Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and Nectin-2 (PRR-2/CD112)

Running Title; DNAM-1 interaction with its ligands PVR and Nectin-2

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Abstract

CD226 (DNAM-1) is an adhesion molecule involved in NK and T-cell-mediated cytotoxicity against certain tumors. Here we have identified the human poliovirus receptor related (PRR) family members CD155 (poliovirus receptor; PVR) and CD112 (nectin-2/PRR-2) as the ligands for human CD226. Ectopic expression of human CD155 and/or CD112 rendered mouse BW5147 T cells more susceptible to IL-2-activated T cell and NK cell-mediated cytotoxicity, and killing was specifically inhibited by anti-CD226 mAb, demonstrating functional interactions of CD226 with CD155 and CD112. Although the binding affinities between soluble CD226 and CD155 or CD112 were comparable, the homophilic interaction of cell surface CD112 may adversely affect CD226 binding to CD112. We also demonstrate that ligation of CD226 and LFA-1 with their respective ligands cooperate in triggering cytotoxicity and cytokine secretion by T cells and NK cells.

Introduction

Intercellular adhesion molecules play important roles in lymphocyte-mediated immune responses (reviewed in (1,2)). In order to mediate their immune functions, CTL and NK cells require engagement of intercellular adhesion molecules on these effector cells to their respective ligands expressed on target cells.

We previously reported an adhesion molecule DNAM-1 (CD226) that is a member of immunoglobulin superfamily containing two Ig-like domains of the V-set and is encoded by a gene on human chromosome 18q22.3 (3,4). CD226 is a ~65 kDa glycoprotein expressed on the majority of NK cells, T cells, monocytes and platetelets and a subset of B lymphocytes. We observed that anti-CD226 monoclonal antibody (mAb) blocked CTL and NK cell-mediated cytolysis of some, but not all, tumor targets, suggesting that certain tumor cells may express cell surface ligands for CD226 (3). CD226 is co-immunoprecipitated with LFA-1 from NK cells and anti-CD3-stimulated T cells (5). Cross-linking LFA-1, but not CD226, on CD3-stimulated T cells resulted in tyrosine phosphorylation of CD226, which is mediated by the Src-family tyrosine kinase Fyn. Recently, we have reported that CD226 is involved in LFA-1-mediated

costimulatory signals for triggering naive T cell differentiation and proliferation (6). However, it has been uncertain how adhesion and intracellular signaling are mediated by the complex of LFA-1 and CD226. To address this issue, it is necessary to identify the CD226 ligand and determine the structural requirements involved in CD226 binding. In the present study, we describe the structural characteristics of CD226 interaction with its ligands and the functional relationship of CD226 with LFA-1.

Materials and Methods

Antibodies, cells and cDNA

The anti-CD155 mAb TX-24 and the anti-CD112 mAb TX-31 were generated by standard methods. Anti-CD155 (P286) and anti-CD226 (DX11) were generously provided by Dr. Akio Nomoto (University of Tokyo) and by Dr. Joe Phillips (DNAX, Palo Alto, CA), respectively. Anti-CD111 (R1.302.12) and anti-CD112 (R2.477.1) were purchased from Coulter/Immunotech (Marseille). Anti-CD18 (TS1/18) and anti-mouse ICAM-2 (3C4) were obtained from ATCC and BD Pharmingen, respectively. Human CD3+ T cells and CD3-CD56+ NK cells were purified from peripheral blood by using a MACS Magnetic cell sorting system (Miltenyi Biotec) or by sorting using flow cytometry and were cultured in the presence of IL-2 (100 U/ml). CD155α and CD155δ cDNAs and CD111 cDNA were kindly provided by Dr. Akio Nomoto and Dr. Yoshimi Takai (Osaka University, Osaka), respectively.

Expression of the Fc Fusion Proteins

CD226-Fc fusion protein was described previously (7). CD155-Fc and CD112-Fc

fusion proteins were also generated with chimeric cDNAs of the entire extracellular domain of CD155 or CD112 with the human IgG_1Fc, as described (7). The ICAM-1-Fc was purchased from R & D Systems (Minneapolis).

