Transformation-associated 86 kDa Natural Killer Target Molecule Expressed on the Mouse, Rat and Human Cell Surface

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We previously reported on the 86 kDa natural killer target molecule associated with transformation of the oncogene-transfected rat fibroblasts. This molecule may participate in the lethal hit phase of cytotoxicity by natural killer (NK) cells. Originally, this molecule was defined by mAb109, but mAb109 could react only with rat tumor lines. In this report, to determine whether the 86 kDa molecule could be utilized as a natural killer target molecule in mammalian cells, we developed a polyclonal anti-86 kDa antibody (pAb109). Our data indicated that pAb109 preferentially reacted with NK-susceptible lines such as mouse YAC-1, rat W31 and human K562 cells, but reacted only weakly with NK-resistant mouse EL-4, rat fetal fibroblast WFB and human fetal fibroblast HEPM. In a cytotoxicity experiment, pAb109 F(ab')₂ fragments could inhibit the cytotoxicity by NK cells of W31 and K562 cells. However, these fragments did not inhibit the cytotoxicity of non-NK cells such as CD3⁺, CD4⁺, CD8⁺ T cell receptor $\alpha\beta$ ⁺ T cells (presumably $\gamma\delta$ T cells) to W31 cells. Taken together, these data suggest that the cell transformation-associated 86 kDa molecule may be critical in NK cytotoxicity, and a candidate for the NK target structure in mammalian tumor cells.

Key words: NK cells — Target molecule — 86 kDa

It is generally accepted that NK² cells comprise an important effector population which seems to be implicated in the host's surveillance against neoplastic cells.¹⁻⁴ However, the molecular mechanism by which NK cells recognize and destroy these neoplastic cells is unknown. In the past three years, several candidates for NK receptors were cloned.⁵⁻⁷ Furthermore, recent studies have shown that susceptibility to cytolysis by NK cells is influenced by several cell surface molecules.⁸⁻¹⁰ For example, the expression of certain MHC class I molecules may strongly reduce the susceptibility to NK cells. On the other hand, it is speculated that the cell surface expression of NK target molecules is important. Harris *et al.* suggested the expression of a candidate on an NK-susceptible K562 line.¹¹ However, the molecular nature of NK target molecules has not been clarified.

We previously demonstrated that the expression of the 86 kDa glycoprotein, composed of a single polypeptide chain, was clearly enhanced with the transformation of a rat fetal fibroblast WFB.² This protein appeared to be an NK target molecule, since mAb109^{2, 12} that recognizes this 86 kDa antigen could block the cytotoxicity of rat NK cells to an *H-ras* oncogene-induced transformant,

W31, of the WFB parental line. The blocking by mAb109 was observed even at 60 min after mixing with the NK effector and target cells, suggesting that the 86 kDa molecule may participate in the lethal hit phase of cytotoxicity by NK cells rather than the conjugation phase between NK and target cells.

Thus, the mAb109-defined 86 kDa molecule appeared to be critical as a target structure in the cytotoxicity by rat NK cells. However, mAb109 could only react with rat cells, and not with human and mouse cells, so it was not determined whether this molecule could be used as an NK target molecule in the mouse and human system as well. For this reason, we have developed a rabbit polyclonal anti-86 kDa antibody (pAb109), to examine whether the 86 kDa molecule plays a role in the cytotoxicity of NK cells. Our data demonstrated that the cell surface expression of this molecule was enhanced in NK-susceptible lines of mouse, rat and human origins, and that pAb109 F(ab')₂ fragments inhibited the cytotoxicity of NK cells against W31 and K562 targets. Collectively, our present results suggest that the transformation-associated 86 kDa molecule may be important in the host's anti-tumor surveillance mechanism by NK cells. Moreover, this molecule may act as a general NK target structure in mammalian cells.

MATERIALS AND METHODS

Animals, cells and production of polyclonal anti-86 kDa antibody (pAb109) All animals were obtained from CLEA Japan Inc., Shizuoka. W31 and W14 cells are

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² Abbreviations used in this paper: NK, natural killer; DNT, CD3⁺, CD4⁺ CD8⁺ T cell receptor $\alpha\beta$ ⁺ double-negative T cells; FACS, fluorescein-activated cell sorter; mAb, monoclonal antibody; NRS, normal rabbit serum; pAb109, rabbit anti-86 kDa antibody; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCR, T cell receptor.

