Fas-mediated Cytotoxicity by Freshly Isolated Natural Killer Cells

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Summary

The expression of Fas ligand on natural killer (NK) cells and Fas-mediated cytotoxicity by NK cells was investigated. Fas ligand mRNA was expressed in freshly isolated NK cells but not in T cells. Furthermore, the Fas ligand was detected on the cell surface of NK cells by staining with soluble Fas molecule. We analyzed the cytolytic activity of NK cells against thymocyte targets from normal and lpr mice, and found that the NK cells killed thymocytes from normal mice but not from lpr mice. On the other hand, splenic T cells did not show any cytotoxicity against either of the thymocyte targets. Similarly, NK cells exhibited cytotoxicity against transfectants expressing Fas antigen but not against parental cells or transfectants expressing a mutant Fas antigen with deleted cytoplasmic region. These results demonstrated that NK cells express Fas ligand and possess the capability of killing target cells expressing Fas antigen on their surface. This finding suggests that NK cells play an important role by eliminating Fas-expressing cells either constitutively or inducibly in peripheral lymphoid organs.

Materials and Methods

Mice. MRL/Mpj+/+ (MRL +/+ ) and MRL/Mpj-lpr/lpr (MRL lpr/lpr) mice were obtained from the Shizuoka Laboratory Animal Corporation (Hamamatsu, Shizuoka, Japan). C57BL/6J Jcl (B6) mice were obtained from the Japan Clea Animal Corporation (Tokyo, Japan).

Cell Preparation. Cells were purified as previously described (13). Briefly, splenocytes from 6-8-wk-old B6 mice were treated with magnetic beads (Advanced Magnetics, Inc., Cambridge, MA) coupled with goat anti-mouse-IgG Ab (Cappel, Organon Teknika Co., West Chester, PA) to remove surface Ig (sIg) + B cells. The sIg- splenocytes, >90% being CD3 + cells, were used as the T cell population. To isolate NK cells, the sIg- splenocytes were mixed with anti-CD4 mAb (GK1.5) and anti-CD8 mAb (53.6.7) followed by incubation with magnetic beads coupled with goat anti-rat IgG Ab (Cappel) to enrich NK cells. The residual cells were then incubated with PE-anti-NK1.1 mAb and biotin-anti-CD3 mAb (PharMingen, San Diego, CA) followed by Tri-Color-streptavidin (Caltag Laboratories, San Francisco, CA). The stained cells were sorted into NK1.1 + CD3- cells by FACStar plus (Becton Dickinson & Co., Mountain View, CA). The purity of the sorted cells was always >99%.

Reverse Transcription (RT)-PCR. Total cellular RNA was extracted with guanidinium-isothiocyanate method. Single strand cDNA was synthesized with reverse transcriptase from 0.5 μg RNA and was used for PCR reaction. Primer sequences were as follows: β-actin: 5't-ATGTAATCATCCCTCTGAGCTCATGAAC and 3't-GAAAACGCACACCTAGAACAAC; Fas ligand: 5't-ATGTTTCTGGCTGTCTGTGCTGCT and 3't-ATTTGTGAGGGGTCTGTTGCTGTC. Various amounts of cDNAs were amplified in PCR under the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min with 20 cycles for β-actin or with 24 cycles for Fas ligand. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized by UV light illumination.
Fas Ligand Detection by Soluble Fas-Ig Molecule. Soluble Fas-Ig chimeric molecule was kindly provided by Dr. S. Nagata (Osaka Bioscience Institute, Suita, Japan) (5). Slg- splenocytes were mixed with the Fas-Ig molecule or human myeloma IgG1 control Ab (The Binding Site Ltd., Birmingham, UK) followed by FITC-anti-human IgG Ab (Cappell). After blocking nonspecific binding sites of anti-human IgG Ab with mouse and rat Ig, cells were stained with PE-anti-NK1.1 and biotin-anti-CD3 mAb followed by Tri-Color-streptavidin. Stained cells were analyzed by FACScan® (Becton Dickinson & Co.).

