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The participation of the Fas-mediated cytotoxic pathway by natural killer cells is tumor-cell-dependent

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Abstract Cytotoxic T lymphocytes and natural killer (NK) cells kill target cells by two main mechanisms, namely, the perforin/granzymes and the Fas ligand (Fas-L) pathways. The preferential activation of either of these two mechanisms by target cells is not known. This study examined whether various NK stimuli regulate preferentially the perforin/granzyme or the Fas pathways during the NK-cell-mediated cytotoxic reaction (NK-CMC). Purified peripheral-blood-derived NK cells were stimulated with interleukin-2 (IL-2), IL-12, or interferon α (IFNα) and their response was analyzed by the reverse transcriptase/polymerase chain reaction (RT-PCR) for NK-associated gene expression and by the ^{51}Cr-release assay for cytotoxic function. RT-PCR data revealed that the perforin, granzyme A and granzyme B mRNAs were constitutively expressed in unstimulated NK cells and the level of perforin mRNA was augmented following activation. IL-2 enhanced the level of Fas-L mRNA in NK cells; however, the Fas-L level was much lower than that obtained in activated T cells. NK-CMC against Fas-sensitive cells was examined in the presence of neutralizing anti-(Fas antigen receptor) (Fas-R) antibody (ZB-4) or EGTA/Mg^{2+}, which inhibits the perforin/granzyme pathway but not the Fas Fas-L interaction. The human colon adenocarcinoma HT-29 cells were sensitized to anti-Fas-R antibody (CH-11) cytotoxicity following treatment with IFNγ. NK-CMC against untreated HT-29 cells was completely inhibited by EGTA/Mg^{2+} and was unaffected by ZB-4, while both EGTA/Mg^{2+} and ZB-4 partially inhibited NK-CMC against IFNγ-treated HT-29 cells. Similar findings to those obtained with untreated NK cells were observed with NK cells stimulated with IL-2, IL-2 plus IL-12 or IFNα. In contrast to IFNγ-treated HT-29 cells, the neutralizing anti-Fas antibody ZB-4 did not inhibit NK-CMC against Fas-sensitive U937, CEM or Jurkat tumor cells. These findings demonstrate that the Fas pathway is involved in NK-CMC against certain target cells but not all. Further, the data demonstrate that activation of NK cells by IL-2, IL-2 plus IL-12 or IFNα does not preferentially modulate the Fas-L-mediated killing by NK cells.

Key words Interferon-γ · NK cells · Killing mechanism · Fas ligand · Perforin

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Introduction

Cytotoxic T Iymphocytes (CTL) and natural killer (NK) cells play a crucial role in immune surveillance, including elimination of tumor cells and virus-infected cells. These two types of effector cells differ in specificity but share similar characteristics in that both have cytotoxic activity, secrete cytokines, and are rich in granules. During the cytotoxic reaction, degranulation takes place and several molecules in the granules participate in cytotoxicity, namely perforin and granzymes A and B [3]. Recently, it has been documented that, in addition to perforin, another mechanism is involved in cell-mediated cytotoxicity. Evidence for this second mechanism emanated from studies demonstrating that alloimmune peritoneal exudate lymphocytes kill target cells although such cells are devoid of perforin in their cytoplasmic granules [3]. Furthermore, whereas perforin-mediated killing requires calcium ions, cloned CTL can lyse target cells in the absence of calcium ions [21, 23].

The Fas antigen receptor (Fas-R) was identified as a receptor that transduces an apoptotic signal to susceptible cells [12, 29]. Several studies have shown that the Fas

Table 1 Primers used in the reverse transcriptase/polymerase chain reaction, *bp* base pairs, *Fas-L* Fas ligand

Name of gene	Sequences	Size of DNA (bp)	Reference
Perforin	5′-GAGGCCCAGGTCAACATAGGCA-3′ 5′-TCACCACACGGCCCCACTCCG-3′	888	[16]
Granzyme A	5′-GGAGGAAATGAAGTAACTCCTCA-3′ 5′-AGGCCCACGAGGGTCTCCGC-3′	630	[7]
Granzyme B	5′-AACAGGAGCCGACCCAGCAG-3′ 5′-GCTCGTGGAGGCATGCCATTG-3′	445	[25]
Fas-L	5′-CCTGACTCACCAGCTGCCATGC-3′ 5′-CTCTTAGAGCTTATATAAGCCG-3′	867	[27]
β-Actin	5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′	838	[20]

pathway plays an important role in CTL-mediated cytotoxicity [9, 14, 17, 24]. The Fas ligand (Fas-L) [26, 27] on the killer cells recognizes the Fas-R on the target cells and triggers a signal for cell death. To determine the effect of perforin on cytotoxicity by CTL and NK cells, Kägi et al. developed perforin-deficient mice [13] and indicated that both the perforin and the Fas systems are major pathways in CTL-mediated cytotoxicity [13, 14]. Furthermore, recent studies revealed that granzymes A [10] and B [11], which are the granular contents of CTL and NK cells, function as co-factors in the induction of apoptosis in target cells.

