rat NKR-P1 and human CD69, respectively. The CRD is depicted as a box, with intramolecular disulphide bonds marked by solid lines and interchain bonds by dotted lines; C represents the position of cysteines. The number of the most N-terminal amino acid present in the two recombinant proteins is circled. (d) and (f) Analysis of refolded soluble NKR-P1 and CD69 proteins, respectively, by gel filtration. A TSK G3000 SW column was calibrated in 20 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl and 10 mM CaCl₂ and calibrated with protein markers including 1, immunoglobulin G (150 kDa); 2, BSA (65 kDa); 3, ovalbumin (44 kDa); 4, carbonic anhydrase (30 kDa); 5, myoglobin (17 kDa); and 6, aprotinin (6 kDa). One hundred micrograms of the respective recombinant proteins was injected, and the elution monitored at 280 nm. (e) and (g) Analysis of the purified soluble dimeric NKR-P1 and CD69 proteins, respectively, by SDS-PAGE performed under non-reducing (left lanes) and reducing (right lanes) conditions [22]. Five micrograms of each protein was applied and gels were stained by Coomassie Brilliant Blue R-250. Positions of molecular mass marker proteins are given in kDa.
Carbohydrate Recognition Proteins

Figure 2
Analysis of monosaccharide-binding specificity of NKR-P1 by neoglycoprotein overlays

Neoglycoproteins (1 μg/lane) were separated on 10% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R-250 (a). Binding of NKR-P1 was detected by autoradiography after electrophoretic transfer of the neoglycoproteins onto nitrocellulose sheets and overlay with 125I-labelled soluble dimeric NKR-P1 in the presence of 10 mM CaCl₂ (b) or 10 mM EGTA (c). Alternatively, binding was detected with an enhanced chemiluminescence system (Amersham Corp.) after electrophoretic transfer and overlay with soluble NKR-P1 followed by antibody against NKR-P1 [3,2,3] (d). Lane 1, BSA; lane 2, α-o-Glc-BSA; lane 3, β-o-Glc-BSA; lane 4, α-o-Gal-BSA; lane 5, β-o-Gal-BSA; lane 6, β-o-lactosyl-BSA; lane 7, α-o-L-Fuc-BSA; lane 8, α-o-Man-BSA; lane 9, β-o-GlcNAc-BSA; lane 10, β-o-GalNAc-BSA. Reproduced from [12] with permission.

The role of NKR-P1 protein in adhesive reactions of NK rat cells

Three stages in the killing process of NK cells can be clearly distinguished: (i) recognition and binding to the target cell, (ii) triggering of killer cell activation and (iii) cytolytic action [1]. In order to investigate the participation of rat NKR-P1 in the adhesive reactions of NK cells, we measured the effects of soluble dimeric NKR-P1 protein or one of its high-affinity carbohydrate ligands (heparin IS disaccharide [18]), on the proportions of freshly isolated NK cells or activated NK cells that form conjugates with the appropriate targets (Figure 3a). With fresh NK cells, no inhibitory effects were observed by either of the above inhibitors under the conditions of our assay (see legend to Figure 3). As NKR-P1 is known to increase dramatically in NK cells upon their activation [4], we performed parallel experiments with rat NK cells which had been stimulated by interleukin-2 (IL-2) for 120 h. After this treatment, the cell surface levels of several membrane antigens, such as CD8 or CD5, did not change (Figures 3d–g). However, cell surface expression of NKR-P1 was remarkably increased (Figures 3b and c), and there was a significant increase in the ability of these cells to form conjugates (from 11% to approximately 23% of total NK cells, Figure 3a). The fact that all the increased adhesion activity could be inhibited by both inhibitors (Figure 3e) indicated that it is indeed mediated by NKR-P1 or related proteins. Collectively these results suggest that, while on freshly isolated NK cells the level of expression of the lectin receptors may not be sufficient for mediating sustained adhesion, on the activated NK cells, NKR-P1 or related molecules may have an important adhesive role.

sequences. In addition to blood-group-related carbohydrate sequences, certain oligosaccharides of the ganglio and the glycosaminoglycan families were also strongly bound. The highest affinity ligands among the structures investigated were the highly sulphated oligosaccharides derived from heparin [18]. Moreover, we obtained evidence that oligosaccharide sequences in the form of cell surface gangliosides and proteoglycans constitute physiological ligands for NKR-P1 on target cells. Indeed, such saccharides were found to be abundantly expressed in plasma membranes of NK-sensitive tumour cells, but only sparsely in those of NK-resistant cells [18].

the external calcium concentrations has been observed for CD69 [13], Ly-49A and Ly-49C [15,16].