H-*ras* oncogene transformants of WKAH rat fetus fibroblast WFB.^{2,12} Bras-h is also a H-*ras* oncogene transformant of BALB3T3 cells.³ W31, W14 and Bras-h are NK-sensitive, and WFB and BALB3T3 are resistant.¹² NK-sensitive mouse YAC-1 and human K562 and resistant mouse EL-4 and human HEPM were also used. In FACS analysis, we employed several mouse and human cell lines as listed in Table I. SEBV and QEBV are Epstein-Barr virus-transformed lines.

To develop pAb109, W31 cells were inoculated subcutaneously into 10- to 20-week-old WKAH rats, and W31 tumors were resected. The tumors were homogenized and lysed with 0.5% NP40. The lysate was applied to a column packed with 5 ml of Affigel 10 (BioRad Laboratories, Richmond, CA) which was conjugated with 20 mg of mAb109^{2,12} as previously described.^{13,14} The immunoaffinity-purified mAb109-defined 86 kDa antigen was separated by boiling for 5 min, and the 86 kDa protein was confirmed by SDS-PAGE and subsequent staining with Coomassie Brilliant Blue. Then the antigen was administered five times at weekly intervals to 10-week-old NZW rabbits. Animals were bled, and the pAb109 was obtained. The IgG fraction (2.0 mg/ml) of pAb109 was purified with Sepharose CL-4B coupled with protein A (Pharmacia, Sweden) as described elsewhere.^{2,12} Then, 1 ml of 50× diluted IgG of pAb109 was absorbed for 1 h at 4°C with an equal volume of the homogenized heart tissue of WKAH rats.

F(ab')₂ fragment separation F(ab')₂ fragments of pAb109 IgG as well as preimmune NRS IgG were prepared by pepsin digestion according to the method previ-

ously described.¹² F(ab')₂ fragments of mAb109 (mouse IgG1) and mAb067 (mouse IgG1)¹⁵ were also obtained in a similar manner.

Radiolabeling of proteins and immunoprecipitation The method was as described elsewhere² with slight modifications. W31 cells were surface-labeled with ¹²⁵I using lactoperoxidase, which selectively labels surface proteins. Briefly, 2×10⁷ cells were washed and resuspended in 850 μl of potassium-free PBS. Approximately 1 mCi of ¹²⁵I (New England Nuclear, Boston, MA) was added, followed by 100 μl of lactoperoxidase (Sigma Chemicals Co., St. Louis, MO). Cells were washed extensively with PBS and lysed with a lysis buffer (0.5% NP40, 50 mM Tris, 20 mM iodoacetamide, pH 7.4). Proteins were immunoprecipitated from cell lysates with purified mAb109 and pAb109. We used mAb081 and NRS as the system controls. Goat anti-mouse IgG and protein A-Sepharose CL-4B were allowed to react, and then treated with a washing buffer containing 0.05 M Tris-HCl, pH 7.6, 0.25 M NaCl, 5 mM EDTA and 0.1% Triton X-100. Samples were analyzed by SDS-PAGE on 8% acrylamide gels. For the sequential immunoprecipitations, the W31 cell lysates were first absorbed with pAb109, mAb109 (mouse IgG1), mAb081 (mouse IgG1)⁴ and NRS for 4 h at 4°C, followed by precipitation with mAb109 and pAb109. mAbY-1 (mouse IgG1) was also used as a negative control.

FACS and cytotoxicity assays The procedures of FACS analysis and cytotoxicity assays were as described.^{2,12,15} The FACS scoring system followed that in our previous paper.² In the cytotoxicity blocking experiment, ⁵¹Cr-

Fig. 1. Sequential immunoprecipitation of pAb109-defined antigen. W31 cells were labeled with ¹²⁵I using lactoperoxidase, and were immunoprecipitated with antibodies (lanes a-e). mAb Y-1 (IgG1) and mAb081 (IgG1) were used as negative and positive controls in the experimental system, respectively. The same W31 cell lysates were extensively absorbed with pAb109 (lanes f and j), NRS (g and k), mAb109 (h and l) and mAb081 (i and m), and the supernatants were immunoprecipitated with mAb109 (lanes f-i) and pAb109 (lanes j-m). The immunoprecipitates were subjected to 8.0% SDS-PAGE with 5% 2-mercaptoethanol, and were autoradiographed by exposing the gel to the film for 3 days. An arrow indicates a molecular size of approximately 86 kDa.

