Preferential Interaction of a Novel Tumor Surface Protein (p38.5) with Naive Natural Killer Cells

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Summary

A receptor–ligand interaction exclusive to natural killer (NK) cell–mediated recognition and triggering of tumor cell destruction has not yet been identified. In contrast, molecules that are involved in cellular adhesion and regulation of NK cytolysis have been well studied. In this report, a novel tumor surface protein is identified that exhibits characteristics of a recognition structure for naive NK cells. A tagged ligand–cell adsorption technique revealed a 38.5-kD plasma membrane protein (p38.5) from a prototypical NK-susceptible cell line (K562) that preferentially bound to NK cells ($CD3^-CD5^-CD16^+$) relative to T lymphocytes ($CD3^+CD5^+CD16^-$). The molecule was purified to apparent homogeneity for further characterization. An amino acid sequence of an 11-mer internal peptide of p38.5 did not exhibit homology to known proteins. Affinity-purified antibody generated against this peptide (anti-p38.5) reacted with a single protein of 38.5 kD on Western blots of whole cell extracts of K562. Flow cytometry and immunoprecipitation studies of surface-labeled tumor cells demonstrated expression of p38.5 on NK-susceptible tumor cell lines (K562, MOLT-4, Jurkat), whereas p38.5 was not detected on NK-resistant tumor cell lines (A549, Raji, MDA-MB-231). Significantly, p38.5 loss variants derived from wild-type Jurkat and Molt-4 cell lines exhibited decreased susceptibility to NK cell–mediated lysis demonstrating a strong association between cell surface expression of p38.5 and cytotoxicity. Purified p38.5 retained preferential binding to NK cells and inhibited NK activity in a dose-dependent manner, thereby providing direct evidence of a role in the lytic process. Binding studies identified a 70-kD membrane protein from NK cells as a possible receptor for the p38.5 tumor ligand. Consistent with cellular adsorption studies, the 70-kD, p38.5 binding protein was not detected on T lymphocytes. Based on studies demonstrating selective binding of p38.5 to NK cells, lack of expression on NK-resistant tumor cell lines and ability of the purified molecule to block cytolysis, we conclude that p38.5 may serve as a recognition/triggering ligand for naive human NK cells.

Naive NK cells ($CD3^-$, $CD16^+$, TCR^-), unlike CTL ($CD3^+$, $CD16^-$, TCR^+) provide cell-mediated lytic activity against virus-infected cells and certain tumors without requirement for activation (1–3). Such unactivated lymphocytes, also referred to as resting or naive NK cells, are capable of destroying a relatively limited spectrum of tumor cells (1, 4). Upon activation with lymphokines such as IL-2, NK cells acquire broad anti-tumor lytic activity (lymphokine-activated killer cells, i.e., LAK cells). The mechanism(s) by which naive NK cells recognize their target cells is not completely understood. Interaction of cellular adhesion molecules and recognition of specific target structure(s) have been proposed as critical initial events in the cell-mediated lytic process (1, 5). For example, it is well known that the initiation of target cell lysis by CTL is due to interaction of the MHC class I molecules (plus bound peptide) with the TCR (6, 7). Analogous molecular structures that initiate the lytic process between NK cells and tumor cells have not been defined. Although MHC molecules may serve a regulatory function for NK cells (8–11), it is clear that their presence on the surface of tumor cells is not required for cytolysis, because NK-susceptible cell lines do not express MHC gene products (11, 12).

A possible feature of NK cell tumor–specific recognition structures is that the molecules may be exclusively expressed on NK cells and their susceptible target cells, respectively. We examined this possibility by using a tagged ligand–cell adsorption technique (13) to reveal surface molecules of human tumor cells that preferentially bind to NK cells. Results from these studies identified a 38.5-kD tumor membrane protein (p38.5) that bound to NK cells and not at all to T lymphocytes. Functional studies suggest that this

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interaction is necessary for naive NK cell–mediated cytotoxicity.

Materials and Methods

Chemicals, Antibodies, and Cell Lines. N-hydroxy succinamide ester of biotin (biotin–NHS) was purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA). Streptavidin alkaline phosphatase and all cell culture media and reagents were from GIBCO BRL (Gaithersburg, MD). Electrophoresis reagents and chemicals were purchased from Bio-Rad Laboratories (Melville, NY). All other chemicals used were from Sigma Chemical Co. (St. Louis, MO).

Mouse anti–rabbit IgG was purchased from Pierce (Rockford, IL), goat anti–rabbit alkaline phosphatase was obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti–rabbit IgG conjugated to FITC was obtained from Tago, Inc. (Burlingame, CA). Antibody recognizing p38.5 was raised in a rabbit against an 11-mer synthetic peptide derived from the amino acid sequence of an internal peptide of the molecule (see below). The synthetic peptide (250 μg) was mixed with Titermax (CytRx Co. Atlanta, GA) (1:1 vol/vol) and injected subcutaneously into a rabbit at four sites as instructed by the manufacturer. Subcutaneous booster injections (100 μg peptide in IFA) were given to the rabbit at bimonthly intervals. Serum was collected 1 wk after the third booster injection. Anti-p38.5 antibodies were affinity purified from sera using BSA, 11-mer peptide conjugate linked to CNBr–Sepharose 4B. Affinity-purified antibody had a titer of 1:2,000 and reacted with a single protein of 38.5 kD on Western blots (see results). Cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in media according to instructions provided.