Surface Plasmon Resonance Experiments

Binding experiments were carried out using surface plasmon resonance as implemented in the BIAcore 3000 (BIAcore AB, St. Albans, UK). For experiments to determine the binding affinity of CD226 for its ligands, CD226-Fc (20 μg/ml in 10 mM sodium acetate, pH 4.5) was directly immobilized to the CM5 sensor chip surface by ammine coupling using the manufacture's kit (BIAcore AB) and an activation time of 5 min, resulting in 3261-3640 resonance units (RU) of immobilized ligand. Equilibrium binding analysis was performed as described (8). The following equation was used for calculation of dissociation constants based on equilibrium binding analysis.

$K_D=C(1-Req)/Req$, where K_D, C and Req are equilibrium dissociation constant, concentration of analytes and resonance unit at equilibrium.

Cytotoxicity assay and ELISA

Cytolytic activity was determined by a standard 4-h ^{51}Cr-release assay, as described (3).

Interferon-γ concentrations in culture supernatants were determined using an ELISA kit (Biosource International, Camarillo, CA).

Results

Cloning of CD226 ligands

To characterize the CD226 ligand, we examined the expression of CD226 ligand on several tumor cell lines by staining with the CD226-Fc. As shown in **Fig 1A**, CD226-Fc bound to non-hematopoietic tumor cell lines, including the osteosarcoma cell HOS, the microglioma cell U87-MG and the rhabdomyosarcoma cell RD, but not to the EBV-transformed B lymphoblastoid cells, T cell leukemia H9 and monocytic leukemia U937.

To identify a cDNA encoding the CD226 ligand, we screened Ba/F3 cells infected with a retroviral cDNA library prepared from the HOS cells, using CD226-Fc as a probe. We isolated a cDNA that encodes the human poliovirus receptor (PVR) α (CD155). CD226-Fc stained Ba/F3 cells transfected with the isolated *PVRα*cDNA, indicating that PVRα is capable of binding with CD226 (Fig 1B). We also found that CD226-Fc stained the transfectant expressing PVRδ (Fig 1B), an isoform of CD155 with the same ectodomain sequence as PVRα (9,10). CD155 is a member of the poliovirus receptor-related (PRR) family, which consists of CD111 (nectin-1/PRR-1),

CD112 (nectin-2/PRR-2), PRR-3 and PRR-4, in addition to CD155. Because the ectodomains of the PRR family molecules are composed of three Ig-like domains that share 30 to 55% amino acid identity (11), we examined whether CD111 and CD112 also can bind to CD226. As demonstrated in **Fig 1B**, CD226-Fc bound to the transfectant expressing CD112, but not CD111.

To confirm cell surface expression of CD226 ligands CD155 and/or CD112 on tumor cell lines that bind CD226-Fc, we generated mAbs against CD155 (TX-24) and CD112 (TX-31). Tumor cell lines, including HOS, U87MG, RD and Colo-205 that bind CD226-Fc (**Fig 1A** and data not shown), express both CD155 and CD112. However, EBV-B cells that did not bind CD226-Fc also did not express CD155 or CD112 (**Fig 1C**).

Affinity of CD226 for its ligands

To confirm the specific interaction of CD226 with CD155 and CD112, we generated human CD155-Fc and CD112-Fc proteins. These soluble fusion proteins bound to the BW5147 transfectant expressing human CD226, as determined by flow

cytometry (**Fig 2A**). However, we observed that the mean fluorescence intensity of the CD155-Fc-binding transfectant was significantly higher than the CD112-Fc–binding transfectant, although the same amounts of the fusion proteins were used for staining (**Fig 2A**). Conversely, the mean fluorescence intensity of the CD226-Fc-binding transfectant expressing CD155 was higher than that of the transfectant expressing CD112 (**Fig 2A**), in spite of the similar amount of expression of CD155 and CD112 on each transfectant (data not shown). These results suggest that the affinity of CD155 to CD226 may be higher than that of CD112 to CD226.

We therefore analyzed CD226-Fc: ligands (i.e., CD155-Fc and CD112-Fc) interactions by using surface plasmon resonance. The affinities were determined by equilibrium binding. To this end, CD155-Fc or CD112-Fc at a wide range of concentrations were injected sequentially over immobilized CD226-Fc (**Fig 2B**, upper panel). A plot of the binding at equilibrium versus concentration indicated that the binding of CD226-Fc to both CD155-Fc and CD112-Fc was saturable (**Fig 2B**, lower panel). Based on the equilibrium binding analysis, we determined the binding affinities between CD226-Fc and CD155-Fc or CD112-Fc (Kd=2.3 x 10^{-7} M and 3.1 x