labeled target cells were pretreated with a saturating amount of antibodies before mixing with effector and target cells. In the rat system, poly I:C (Sigma Chemical Co.)-induced rat splenic NK cells were used as effector cells.¹²⁾ In human systems, human resting PBL NK cells were obtained by the method previously described.¹⁶⁾ We also utilized rat DNT cells as effector cells to assess whether the 86 kDa molecule is involved in the cytotoxicity of DNT cells. Separation of DNT cells was described earlier.¹⁵⁾

RESULTS

Sequential immunoprecipitation of pAb109 The specificity of pAb109 was assessed by means of sequential immunoprecipitation experiments. W31 cells were surface-labeled with ¹²⁵I using lactoperoxidase, and immunoprecipitated with various antibodies. As shown in Fig. 1, pAb109 and mAb109 formed immunoprecipitates with a molecule of approximately 86 kDa (lanes b and d, respectively). In contrast, mAb081 detected a cell surface molecule of approximately 105 kDa (lane e). Lane a shows the reactivity of preimmune NRS when used as a negative control for pAb109. We also used Y-1 mAb (lane c) as a negative control for mAbs109 and 081. A band with a similar molecular size (86 kDa) to that with pAb109 is seen in lanes (c) and (e). Similarly, a band with a similar size (105 kDa) to that with mAb081 is also observed in lanes (c) and (d). However, these bands are considered to represent background activity, since the band intensity with 86 kDa in pAb109 (lane b) and mAb109 (lane d) and that with 105 kDa in mAb081 (lane e) were clearly stronger than in the other lanes.

Next, the same W31 cell lysates were extensively absorbed with pAb109, NRS, mAb109 and mAb081, and the supernatants were immunoprecipitated with mAb109 (lanes f-i) and pAb109 (lanes j-m). Although mAb109 could form immunoprecipitates with the cell lysates absorbed with NRS (lane g) and mAb081 (lane i), it did not react with those absorbed with pAb109 (lane f) and mAb109 (lane h). This was found to be so when pAb109 was used as an immunoprecipitating antibody. The cell lysates absorbed with mAb109 (lane l) did not develop immunoprecipitates with pAb109. It was clear that when the lysates were absorbed with pAb109 (lane j), the supernatants almost completely lost their reactivity with pAb109. These data indicated that pAb109 could react with the same antigen defined by mAb109.

Cell surface expression of pAb109-defined antigen The reactivity of pAb109 against various cells was defined by FACS analysis. As shown in Fig. 2, a pAb109-defined antigen was expressed strongly on the cell surface of highly tumorigenic W31, a *H-ras* oncogene transfectant of the parental rat fibroblast line WFB. Moderately

tumorigenic W14 cells also expressed a pAb109-defined antigen. In contrast, WFB cells showed a decreased expression of this antigen. This finding is consistent with that of mAb109.^{2, 12)} Furthermore, Bras-h, an *H-ras* oncogene-transformant of BALB3T3 cells, also showed enhanced expression of this antigen, as compared with the expression on parental BALB3T3 cells (Table I). These data suggested that the 86 kDa antigen expression was associated with the cell transformation.

Our previous study with rat tumor lines indicated that mAb109-defined 86 kDa antigen may be involved in the cytotoxicity of NK cells as a target molecule. Therefore, we determined the reactivity of pAb109 against NK-susceptible or resistant mouse and human tumor lines by FACS analysis. As summarized in Table I, pAb109 reacted strongly with NK-susceptible mouse YAC-1 but not with NK-resistant EL-4 cells. This antibody also reacted clearly with human NK-susceptible K562 cells, but NK-resistant human palatal fibroblast cell line HEPM did not express pAb109-defined antigen on the cell surface. However, it was determined that certain

Fluorescence intensity

Fig. 2. FACS profiles of pAb109 reactivity (—) against WFB (a), and its *H-ras* transformants W14 (b) and W31 (c), (·····) is a control with NRS.