Lymphocytes. Human peripheral blood lymphocytes (HPBL)[1] were isolated as described previously (13). In brief, blood obtained from healthy volunteers was diluted 1:1 with HBSS, layered on Ficoll–Hypaque (Pharmacia, Piscataway, NJ) and centrifuged at 200 *g* for 20 min. The mononuclear leukocyte fraction (Ficoll–Hypaque interface) was passed through nylon wool columns to obtain HPBL depleted of B cells and monocytes.

Isolation of Naive NK Cells and T Lymphocytes. Naive human NK cells are defined as freshly isolated $CD3^-$, $CD5^-$, $CD16^+$ that exhibit limited target cell specificity, i.e., lytic activity against K562, but not A549 tumor cells. Activated NK cells, i.e., those that are stimulated with lymphokines such as Il-2 (LAK), are able to lyse NK-resistant tumor cells such as A549 (1, 4). Only those preparations of HPBL that lacked LAK cell activity were used for the isolation of naive NK cells and T lymphocytes. Isolation of naive NK cells and T lymphocytes was performed as follows. Freshly isolated HPBL were suspended in PBS containing 2% fetal bovine serum (FBS) at 20×10^6 cells/ml and incubated (4°C for 30 min) with anti-CD5, 20 μl/1×10^6 cells (Becton-Dickinson, San Jose, CA). Anti-CD5 reactive lymphocytes were captured by incubating the mixture with sheep anti–mouse IgG-coated magnetic beads, 30 μl/1×10^6 cells (Dynal Inc., Lake Success, NY). The magnetic beads containing adherent $CD5^+$ cells were washed, suspended in RPMI supplemented with 15% FBS, and incubated overnight at 37°C in 5% CO_2. Subsequently, CD5-enriched lymphocytes (T lymphocytes) were separated from the magnetic beads by vortexing 2 min and placing the mixture in a magnetic field. The supernatant containing $CD5^-$ HPBL was incubated with anti-CD3, 20 μl/10^6 cells (Becton-Dickinson, San Jose, CA) at 4°C for 30 min. Anti-CD3–reactive lymphocytes were separated using magnetic beads as described above. The supernatant was centrifuged to obtain cells that were depleted of $CD3^+$ and $CD5^+$ lymphocytes (i.e., CD 16^+ enriched naive NK cells). The average phenotype of the T lymphocyte enriched fraction was $<3\%$ $CD16^+$, 50% $CD4^+$, and 93% $CD3^+$. These cells did not exhibit NK or LAK cytolytic activity. The average phenotype of the negatively selected naive NK cell enriched fraction was 85% CD 16^+, $<3\%$ $CD3^+$. These cells exhibited increased NK cell lytic activity (60% cytotoxicity at an effector to target E/T ratios of 100:1) compared with unfractionated HPBL (32% cytotoxicity).

Binding of Tumor Plasma Membrane Proteins to Lymphocytes. Plasma membrane proteins of viable tumor cells were labeled with biotin as described previously (13). In brief, cells (>99% viable by trypan blue dye exclusion) were washed three times with a solution containing 10 mM Hepes, 145 mM NaCl, 4 mM KCl, 11 mM glucose, pH 8.0 (buffer A) and then incubated in the same buffer with biotin–NHS (1 mM biotin–NHS, 10×10^6 cells/ml) for 1 h at 4°C. Cells were then washed three times with 20 vol of buffer A to remove unreacted biotin. The biotinylated cells were suspended in 250 mM sucrose with 10 mM Hepes (pH 8.0) and disrupted by N_2 cavitation (1,000 psi) in a Parr's chamber. The resulting suspension was centrifuged at 200 *g* for 5 min to remove undisrupted cells and the supernatant was centrifuged at 30,000 *g* for 20 min to obtain a crude membrane fraction. Membranes were washed once with 10 mM sodium borate, 10 mM benzamidine, 1 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, pH 8.0 (buffer B), and incubated in the same solution with 1% Triton X-100 at 4°C overnight. Solubilized membrane proteins were obtained as the 30,000 *g* supernatant of the detergent treated membranes and dialyzed extensively against buffer B containing 0.05% Triton X-100, as described previously (13).

Biotin-labeled solubilized tumor plasma membrane proteins were reacted with freshly isolated naive NK cells and T lymphocytes at a ratio of 2:1 (on the basis of cell numbers) in RPMI-1640 supplemented with 15% FBS at 4°C for 2 h. The reacted cells were washed twice with media and three times with buffer A. Finally, cells were solubilized in Laemmli sample buffer and the solubilized proteins were subjected to SDS-PAGE and Western blotting. Biotinylated tumor membrane proteins that bound to lymphocytes were identified with a streptavidin biotin detection system as described previously (13).