Cytotoxic Assay. Target thymocytes were prepared from 3-4-wk-old mice. Fas transfectants were prepared as described previously (9, 11, 13). Cytotoxic assay was carried out as previously reported (13-15). Briefly, target cells were labeled with PKH-2 green fluorescence dye (Zynaxis Cell Science, Inc., Malvern, PA). After the labeling, living cells were purified by lymphoseparator (Immuno-Biological Laboratories, Fujioka, Japan). The stained cells were mixed with various numbers of effecter cells and incubated for 12 h after centrifugation in a U-bottomed 96-well microplate (Falcon, Becton Dickinson & Co.) in 200 µl of RPMI-1640 supplemented with 10% FCS, kanamycin (100 µg/ml) and 5 × 10⁻⁵ M 2-ME. After the culture, the proportion of dead cells among PKH-2-stained cells was determined via FACScan® by staining the cultured cells with propidium iodide (PI) red fluorescence dye (Sigma Chemical Co., St. Louis, MO). Data are presented as mean ± SD from triplicate cultures.

Results and Discussion

To analyze the expression of Fas ligand transcripts on NK cells, the NK1.1⁺ CD3⁻ population, which is thought to be mouse NK cell population (16), was sorted from CD4⁻ CD8⁻ slg⁻ splenocytes. The purity of the sorted NK1.1⁺ CD3⁻ cells was constantly >99%. However, since the preparation of sufficient numbers of cells with this purity for performing Northern blot analysis is very difficult, we employed RT-PCR to detect Fas ligand mRNA in NK cells. Fig. 1 shows the expression of Fas ligand mRNA and β-actin mRNA as a control in purified NK cells and slg⁻ splenocytes, most of which are T cells (>90%). Whereas the expression level of β-actin mRNA was almost the same between T and NK cells, Fas ligand mRNA was detected only in NK cells under our experimental conditions. Predicted patterns of restriction enzyme digestion proved that the amplified fragment corresponded to Fas ligand (data not shown). The expression of Fas ligand on the cell surface of NK cells was then analyzed by staining with the soluble Fas-Ig chimeric molecule. As shown in Fig. 2, NK1.1⁺ CD3⁻ splenocytes were significantly stained by the Fas-Ig molecule, whereas NK1.1⁺ CD3⁺ cells, as conventional spleen T cells, were not stained. From these observations, it was demonstrated that NK cells express Fas ligand on their cell surface.

To investigate whether the Fas ligand expressed on NK cells is functional, the cytotoxicity of NK cells against Fas-expressing cells was analyzed. Since thymocytes express Fas and have been used as targets for Fas-mediated cytotoxicity by T cells (6, 10, 13) and since thymocytes are known to be sensitive to cytotoxicity by NK cells (17), we used thymocytes from normal and lpr mice as targets. As shown in Fig. 3, NK cells showed significant cytotoxicity against thymocyte targets from MRL lpr/lpr mice. Furthermore, NK1.1 mAb did not affect the cytotoxicity of the slg⁻ CD4⁻ CD8⁻ CD3⁻ population, ~50-60% of which was NK cells, showed similar cytotoxicity against thymocyte targets (data not shown). These observations exclude the possibility that the cytotoxicity of NK cells against thymocytes resulted from the additional activation of NK cells by Fas-Ig mAb used for purification.

We confirmed Fas-mediated cytotoxicity of NK cells by using Fas transfectants. L5178Y (a Fc receptor-negative T lymphoma line derived from DBA/2 mice) was transfected with either a full-length Fas cDNA or a mutated Fas cDNA lacking cytoplasmic domain (90 amino acids had been removed from the COOH-terminal end) (13). NK cells significantly killed Fas-transfected lymphoma cells (A-1 cells), but not parental lymphoma cells (L5175Y) or mutated-Fas transfected cells (F-10) (Fig. 4). Splenic T cells did not reveal any cytotoxicity against these cells. In addition, these transfectants have been shown to be similarly sensitive to the cytotoxicity by allogeneic CTL (13). From these results, it can be concluded that

|       | T cell | NK cell |
|-------|--------|---------|
| Slg⁻ | 50      | 50      |
| Fas ligand | 25      | 25      |
| β-actin | 12      | 12      |
|       | 6       | 6       |