Complex oligosaccharides as physiological ligands for rat NKR-P1 protein

The specificity of soluble dimeric NKR-P1 protein for complex oligosaccharide ligands has been assigned using neoglycolipid technology [17]. Multiple structurally related oligosaccharides have been identified as high-affinity ligands for this protein. The specificity follows certain clear rules as deduced from binding experiments in which a series of structurally defined oligosaccharide sequences based on the trisaccharide GlcNAcβ1→3Galβ1→4Glc have been examined [18]. It was observed that substitution with galactose β1→3 linked to the terminal GlcNAc of this trisaccharide (as in type 1 chains) did not prevent binding, but β1→4-linked galactose (as in type 2 chains) abolished binding. Additional substitution with fucose, sialic acids (3-linked but not 6-linked) or sulphates resulted mostly in enhanced binding to such carbohydrate
Investigation of the involvement of NKR-PI at the surface of fresh and activated NK cells in conjugate formation with target cells

(c) Fresh NK cells were prepared according to the previously published procedure [23], and in some experiments activated by culturing for 120 h in the presence of recombinant IL-2, 1000 units/ml. For the evaluation of conjugate formation [24], NK cells were labelled with calcein-AM and NK-susceptible tumour cells, YAC-1, were labelled with hydroethidine (both from Molecular Probes). Cells were washed and suspended at 2 x 10⁶/ml in PBS containing 10 mM MgCl₂, and 10⁵ NK cells were mixed with an equal number of tumour cells in triplicate experiments in the absence of additives or in the presence of soluble NKR-PI protein (sNKR-PI, 10⁻⁹ M) or heparin disaccharide IS (Sigma, 10⁻⁹ M). The cells were pelleted at 4°C, incubated at 37°C for 10 min, and the percentage of conjugate-forming cells determined by two-colour flow cytometry (FACScan, Becton Dickinson). For flowfluorimetric analysis of surface expression of NKR-P1 (b and c), CD8 (d and e) and CD5 (f and g) on fresh or activated NK cells, respectively, cells were incubated for 1 h at 23°C with saturating concentrations of monoclonal antibodies 3.2.3 (anti-NKR-P1, [4]), OX8 (anti-CD8, Serotec) or OX19 (anti-CD5, Serotec) in PBS containing 3% BSA and 0.1% NaN₃, washed and incubated for 1 h at 23°C in PBS with 0.1% NaN₃ containing fluorescein-labelled rabbit anti-mouse immunoglobulins (Cappel Inc., 1 µg/ml), washed with Ca²⁺/Mg²⁺-free PBS containing 0.02% EDTA and analysed by flow cytometry (K. Bezouska and T. Feizi, unpublished work).

Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity

In order to evaluate the role of oligosaccharide ligands for NKR-P1 in NK cell activation, we prepared liposomes expressing high-affinity ligands and used them as target cell 'decoys' in cellular activation experiments [18]. Incubation of such liposomes with rat NK cells resulted in production of inositol phosphates and an increase in intracellular calcium, as observed with target cell preincubation. No such activation was observed with control liposomes containing an irrelevant oligosaccharide, lactose. These experiments provide evidence that biologically relevant signals are generated when NKR-P1 molecules on the surface of NK cells are cross-linked by their interactions with oligosaccharide ligands. Moreover, this activation was shown to be dependent on an optimal cell surface density of the oligosaccharide ligands, both lower and higher densities resulting in suboptimal levels of NK cell activation [18]. A complete functional reconstitution of this system has been success-
fully achieved in experiments in which NK-resistant tumour cells could be rendered susceptible to natural killing by preincubation with liposomes containing oligosaccharide ligands for NKR-P1 [18]. The possibility of manipulating the activity of NK cells is potentially useful in immunotherapies for purging of tumour or virally infected cells in vivo.

Conclusions
Rapid progress is being made in our understanding of NK cell receptors related to C-type animal lectins [19,20]. An increasing number of molecules belonging to the NK cell gene complex are being cloned and sequenced, thus opening the way to their recombinant expression and investigation of their ligands and functional properties. Definitive evidence has been obtained that both calcium and carbohydrates are ligands for at least three of these proteins. The interaction of oligosaccharide structures containing carbohydrate ligands on the target cell surface with NKR-P1 (and possibly other related receptors) on the killer cell surface is crucial for target cell recognition and killing. Experimental techniques and models such as those elaborated in the present studies provide good prospects for addressing many intriguing questions related to the detailed oligosaccharide specificities of individual isoforms of these NK cell lectins, and the ways in which fine tuning of the activation and inhibitory signals transmitted by these molecules occurs. The ultimate aim of these investigations is to understand molecular mechanisms regulating the biological activities of NK cells in sufficient detail to allow us to use them in a targeted manner in anti-tumour and anti-microbial immunotherapies.

I would like to thank Ten Feizi for many helpful discussions and crucial reading of this manuscript and Mark S. Stoll for his help with the preparation of figures. This work was supported by grant 5R01TW 00275-02 from the Fogarty International Center of the National Institute of Health, grants 310/93/0207, 310/94/1533 and 312/93/0593 from The Grant Agency of Czech Republic, and the research funds of the MRC Glycosciences Laboratory, Harrow.


Received 8 August 1995