Purification of Tumor Membrane Proteins. Membrane proteins were purified as described previously (13). Approximately 10^{11} cells of erythroleukemia cell line (K562) were washed three times with PBS and used to prepare plasma membrane proteins as described above. Solubilized membrane proteins were extensively dialyzed against buffer B and subjected to preparative SDS-PAGE. A vertical portion of the gel was sliced and stained with Coomassie blue to locate desired proteins. Protein bands of interest were cut from the gel, eluted, and further purified by SDS-PAGE. A portion of the purified protein was extensively dialyzed against 10 mM Tris–HCl, pH 8.0 and used for cytotoxicity inhibition studies or dialyzed against buffer B and labeled with biotin as described above for study of its binding properties to various lymphocyte subsets. A portion of the purified protein was also isolated on nitrocellulose paper after SDS-PAGE using the method described by Aebersold et al. (14) for the purpose of internal amino acid sequence analysis of the protein subsequent to in situ protease digestion (Harvard Microchemistry Laboratory, Cambridge, MA).

Immunoprecipitation of Surface-labeled Proteins. Immunoprecipitation of plasma membrane proteins was performed by initially

[1]*Abbreviations used in this paper:* FBS, fetal bovine serum; HPBL, human peripheral blood lymphocytes; Hsp, heat shock protein.

incubating anti-p38.5 with anti–rabbit IgG coupled to CNBr–Sepharose 4B at 4°C for 90 min in PBS containing 1% BSA (13). The reacted beads were then washed three times with buffer and incubated at 4°C for 90 min with surface-biotinylated tumor plasma membrane proteins. Beads were then washed three times with PBS containing 1% BSA and two times with PBS alone, suspended in Laemmli SDS-PAGE sample buffer, and boiled for 10 min. The supernatant was subjected to SDS-PAGE and proteins transblotted to Immobilon-P membrane (Millipore Corp., Bedford, MA). Anti-p38.5 immunoprecipitated biotinylated tumor membrane proteins were identified on Western blots by reaction with streptavidin–biotin detection system.

Fluorescence-activated Flow Cytometry. Approximately, 10^6 cells were washed three times with PBS containing 0.1% BSA (PBS–BSA) and incubated with appropriately diluted anti-p38.5 at 4°C for 30 min. Cells were then washed three times with PBS–BSA and reacted with FITC–F(ab′)$_2$ goat anti–rabbit IgG for 30 min at 4°C. Finally, cells were washed three times with PBS–BSA, resuspended to a concentration of 10^6/ml, and analyzed on a FACSort® Flow Cytometer (Becton-Dickinson, San Jose , CA).

Lymphocyte-mediated Cytotoxicity. Cytolysis of tumor cells by lymphocytes was measured as described previously (13, 15). In brief, 100 μl of ^{51}Cr-labeled target cells (2×10^4 cells/ml) was mixed with 100 μl of lymphocytes (2×10^6 cells/ml) to yield serial twofold dilutions of E/T cell ratios of 100:1 to 6:1. Cells were incubated at 37°C for 3 h in 5% CO_2. Incubation of target cells without the effector cells and in media alone served as control for spontaneous release of ^{51}Cr, and wells without effector cells but with 1% SDS provided the maximum amount of radioactivity present. Cytolysis was calculated as percent cytotoxicity = $100 \times ([CPM_{exp} - CPM_{spont.}] / [CPM_{max} - CPM_{spont.}])$.

Results

Binding of Tumor Plasma Membrane Protein p38.5 to Human Lymphocytes. A tagged ligand–cell adsorption technique (13) was used to identify membrane proteins from a susceptible tumor cell line that preferentially bind to NK cells. Solubilized membrane proteins from surface biotin-labeled K562 cells were reacted with immunomagnetic bead–enriched freshly isolated naive NK cells or T lymphocytes. Approximately 14 common bands were detected on Western blots of K562 plasma membrane proteins that bound to both NK cells and T lymphocytes (Fig. 1). However, a single band of 38.5 kD (p38.5) was detected from NK cell adsorbed preparations, but was not observed in T lymphocyte adsorbed preparations. This initial result suggested the possibility that p38.5 interacted with a receptor on the surface of naive NK cells that was not expressed on T lymphocytes. Accordingly, p38.5 was purified by preparative SDS-PAGE to apparent homogeneity for additional studies. The purified protein resolved as a single band of 38.5 kD molecular mass upon SDS-PAGE (Fig 2, lane *A*). p38.5 was labeled with biotin for use in studying its binding property to lymphocyte subsets (in a similar manner as that described for proteins from the crude membrane preparation). Biotinylated p38.5 bound extensively to the NK cell–enriched fraction (Fig. 2, lane *D*) and not at all to the enriched T lymphocytes (Fig. 2, lane *C*). In this experiment, unfractionated cells bound a slight amount of p38.5 (Fig. 2, lane *B*), which is consistent with the low percentage of NK cells in peripheral blood lymphocyte specimens. These data demonstrate that SDS-PAGE–purified p38.5 retained its preferential binding property for naive NK cells.