10^{-7} M, respectively). This small difference in the binding affinity did not seem to be enough to account for the difference in the bindings of CD226-Fc to the BW5147 transfectants expressing CD155 or CD112. It is of note that CD112-Fc also bound to the transfectant expressing CD112 itself (Fig 1A), consistent with the previous report that CD112 can also mediate homophilic binding (12,13). In fact, we observed homotypic cell aggregation of the BW5147 transfectant expressing CD112, but not of the BW5147 transfectants expressing CD155 or CD226 (data not shown). To examine whether the homophilic interaction of CD112 adversely affects CD226-Fc binding to the BW5147 transfectant expressing CD112, the transfectants was pretreated with the anti-CD112 mAb (R2.477.1), which recognizes an epitope at one of the C-set Ig-like domains of CD112 (14) and blocks homophilic binding of CD112 (13), and then stained with CD226-Fc. As shown in **Fig. 2C**, CD226-Fc binding to the CD112 transfectant was augmented substantially after pretreatment with the R2.477.1mAb, while TX-31 anti-CD112 mAb partially inhibited CD226-Fc binding to the CD112 transfectant. These results suggest that CD226 binding to CD112 on cell surface may be impaired by homophilic interaction of CD112.

Interactions of CD226 and its ligands induce NK and T-cell-mediated cytotoxicity

To determine whether expression of CD226 ligands renders cells more susceptible to cytolysis, we performed cytotoxicity assays using IL-2-activated T cells and NK cells against mouse BW5147 transfectants expressing human CD155, CD112 or both. As demonstrated in **Fig 3A**, T and NK cell-mediated cytotoxicity was significantly augmented when target cells acquired cell surface expression of CD226 ligands. Moreover, anti-CD226 mAb specifically inhibited the cytotoxicity mediated by T and NK cells. These results demonstrate the functional interaction of CD226 with its ligands CD155 and CD112. It is of note that anti-CD155 (TX-24) and anti-CD112 (TX-31) mAbs partially, rather than completely, inhibited the cytotoxicity (Fig 3A), probably because these mAbs incompletely blocked interaction of CD226 with the ligands (Fig 2C and data not shown). In contrast to TX-31 anti-CD112 mAb, R2.477.1 anti-CD112 mAb augmented the CD226-Fc binding to CD112-expressing transfectant by abrogating CD112 homophilic interaction (Fig 2C), raising a question whether R2.477.1 anti-CD112 mAb increases cytotoxicity of T cells or NK cells against

CD112-expressing target cells. Unexpectedly, however, cytotoxicity by activated T cells was significantly inhibited, rather than augmented, in the presence of R2.477.1 anti-CD112 mAb (Fig 3B), in spite of increase of CD226-Fc binding to CD112-expressing transfectant (Fig 2C). The molecular mechanism of this observation is unclear at present. Nonetheless, these results indicate that CD112 as well as CD155 is a functional ligand for CD226.

CD226 and LFA-1 cooperate in cytotoxicity and cytokine secretion mediated by T cells and NK cells

BW5147 cells express murine ICAM-2 (data not shown) that interacts with LFA-1 (CD11a/CD18) on human T and NK cells (15). As described above, acquisition of CD226 ligand expression rendered BW5147 cells more susceptible to IL-2-activated T cell and NK cell-mediated cytotoxicity **(Fig 3 and Fig 4A).** Interestingly, addition of anti-LFA-1 or anti-murine ICAM-2 mAbs that prevent interaction between human LFA-1 and mouse ICAM-2 inhibited the IL-2-activated T cell-mediated cytotoxicity against the BW5147 transfectants expressing CD155, CD112 or both (**Fig 4A**). These

results suggest that LFA-1 and CD226 cooperate in triggering cytotoxicity by IL-2-activated T cells.

To further examine functional relationship between LFA-1 and CD226, T cells and NK cells were directly stimulated with plate-coated ICAM-1-Fc, CD155-Fc or both. Although T cells did not secret detectable amount of cytokines, including interferon-γ and IL-2, after the stimulation, we observed that interferon-γ secretion from IL-2-activated NK cells slightly increased after ligation of either LFA-1 or CD226 with the respective ligand (Fig 4B). However, it was dramatically augmented when LFA-1 and CD226 were stimulated simultaneously with their ligands ICAM-Fc and CD155-Fc, indicating the cooperation of LFA-1 and CD226 in cytokine secretion from NK cells.