Table I. FACS Analysis of the Expression of pAb109-defined 86 kDa Antigen on Various Cell Lines

		Intensity of antigen expression		
		—~+ ^{a)}	++	+++
Rat	WFB		W14	W31
Mouse	EL-4		P815	YAC-1
	BALB3T3		Bras-h	
Human	HEPM		Daudi	K562
			SEBV, QEBV	

a) FACS analysis and the scoring system were as described in our previous paper.²⁾ Briefly, the median channel number was set at approximately 110 in FACS, and the following symbols represent the results: —~+, up to 25%; ++, 25 through 75%; +++, more than 75% fluorescent cells.

Fig. 3. Blocking activity of pAb109 on the cytotoxicity of poly I:C-induced rat NK cells to W31 cells. A saturating amount of $F(ab')_2$ fragments of pAb109, NRS, mAb109 and mAb081 was allowed to react for 60 min at 4°C with ^{51}Cr -labeled W31 cells, which were washed. Then, NK cells were added at a predetermined E/T ratio. Cytotoxicity assays were performed for 10 h. Bars represent mean $\pm$ SE.

Epstein-Barr virus-transformed lines such as Daudi, QEBV and SEBV clearly expressed this antigen, although these lines showed a low susceptibility to the cytotoxicity of NK cells.

pAb109-defined 86 kDa antigen in the cytotoxicity mechanism of mammalian NK cells To determine whether pAb109-defined 86 kDa antigen could be involved in the cytotoxicity mechanism of mammalian NK cells, we studied the blocking capability of pAb109 on the cytotoxicity of NK cells. As shown in Fig. 3, $F(ab')_2$ fragments of pAb109 clearly inhibited (though not completely) the cytotoxicity of poly I:C-induced rat splenic NK cells to W31 fibrosarcoma target cells. This inhibi-

Fig. 4. Blocking activity of pAb109 on the cytotoxicity of human NK cells to K562 cells. A saturating amount of $F(ab')_2$ fragments of pAb109 and NRS was allowed to react, as in Fig. 3. Cytotoxicity assays were performed for 5 h. Bars represent mean $\pm$ SE.

Fig. 5. Blocking activity of pAb109 on the cytotoxicity of poly I:C-induced rat NK cells to mouse YAC-1 cells. A saturating amount of $F(ab')_2$ fragments of pAb109 and NRS was allowed to react, as in Fig. 3. Cytotoxicity assays were performed for 5 h. Bars represent mean $\pm$ SE.

tion was similarly observed with $F(ab')_2$ fragments of mAb109. Control $F(ab')_2$ fragments of preimmune NRS and mAb081 did not affect the cytotoxicity. We also examined whether pAb109 could inhibit the cytotoxicity in the human and mouse NK system. Our data demonstrated that $F(ab')_2$ fragments of pAb109 clearly blocked the cytotoxicity of human allogeneic peripheral NK cells to K562 target cells (Fig. 4). However, pAb109 $F(ab')_2$ fragments failed to inhibit the cytotoxicity of poly I:C-induced rat splenic NK cells to YAC-1 target cells

Fig. 6. Blocking activity of pAb109 on the cytotoxicity of DNT cells. A saturating amount of $F(ab')_2$ fragments of pAb109, mAb109 and mAb067 was allowed to react with ^{51}Cr -labeled W31 cells, as in Fig. 3. Then, DNT cells and poly I:C-induced NK cells were added at an E/T ratio of 100, and the % cytotoxicity (% CTX) was determined. Cytotoxicity assays were performed for 10 h. Bars represent mean $\pm$ SE.

(Fig. 5). This was also true when we utilized poly I:C-induced mouse splenic NK cells (data not shown).

pAb109-defined 86 kDa antigen may be selectively involved in the cytotoxicity of NK cells The above data suggested that the pAb109-defined 86 kDa molecule may act as a target ligand in NK cytotoxicity. Next, we determined whether the pAb109-defined 86 kDa antigen could participate in cytotoxicity other than that of NK cells. Our previous study demonstrated that $CD3^+ CD4^- CD8^- TCR\alpha\beta^-$ DNT cells were cytotoxic to W31 cells.¹⁵⁾ This cytotoxicity was blocked by mAb067, which detects the 70 kDa heat shock protein. In the present experiment, we utilized DNT cells, and determined whether pAb109 could affect the cytotoxicity of DNT cells. As shown in Fig. 6, $F(ab')_2$ fragments of pAb109 as well as mAb109 could not block the cytotoxicity of rat splenic DNT cells to W31 target, although these fragments could strongly inhibit the cytotoxicity of poly I:C-induced rat splenic NK cells.