Structural Characteristics of p38.5. Initial attempts to obtain a partial amino acid sequence of the purified p38.5 were unsuccessful because the NH_2 terminus of the molecule was blocked. Consequently, p38.5 was transferred onto nitrocellulose paper and subjected to in situ protease digestion (14). Amino acid sequence analysis of resultant peptides was performed at the Harvard Microchemistry Laboratory (Cambridge, MA). An 11-mer amino acid sequence of an internal peptide was obtained with a high degree of confidence (Table 1). Comparison of this sequence with known sequences of proteins in Gen Bank did not reveal significant homology. Treatment of p38.5 with sulfhydryl reducing agents (2-mercaptoethanol and dithiothreitol) or deglycosylating enzymes (*O*-glycosidase, *N*-acetylneuramin-

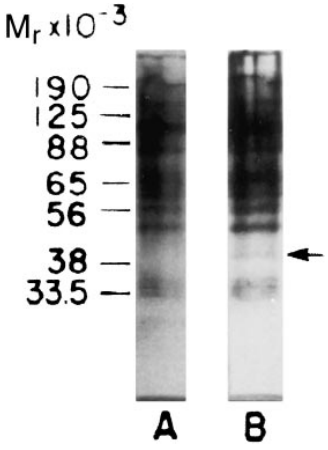

Figure 1. Binding of K562 membrane proteins to subsets of HPBL. Membrane proteins from surface biotinylated K562 cells were reacted with freshly isolated viable CD3$^-$, CD16$^+$ enriched naive NK cells and CD3$^+$ CD16$^-$-enriched T lymphocytes. Reacted lymphocytes were washed, solubilized in Laemmli sample buffer, and proteins resolved by SDS-PAGE. Lymphocyte-bound biotinylated tumor plasma membrane proteins were detected on Western blots with streptavidin–alkaline phosphatase. Lane *A*, a representative blot of surface-labeled K562 membrane proteins that bound to enriched T lymphocytes. Lane *B*, a representative blot of surface-labeled K562 membrane proteins that bound to enriched NK cells. Densitometry analysis employing Gelbase software (UVP System 5000; Upland, CA) identified 14 common bands in lanes *A* and *B* of the original blot. A protein peak of 38.5 kD was present only in the NK cell adsorbed sample.

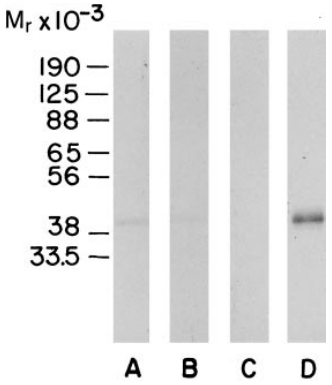

Figure 2. Binding of purified p38.5 to HPBL. The binding reaction with purified p38.5 and detection of bound biotin-labeled p38.5 was performed as described for experiments in Fig. 1. Lane *A*, a Western blot of Coomassie blue–stained purified p38.5 K562 membrane protein. Lane *B*, a Western blot of an extract of viable HPBL that were reacted with purified biotin-labeled p38.5 showing slight reactivity. Lane *C*, a Western blot of an extract of viable enriched T lymphocytes that were reacted with purified biotin-labeled p38.5 revealing little or no reactivity. Lane *D*, a Western blot of an extract of viable enriched NK cells that were reacted with purified biotin-labeled p38.5 demonstrating extensive reactivity.

Table 1. *Amino Acid Sequence of an Internal Peptide of K562 Membrane Protein, p38.5*

1					5					10
F	V	N	W	Q	V	D	G	E	Y	R

p38.5 was purified from an extract of K562 membrane proteins by preparative SDS-PAGE as described in Materials and Methods and the purified protein was electroblotted onto nitrocellulose paper. In situ protease treatment of the blotted protein and HPLC chromatography of the p38.5 digest were performed according to Aebersold et al. (14). A total of 152 peaks were detected on the chromatogram but only a few appeared to be ammenable to sequencing (based on the intensity and the homogeneity of the peaks). Peaks 17, 42, 72, and 102 were sequenced, but only peak 72, as shown above, provided unambiguous amino acid data.

idase II and peptide-*N*-glycosidase F, deglycosylation kit; Glyko, Inc., Novato, CA) did not alter the molecular mass of the molecule, suggesting that the isolated protein is a monomer and nonglycosylated (Das, B., unpublished data).

Expression of p38.5 on Established Tumor Cell Lines. Because p38.5 (derived from K562 cells) exhibited selective binding to naive NK cells, it was of interest to examine the expression of this molecule on the plasma membrane of NK-resistant cell lines as well as other NK-sensitive tumor cell lines. Affinity-purified antibody raised against the internal 11-mer synthetic peptide of p38.5 (anti-p38.5) was used for this purpose. Anti-p38.5 was reacted with three NK-sensitive and three NK-resistant tumor cell lines and analyzed separately by flow cytometry. As shown in Fig. 3 *A*, anti-p38.5 reacted with the surface of NK-sensitive tumor cell lines, K562 (erythroleukemia), Jurkat (T cell lymphoma), and Molt-4 (T cell leukemia). In contrast, anti-p38.5 did not bind to the three NK-resistant tumor cell lines A549 (lung adenocarcinoma), Raji (Burkitt lymphoma), and MDA–MB231 (breast carcinoma). Plasma membrane expression of p38.5 was also studied by anti-p38.5 immunoprecipitation of surface-labeled (biotin) membrane proteins of the above tumor cells. Western blot analysis of the immunoprecipitates showed the presence of a single 38.5-kD biotinylated protein from NK-sensitive cell lines, whereas biotinylated proteins were not observed when the NK-resistant cell lines were employed (Fig. 3 *B*). These results demonstrate an association between the expression of p38.5 on the surface of tumor cell lines and their susceptibility to NK cell–mediated lysis.