To examine whether CD226-mediated cytotoxicity requires LFA-1 ligation, we established LFA-1-deficient NK clones derived from a patient with leukocyte adhesion deficiency (LAD) (Fig 4C). While cross-linking CD226 with anti-CD226 mAb induces re-directed cytotoxicity against Fc-receptor-bearing P815 by normal NK cells expressing LFA-1 (3,5), anti-CD226 mAb did not induce the re-directed cytotoxicity by the LAD NK clones (Fig 4D), consistent with our previous report (5),

suggesting a requirement of LFA-1 for CD226-mediated activation signal. However, treatment of the LAD NK clones with phorbol 12-myristate 13-acetate (PMA) specifically restored anti-CD226 mAb-dependent re-directed cytotoxicity (Fig 4D), although it did not affect anti-CD2- or ant-CD16-dependent re-directed cytotoxicity, indicating that CD226-mediated cytotoxicity does not always require LFA-1 ligation and may depend on cell activation status. To confirm this idea, we performed cytotoxicity assays using polyclonal, but not monoclonal, LAD T cells and NK cells cultured in IL-2-containing medium, which may consist of various activation conditions of LAD T clones and NK clones. As demonstrated in Fig 4E, polyclonal LAD T cells and NK cells killed BW5147 transfectants expressing CD155 or CD112 and these cytotoxicities were completely inhibited in the presence of anti-CD226 mAb, indicating that, although CD226 and LFA-1 cooperate in cytotoxicity and cytokine secretion by T cells and NK cells, CD226-mediated cytotoxicity does not always require LFA-1 ligation.

Discussion

By expression cloning using CD226-Fc as a probe, we have identified the PRR family members CD155 (PVR) and CD112 (nectin-2/PRR-2) as ligands for CD226 (Fig 1, 2). We have shown that acquisition of human CD155 and/or CD112 rendered mouse BW5147 cells more susceptible to IL-2-activated T cell and NK cell-mediated cytotoxicity, and killing was specifically inhibited by anti-CD226 mAb (Fig 3A), demonstrating functional interactions between CD226 and its ligands CD155 and CD112. Very recently, Moretta and colleagues have also independently identified CD155 and CD112 as functional CD226 ligands by a different strategy (i.e. generating monoclonal antibodies that block NK cell-mediated cytotoxicity against certain tumors) (16). In this paper, we have explored further the structural and functional characteristics of CD226 interactions with its ligands. We have demonstrated that CD155 and CD112 are often simultaneously expressed on cells (Fig 1C), raising a question how CD226 interaction with each ligand is regulated. Although the binding affinities of CD226-Fc to CD155-Fc and to CD112-Fc were comparable (Fig 2B), homophilic interaction of cell surface CD112 may adversely affect CD226-Fc binding

to CD112 (Fig 2A, C), suggesting that CD226 may prefer CD155 to CD112 as a physiological ligand. However, abrogation of CD112 homophilic interaction by R2.477.1 anti-CD112 mAb inhibited, rather than augmented, CD226-mediated cytotoxicity, in spite of increase of CD226 binding (Fig 3B). These results suggest that, although homophilic interaction of CD112 may decrease affinity of CD226 binding to CD112, it may accumulate CD226 at immunological synapse, resulting in increase of its avidity that is capable of mediating activation signal for cytotoxicity in T cells and NK cells. Further studies are required to explore the molecular and functional relationship between CD226 and CD112.

In this paper, we have formally demonstrated that LFA-1 and CD226 cooperate in triggering T cell and NK cell-mediated cytotoxicity and cytokine secretion, using the transfectants expressing CD226 ligands (CD155 and/or CD112 or the soluble CD155) and LFA-1 ligand (ICAM-1 proteins) (Fig 4A, B). These results were consistent with our observation that cross-linking CD226 with anti-CD226 mAb did not induce re-directed cytotoxicity against P815 by LFA-1-deficient LAD NK clones (Fig 4D), suggesting a requirement of LFA-1 for CD226-mediated cytotoxicity. However,

LAD NK clones acquired ability to mediate anti-CD226-dependent re-directed cytotoxicity after stimulation with PMA (Fig 4D). Furthermore, IL-2-activated polyclonal LAD T cells and NK cells exhibited CD226-mediated cytotoxicity (Fig 4E). These results suggest that whether or not CD226-mediated cytotoxicity essentially requires LFA-1 ligation may depend on activation condition (e. g., of PKC) in each T or NK cell clones. Further studies are required to define the physical and functional relationship between LFA-1 and CD226 and their intercellular signaling in various cell types and conditions.