Figure 1. Expression of Fas ligand mRNA on NK cells. The expression of Fas ligand and β-actin mRNA in slg⁻ CD4⁻ CD8⁻ NK1.1⁺ CD3⁻ splenocytes (NK cell) or slg⁻ splenocytes (T cell) was analyzed by RT-PCR. The amounts of RNA (ng) used for amplification are indicated. Representative data from three independent experiments are shown.
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Introduction

Fas ligand was recently cloned, and it was shown that its expression is upregulated upon stimulation of T cells (7). On the other hand, we have found that freshly isolated NKI.1 + TCR-α/β+ T cells constitutively express Fas ligand and kill CD4+CD8+ thymocytes (13). Collectively, some activated T cells and NKI.1 + T cells express Fas ligand and have the important function of eliminating Fas-expressing cells. In the present study, we found that not only these T cells but also freshly isolated NK cells express functional Fas ligand that induces Fas-mediated cytotoxicity. This finding indicates that NK cells, similar to NKI.1 + T cells, seem to be important for the immune system since they concomitantly express Fas ligand in vivo without additional stimulation.

In both lpr and gld mice, autoantibody production and accumulation of abnormal T cells are observed. Therefore, it is reasonable to speculate that a major role of Fas and Fas ligand interaction in vivo is to prevent the progression of autoimmune or abnormal T cell development. However, the mechanism of the onset of disorders observed in these mice is still unclear. Considering that Fas is expressed on immature thymocytes or activated T cells among lymphoid cells (3), identification of populations that express Fas ligand in thymus and peripheral lymphoid organs is important for clarifying the role of Fas both in these autoimmune disorders and in normal immune responses. In thymus, NK cells and activated conventional T cells are hardly detected, and NKI.1 + TCR-α/β + T cells are the only population expressing Fas ligand (13). These observations have suggested that NKI.1 + TCR-α/β+ thymocytes are important cells regulating thymocyte development. In peripheral lymphoid organs, at least three populations express Fas ligand: NK cells, NKI.1 + T cells, and activated T cells (memory T cells). These populations are possibly involved in regulating the immune system through cytotoxicity against Fas-expressing cells in these organs. At present, it is unclear whether only Fas ligand on these effector cells is responsible for the cytotoxicity against Fas-expressing cells, because there seems to be antigen specificity, even in Fas-mediated cytotoxicity, similar to perforin-mediated cytotoxicity (8, 9). Therefore, these different types of effector cells expressing Fas ligand, depending on the nature of these effector cells, may kill different types of target cells.

Introduction of Fas into tumor cells significantly induced their susceptibility to the cytotoxicity of NK cells. This result suggests that not only perforin-mediated cytotoxicity (18) but also Fas-mediated cytotoxicity is involved in the NK cell cytotoxicity. However, the NK activity in gld mice expressing the mutated Fas ligand did not differ from that in normal mice when NK-sensitive YAC-1 cells were used as targets, which express low levels of Fas (Arase, H., and T. Saito, unpublished observation). In contrast, EL-4 cells also expressing Fas (6) are known to be an NK-resistant cell line. From these observations, the susceptibility of target cells to NK cells...
cannot be merely explained by the expression of Fas. Involvement of Fas in the cytotoxicity by NK cells might be determined by the characteristics of the target cells. For example, bcl-2 expression has been reported to antagonize signals for apoptosis by Fas (19). On the other hand, class I expression on target cells is correlated to their susceptibility to NK cells (20, 21). Therefore, the expression of these molecules as well as Fas on target cells may contribute to the involvement of Fas in the cytotoxicity of NK cells. Further analysis will elucidate the specificity and function of Fas-mediated cytotoxicity by NK cells.

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