Cell Surface Expression of p38.5 on NK-resistant Variants of Jurkat and Molt-4 Cell Lines. Though the expression of p38.5 is associated with susceptibility of well-established tumor cell lines to NK cell–mediated cytolysis, one must interpret such data with caution as the cell lines are derived from different tissues and are likely to have been transformed by different mechanisms. This issue could however be more readily addressed by examining the association of NK susceptibility and p38.5 expression in variants derived from wild-type tumor cells. After long-term culture of Jurkat and Molt-4 cell lines, we observed a substantial reduction in their susceptibility to NK cell–mediated cytotoxicity. These variant cell lines were then analyzed for surface expression of p38.5 by anti-p38.5 immunoprecipitation. As shown in Fig. 4, variant cell lines exhibited decreased susceptibility to lysis by NK cells and expressed significantly lower amounts of p38.5 on their surfaces as compared with the wild-type parental cell lines.

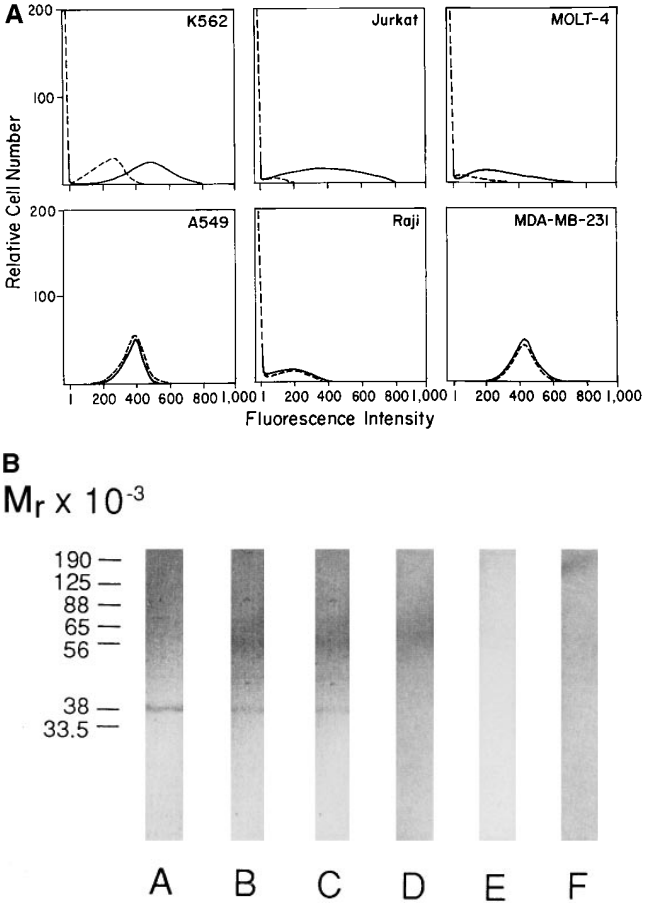

Figure 3. Plasma membrane expression of p38.5 on NK-susceptible and NK-resistant tumor cell lines. (*A*) Flow cytometry analysis of anti-p38.5–treated tumor cell lines. Cells were treated with anti-p38.5 followed by goat anti–rabbit $F(ab')_2$ IgG–FITC (—). Control tumor cells were treated with secondary antibody alone (- - -). Nonimmune rabbit serum had similar reactivity as secondary antibody alone (data not shown). The NK-sensitive cell lines, K562, Jurkat, and Molt-4, were positive for anti-p38.5, whereas NK-resistant cell lines, A549, Raji, and MDA-MB-231, were not reactive with p38.5 antibody. (*B*) Analysis of anti-p38.5 immunoprecipitates from surface-labeled cells. Western blots of anti-p38.5 immunoprecipitated proteins from surface biotinylated cells: K562 (lane *A*), Jurkat (lane *B*), Molt-4 (lane *C*), Raji (lane *D*), A549 (lane *E*), and MDA-MB-231 (lane *F*). Proteins in these lanes were probed with streptavidin–alkaline phosphatase to detect cell surface-biotinylated proteins. Biotin labeling and immunoprecipitation of plasma membrane proteins have been described previously (13).

Effect of Purified p38.5 on NK Cell–mediated Cytolysis. The preferential binding of p38.5 to NK cells suggested that the molecule may be involved as a ligand in the cytolytic process mediated by naive NK cells. To examine this possibility directly, a purified preparation of p38.5 was incubated with human lymphocytes and then tested in a standard cytotoxicity assay against ^{51}Cr-labeled K562 target