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Figure Legends

Fig 1. Identification of CD155 (PVR) and CD112 (PRR-2) as CD226 (DNAM-1) ligands

A. Binding of CD226-Fc fusion protein to tumor cell lines. The tumor cell lines indicated were stained with CD226-Fc fusion protein (bold line) or control human IgG (thin line), followed by FITC-labeled anti-human IgG.

B. Transfectants expressing poliovirus receptor-related family members (CD155, CD111 and CD112) were stained with CD226-Fc fusion protein (bold line) or control human IgG (dotted line), followed by FITC-labeled anti-human IgG (upper panel). Each transfectant was also stained with the FITC-labeled specific mAb indicated (bold line) or control Ig (dotted line) (lower panel).

C. The transformed cell lines indicated were incubated with Alexa Fluor 488-conjugated anti-CD155 (bold line), anti-CD112 (bold line) or control Ig (dotted line) and analyzed by flow cytometry.

Fig 2. Binding Analysis

A. BW5147 transfectants expressing CD226, CD155 or CD112 were stained with the Fc fusion proteins indicated (bold line) or control human IgG (dotted line), followed with FITC-conjugated anti-human IgG. The numbers indicate mean fluorescence intensity of the transfectants stained with the Fc fusion proteins.

B. Binding affinities between CD226-Fc and either CD155-Fc or CD112-Fc were analyzed using surface plasmon resonance as measured by using a BIAcore 3000 instrument. CD155-Fc (upper, left) or CD122-Fc (upper, right) at a range of concentrations (125, 250, 500, 750, and 1000 nM, and 160, 329, 638, 958 and 1280 nM, respectively) were injected at 5 l/min sequentially through a flow-cell containing 3640 RU of directly immobilized CD226-Fc at 25^{o}C. Background responses observed in a control flow-cell were subtracted from the total responses to calculate binding (solid lines). Based on the resonance unit at equilibrium (Req) and corresponding concentration (Conc.) of the analytes (i.e., CD155-Fc or CD112-Fc) produced (upper panel), each dot was plotted (lower panel).

C. The BW5147 transfectant expressing CD112 was pretreated with anti-CD112 (R2.477.1 or TX-31) mAb (bold line) or control Ig (thin line) and then stained with CD226-Fc, followed by staining with FITC-conjugated anti-human IgG. Dotted line indicates the BW5147 transfectant stained with control human IgG, followed by staining with FITC-conjugated anti-human IgG.

Fig 3. Functional interaction of CD226 with the ligands CD155 and CD112

A. B. CD3+ T cells and CD3-CD56+ NK cells isolated from healthy donors were cultured with IL-2. Cytotoxic activities mediated by these T cells or NK cells against BW5147 transfectants expressing CD155, CD112 or both were analyzed in the presence of the mAbs indicated.

Fig 4. Cooperation of LFA-1 and CD226 in IL-2-activated T cell and NK cell-mediated cytotoxicity and cytokine secretion

A. Cytotoxic activities by IL-2-activated T cells against BW5147 transfectants expressing CD155, CD112 or both were analyzed in the presence of the mAbs

indicated.

B. CD3-CD56+ NK cells were pretreated with human IgG to block Fcγ receptor and then stimulated with plate-coated antibodies or fusion proteins indicated for 48 hours. IFN-γsecretion in the culture supernatant was determined by ELISA.

C. CD3+ T cells and CD3-CD56+ NK cells isolated from a healthy donor or a patient with leukocyte adhesion deficiency (LAD) were stained with FITC-labeled anti-CD11a and PE-labeled anti-CD18 mAb and analyzed by flow cytometry.

D. NK clones established from a LAD patient were cultured with ^{51}Cr-labeled P815 cells in the presence of mAbs indicated. Antibody-dependent redirected cytotoxic activities by these LAD NK clones were analyzed. Data are representative in several experiments using several LAD NK clones.

E. Cytotoxic activities against BW5147 transfectants expressing CD155 or CD112 by polyclonal LAD T cells and NK cells cultured in IL-2-containing medium were analyzed in the presence of the mAbs indicated.