DISCUSSION

It has been considered that NK cells may play an important role in various areas of immunobiology, such as the regulation of hematopoiesis, elimination of virally infected cells and transplanted graft rejection.^{17, 18)} Re-

cently, candidates for NK receptors have been reported by several groups, indicating that these receptors are members of the type II transmembrane C-type lectin family.^{5-7, 19)} These studies strongly support the notion that the ligand, which is presumed to be the NK target structure, to these NK receptors must exist in NK susceptible cells. However, the molecular nature of the NK target structures has not been elucidated except in a few studies.^{11, 12)}

We previously reported on the mAb 109-defined 86 kDa cell surface antigen.²⁾ Expression of this antigen was greatly enhanced with the transformation of WFB cells. In cytotoxicity assays, poly I:C-induced rat splenic NK cells were cytotoxic to WFB-H-ras transformants, W14 and W31, but not to parental non-transformed WFB. It was also indicated that $F(ab')_2$ fragments of mAb109 strongly inhibited the cytotoxicity of NK cells. Furthermore, it was suggested that this molecule may participate in the lethal hit phase of cytotoxicity by NK cells rather than the conjugation phase between target and NK cells.¹²⁾ Thus, this molecule appeared to be critically involved in the cytotoxic mechanisms of NK cells, possibly as the target molecule. However, mAb109 could react only with rat cells.

In order to clarify the expression and role in the cytotoxicity of this 86 kDa molecule in the mouse and human systems as well, we developed a polyclonal antibody, pAb109. We found that pAb109 preferentially reacted with NK-susceptible lines such as mouse YAC-1, rat W31 and human K562 cells, but only weakly reacted with NK-resistant mouse EL-4, rat WFB and human fetal fibroblast HEPM. pAb109 $F(ab')_2$ fragments inhibited the cytotoxicity of NK cells to W31 and K562 cells. Thus, the 86 kDa molecule may be involved in the cytotoxicity of NK cells to these rat and human cells.

YAC-1 is highly susceptible to NK cells. However, our present data indicated that pAb109 could not inhibit the cytotoxicity to YAC-1 cells. Perhaps YAC-1 cells express different sets of NK target molecules, rather than simply expressing the 86 kDa molecule alone, on the cell surface. Indeed, our previous study suggested the polymorphism of NK target structures,¹²⁾ since complete inhibition of the cytotoxicity by mAb109 was not seen. Thus, strong inhibition of the cytotoxicity against YAC-1 target may require a mixture of antibodies reacting against these NK target structures.

We previously showed that W31 cells were lysed not only by poly I:C-induced rat NK cells^{2, 12)} but also by other cytotoxic cells such as $CD3^+ CD4^- CD8^- TCR\alpha\beta^-$ rat DNT cells, perhaps of T cell receptor $\gamma\delta$ type.¹⁵⁾ Our present study indicated that pAb109 inhibited the cytotoxicity to W31 cells of rat NK marker 3.2.3.²⁰⁾ antigen-positive cells but not that of DNT cells. Our preliminary study also suggested that the cytotoxicity of

CD8⁺ TCR $\alpha\beta$ ⁺ type cytotoxic T cells was not inhibited by pAb109 (data not shown). These data suggested that the 86 kDa molecule may be selectively involved in the cytotoxicity of NK cells. Moreover, pAb109 did not inhibit the conjugate formation between NK and target cells (data not shown). Taken together, these data support our notion that the 86 kDa molecule acts as the NK target structure.

Currently, it is not known whether the 86 kDa molecule directly interacts with NK cell receptors. Frey *et al.* have studied the mechanism of target cell recognition by NK cells by using a rabbit anti-idiotypic serum against mAb36 that reacted with the cell membrane of K562. They concluded that 80, 110, and 150 kDa proteins seemed to be important for both recognition and triggering of NK-mediated lytic function.²¹⁾ Our preliminary

study suggested that anti-idiotypic mAbs developed against mAb109 could react selectively with the 3.2.3. antigen-positive rat NK cell line RNK16,²²⁾ but not 3.2.3.-negative cells. These anti-idiotypic mAbs may recognize the NK receptor itself. If so, it is probable that the 86 kDa molecule defined by mAb109 and pAb109 interacts directly with the NK receptors. We are now investigating this point.

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