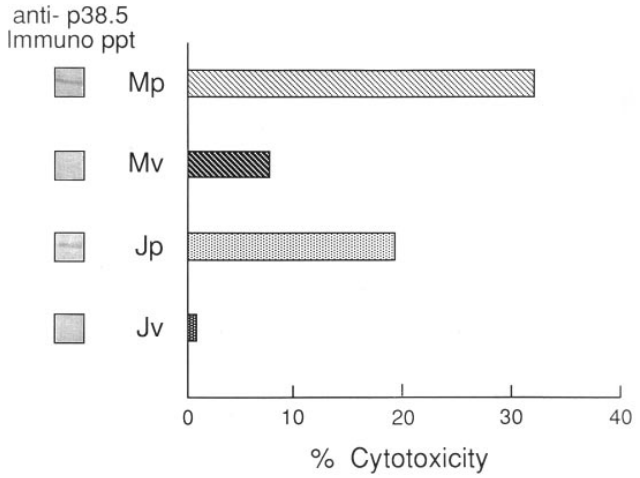

Figure 4. Relationship between cell surface expression of p38.5 on normal and variant tumor cell lines and susceptibility of tumors to NK cell–mediated lysis. Parental cell lines, Mp, Jp; variant cell lines, Mv, Jv. After long term culture of Jurkat (*J*) and Molt-4 (*M*) cell lines, substantial reduction in NK cell–mediated cytolysis was observed. These variant cell lines were analyzed for surface expression of p38.5 by specific antibody as described above. Membrane proteins derived from 1×10^6 cells were loaded in each lane. The level of cytolytic activity was directly related to the apparent degree of expression of surface p38.5. Gelbase analysis (UVP, Upland, CA) of the blots for protein bands from parental cell lines demonstrated relative single peak heights of 28 and 25, respectively. Similar analysis of the blots for protein bands from the variant cell lines did not reveal anti-p38.5 reactive protein. However, a very light band was visible by the naked eye (for Mv).

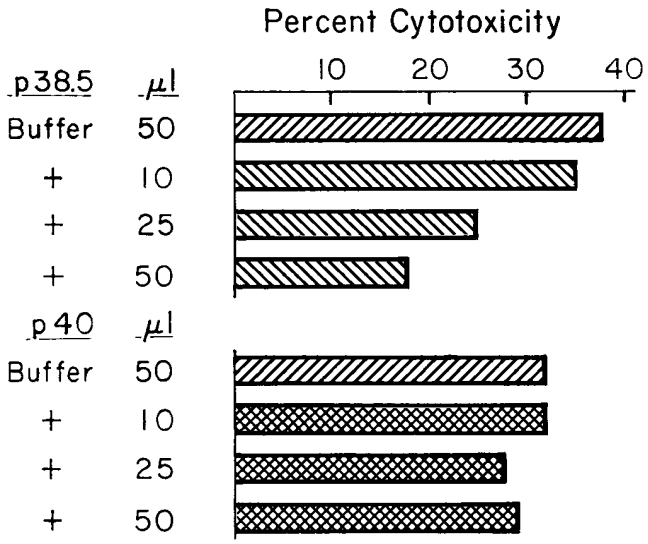

Figure 5. Effect of purified K562 membrane proteins on naive NK cell–mediated cytotoxicity. HPBL were preincubated with the indicated amount of purified proteins (~45 ng/μl based on amino acid sequence data) at 37°C for 30 min and then added to ^{51}Cr-labeled K562 cells at an E/T ratio of 100:1 as previously described. Percent cytotoxicity was determined after 3 h incubation by the release of ^{51}Cr (13, 15). Representative data of two experiments is shown. The cytotoxicity data were analyzed by the Student's *t* test. p38.5, 10 μl, not significant; p38.5, 25 μl, $P < 0.01$; p38.5, 50 μl, $P < 0.001$ compared with buffer control. p40 values compared with buffer control were not significant at any concentration.

cells. Incubation of lymphocytes with the purified p38.5 preparation inhibited naive NK activity in a concentration-dependent manner (Fig. 5). A K562 membrane protein of 40 kD, purified by the same procedure as p38.5, did not affect NK activity. These data provide direct evidence that interaction of tumor surface protein p38.5 with its NK cell receptor is necessary for cytotoxicity, because binding of soluble ligand to the NK cells before contact with K562 cells inhibited their lysis.

Characterization of NK Cell Receptor of p38.5. To delineate further the preferential interaction of p38.5 with NK cells relative to T lymphocytes, we conducted investigations to identify an interactive molecular species on effector cells. In direct binding assays, biotin-labeled purified p38.5 was reacted with either NK cell or T lymphocyte membrane proteins that had been previously immobilized on Western blots. Labeled p38.5 was detected as a single band at 70 kD on blots of NK cell membrane proteins, whereas reactive bands were not detected on blots of T lymphocyte membrane proteins (Fig. 6). This result suggests that a protein of 70 kD from naive NK cells binds cell surface p38.5 from NK cell–susceptible tumors and that the 70-kD protein is either not present or is present in substantially lower amounts in T lymphocytes.

To establish the immunologic identity of the ligand bound to the NK cell receptor, anti-p38.5 serum was used. A Western blot of peripheral blood lymphocyte membrane proteins was initially incubated with a solubilized crude extract of K562 membrane proteins, washed, and then reacted with anti-p38.5. The antiserum reacted with a band at 70 kD rather than at 38.5 kD (Fig. 7, compare lanes *C* and *D* to lane *A*). Western blots of lymphocyte membrane proteins that were not preincubated with the tumor proteins did not react with anti-p38.5 (Fig. 7, lane *B*). These results suggest that the 38.5-kD surface molecule from K562 cells that binds to the 70-kD naive NK cell protein contains the 11-mer peptide to which antibody was raised. We conclude that the 11-mer epitope of p38.5 is probably not directly involved in the receptor–ligand interaction. (Presumably, if the p38.5–70-kD interaction included the 11-mer

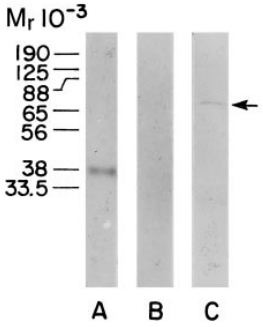

Figure 6. Identification of a p38.5-binding naive NK cell membrane protein by direct interaction on Western blots. Lane *A*, a Western blot of Coomassie blue–stained purified p38.5 K562 membrane protein. Lane *B*, a Western blot of T lymphocyte proteins that were reacted with purified biotinylated p38.5. Bound p38.5 was not detected on this blot indicating the absence of reactive T lymphocyte proteins. Lane *C*, a Western blot of NK cell proteins that were reacted with purified biotinylated p38.5. Labeled p38.5 was detected at ~70 kD demonstrating a preferential interaction with the latter immobilized NK cell protein. The reactivity of p38.5 with the 70-kD NK cell protein was markedly reduced when an extensively denatured (by boiling) preparation of NK cell proteins was used (data not shown).

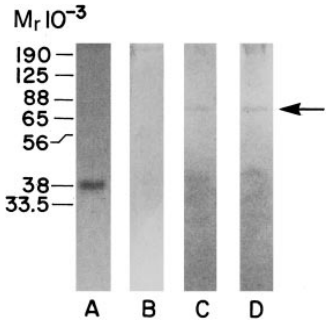

Figure 7. Immunologic identification of the p38.5 ligand bound to the 70 kD NK cell protein. All lanes were developed with goat anti–rabbit alkaline phosphatase. Lane *A*, a Western blot of K562 membrane protein extract that was reacted with affinity-purified anti-p38.5. Lane *B*, a Western blot of HPBL membrane proteins reacted with affinity-purified anti-p38.5. Note the absence of anti-p38.5–reactive proteins. Lane *C*, a Western blot of HPBL membrane proteins reacted with K562 membrane extracts for 2 h at ambient temperature, washed, and probed with anti-p38.5. Note the detection of a band at an approximate molecular mass of 70 kD. Lane *D*, same as lane *C* with twice the amount of K562 membrane proteins.

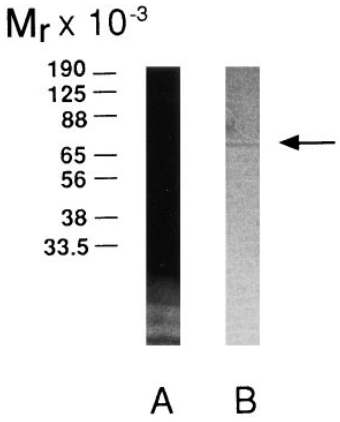

Figure 8. Plasma membrane localization of the 70-kD NK cell tumor binding protein. Solubilized membrane proteins from surface-biotinylated naive NK cells were applied to a p38.5–Sepharose 4B affinity column. After extensive washing with 0.5 M NaCl buffer to remove nonspecifically bound NK cell membrane proteins, remaining proteins were eluted by solubilization of the beads with Laemmli sample buffer and resolved by SDS-PAGE. p38.5–Sepharose 4B–bound NK cell membrane proteins were detected on Western blots using standard streptavidin–alkaline phosphatase reaction. Lane *A*, crude extract of NK cell membrane proteins from surface-biotinylated NK cells before affinity chromatography. Lane *B*, a Western blot of NK cell membrane protein(s) specifically bound to the p38.5 affinity column. A single discreet NK cell band at 70 kD was detected after affinity chromatography.

epitope of p38.5, antibody to the latter determinant could not bind to the complex).

The following experiment was performed to confirm that a 70-kD NK cell protein that specifically binds p38.5 is localized on the plasma membrane. Solubilized membrane proteins from surface-biotinylated naive NK cells were applied to a p38.5-Sepharose 4B column. After extensive washing with high salt buffer, specifically bound protein was extracted in denaturing buffer and subjected to SDS-PAGE and Western blot analysis. A single discreet biotinylated band of 70 kD from NK cells was detected on the blots suggesting that this p38.5 binding protein is located on the exterior of the plasma membrane (Fig. 8, lane *B*). One possibility is that the 70-kD, p38.5 binding protein is the inducible form of heat shock protein (Hsp 70). We performed flow cytometry studies using antibody to Hsp 70 (Stressgen Biotech. Corp., Victoria, BC Canada; Clone C92F3A-5) to determine surface expression of this moleule on T and NK cells. Such experiments indicated that neither NK nor T cells expressed Hsp70 on their plasma membranes in contrast with K562 cells that served as a positive control (Norin, A.J., unpublished data).

Discussion

Previous attempts during the past two decades to identify recognition structures exclusive to NK cell–tumor interaction have been unsuccessful (reviewed in reference 8), though important components on both NK cells and on tumor cells that contribute to cellular adhesion and regulation of the cytolytic process have been revealed (5, 8–12). However, these receptor–ligand interactions do not appear to be unique to NK cells, because they also occur between T lymphocytes and respective target cells (10). The limited number of NK cells available for biochemical studies (<5% in peripheral blood) has undoubtedly contributed to the difficulty in analysis of NK cell–specific receptor–ligand interactions. Many investigators have addressed this issue by the expansion of freshly isolated NK cells in vitro using a variety of culture systems employing growth factors and/or feeder cells (11, 12, 16). Such techniques have provided sufficient numbers of cells for immunologic and biochemical studies but at the expense of altering the phenotype of the freshly isolated NK cells from a naive state to an activated one, as demonstrated by induction of the Lag 3 protein (17). It is possible that activation results in decreased expression of naive NK cell recognition structure(s), thereby preventing their detection. In the present study, several strategies were used to overcome the difficulties of heterogeneity and the low frequency of NK cell in HPBL preparations. Only freshly isolated NK cells were used. Cells from individuals that demonstrated activity against NK-resistant LAK-sensitive tumor cell lines were not used, because the activated NK cells in these fresh preparations might mask the detection of naive NK cell–specific ligands. Finally, a tagged ligand–cell adsorption technique (13) was used with enriched NK cell preparations to enhance the likelihood of detection of tumor proteins that selectively bound to the naive killer cells.

Using the above approaches and additional biochemical and immunologic techniques, we demonstrate a novel 38.5-kD protein on the plasma membrane of certain tumor cell lines that preferentially reacts with a surface component of naive human NK cells. The interaction appears to be unique to NK cells, because T lymphocytes did not bind p38.5. However, binding studies have not been conducted with B cells, monocytes, or polymorphonuclear leukocytes. In further experiments, a 70-kD protein on the plasma membrane of NK cells was identified as a p38.5 binding molecule. Consistent with p38.5 cellular binding studies, a 70-kD receptor was not detected on T lymphocytes. Addition of purified p38.5 to fresh lymphocyte preparations before incubation with K562 target cells substantially decreased NK cell lytic activity. Preferential interaction of p38.5 with NK cells and its blocking activity in functional assays are properties consistent with a role in an early recognition event in NK cell–mediated tumor cytolysis.

Additional evidence of a role for p38.5 as a target ligand in naive NK cell–mediated cytotoxicity is provided by data

demonstrating an association of the expression of this molecule and susceptibility to cytolysis of different tumor cell lines. Flow cytometry and immunoprecipitation studies (of surface labeled cells) revealed that p38.5 is expressed on NK susceptible targets such as K562, MOLT-4, and Jurkat, whereas this molecule was not detected on the plasma membrane of NK-resistant LAK-sensitive targets such as Raji, A549, and MDA-MB-231, suggesting that p38.5 is not involved in LAK-mediated cytotoxicity. The functional role of p38.5 in NK cell–mediated cytolysis was also demonstrated in studies of p38.5 loss variants. After long-term culture of wild-type, NK-sensitive Jurkat and Molt-4 cell lines, variants were isolated that exhibited decreased levels of p38.5 and reduced susceptibility to lysis by NK cells. This property was not due to a phenotypic alteration in the cells as a result of culture conditions, because resistant clones were obtained at limiting dilution (Norin, A.J., unpublished data). These studies clearly establish a strong association between the expression of p38.5 on the tumor plasma membrane and susceptibility to NK cell–mediated cytolysis.

Recent studies suggest that cytolytic activity is affected by recognition of HLA class I polymorphisms on target cells by NK cell receptors of the C-lectin family (CD94) (18) or members of the immunoglobulin multi gene family (p50/p58) (19–24). These receptors, which we note are detected on both T cells as well as NK cells, may downregulate or upregulate cytolytic activity (10, 20, 25) depending on the subtype of receptor and/or the presence of cytosol associated clonotypic signaling molecules (18, 21–24). Inhibitory receptors apparently interfere with proximal signaling events such as Ca^{2+} flux, phospholipase Cγ2 activity, and inhibition of specific tyrosine kinases activities (lck and ZAP-70), whereas the activity of other tyrosine kinases may remain unchanged (18, 26, 27). Experimental results in the current study are likely not influenced by the above families of molecules, because K562 cells do not express HLA class I or class II molecules (11, 12).

Current concepts regarding the mechanism of lymphocyte-mediated cytolysis have focused on three alternative pathways: (*a*) Fas–FasL-induced apoptosis of tumor cells (28–33), (*b*) extracellular ATP-mediated osmotic lysis (13, 34–36), and (*c*) granule exocytosis of effector molecules such as perforin and granzymes (29, 37–40). Clearly, NK cell–induced cytolysis is not mediated by Fas–FasL interaction, because K562 tumor cells do not express Fas and naive NK cells do not express FasL (41; Das, B., unpublished data). Previous reports by us and others have suggested that NK cells use the ATP–osmotic lysis pathway (13, 35) and the granule exocytosis pathway (28, 33). Further studies are likely to reveal which lytic pathway is mediated by the interaction of tumor surface ligand p38.5 with NK cell plasma membrane proteins.

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