Although many studies have documented the involvement of the Fas pathway in CTL-mediated cytotoxicity, the role of the Fas pathway in NK-cell-mediated cytotoxicity (NK-CMC) has been controversial. Poly(I) · poly(C)-activated NK cells from perforin-deficient mice completely lacked cytotoxic activity against YAC-1, a cell line sensitive to Fas-mediated cytotoxicity [13]. On the other hand, Arase et al. [2] demonstrated the involvement of Fas-mediated killing in murine NK cells (NK-CMC). Montel et al. [19] and Tanaka et al. [28] also reported that NK-like tumor cells, such as YT cells, large granular lymphocytic leukemia and NK lymphoma cells, kill Fas-R transformants via the Fas pathway.

Previous studies used NK-cell-like tumor cells as effectors and Fas-R transformants as target cells. In spite of such reports, which implicated the Fas pathway in NK-CMC, the physiological role of the Fas pathway in the elimination of tumor cells by NK cells remains unclear. Further, it is not resolved whether freshly isolated NK cells can kill Fas-sensitive target cells via the Fas pathway.

This study addressed several questions: (1) whether human peripheral-blood-derived NK cells express Fas-L and whether stimuli of NK cells regulate the expression of Fas-L mRNA; (2) whether NK cells kill target cells via the Fas pathway; and (3) whether stimuli of NK cells like interleukin-2 (L-2), IL-12, and interferon α (IFNα) regulate the Fas pathway in NK-CMC. To resolve these questions, we examined the expression of the genes for perforin, granzymes A and B and Fas-L in NK cell; we also analyzed the mechanism of cytotoxicity mediated by IL-2, IL-2 plus IL-12, L-2, IL-2 plus IL-12, and IFNα-activated NK cells.

Materials and methods

Reagents and cells

The isotype mouse IgG and recombinant IL-2 were obtained from R&D Systems (Minneapolis, Minn.). Recombinant IFNα Con I was a generous gift from Dr. L. Blatt (Amgen Inc., Thousand Oaks, Calif.) [22]. Recombinant IL-12 was a generous gift from Dr. G. Trinchieri (Wistar Inst., Philadelphia, Pa.). Recombinant IFNγ was a generous gift from Pepro Tech Inc. (Brooks Hill, N.J.). Phycoerythrin-conjugated IgG was purchased from Biomedia Inc. (Forest City, Calif.). Cell lines U937 (CRL 1593), Jurkat, clone E6-1 (TIB 152), CEM (CCL 119), HT-29 (HTB58), and PA2.6 (HB118) were purchased from the American Type Culture Collection. Anti-Fas-R IgM antibody (CH-11) [29] and anti-Fas-R IgG antibody (ZB-4) [28] were purchased from Kamiya Biochemical Inc. (Tukwila, Calif.).

Human peripheral blood was obtained from healthy volunteers in the laboratory according to Human Subjects Protection Committee approval procedures. NK cells were purified from human peripheral blood mononuclear cells (PBMC) using an NK cell isolation kit (Miltenyi Biotec Inc., Auburn, Calif.). T cells in PBMC were purified by positive selection using CD3 microbeads. Purified NK cells were 80%–90% $CD16^+$ $CD56^+$ and had no contamination with either T or B lymphocytes or monocytes. Purified NK cells were resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum at a concentration of 10^6 cells/ml. A 1-ml sample of cell suspension was seeded in a 24-well culture plate and cultured for 4 h (reverse transcription/polymerase chain reaction, RT-PCR) or 18 h (cytotoxicity assay) in the absence or presence of IL-2 (0.25 μg/ml), IL-2 (0.25 μg/ml) plus IL-12 (1000 U/ml) or IFNα ConI (1000 U/ml) at 37 °C, 5% CO_2 air.

PT-PCR

Poly(A)-rich RNA from NK cells or T cells was purified using $(dT)_{25}$-bound Dynabeads (Dynal Inc., Lake Success, N.Y.) according to the instruction manual, and subjected to cDNA synthesis. The PCR reaction was performed with 5×10^4 NK cell equivalents of cDNA, $(dT)_{25}$-bound Dynabeads under the following conditions: 1 cycle at 94 °C for 3 min, 60 °C for 2 min, and at 72 °C for 3 min, 28 cycles at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min, 1 cycle at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 7 min. A 10-μl sample from the PCR reaction was subjected to analysis. To detect Fas-L mRNA by PCR, 2.5 units Taq DNA polymerase was added to the PCR reaction, followed by amplification for an additional 10 cycles. The primers used in this study are listed in Table 1 [7, 16, 20, 25, 27]. Analysis by densitometry was performed using Bio Analysis Software (London, UK) following scanning of the PCR pictures.

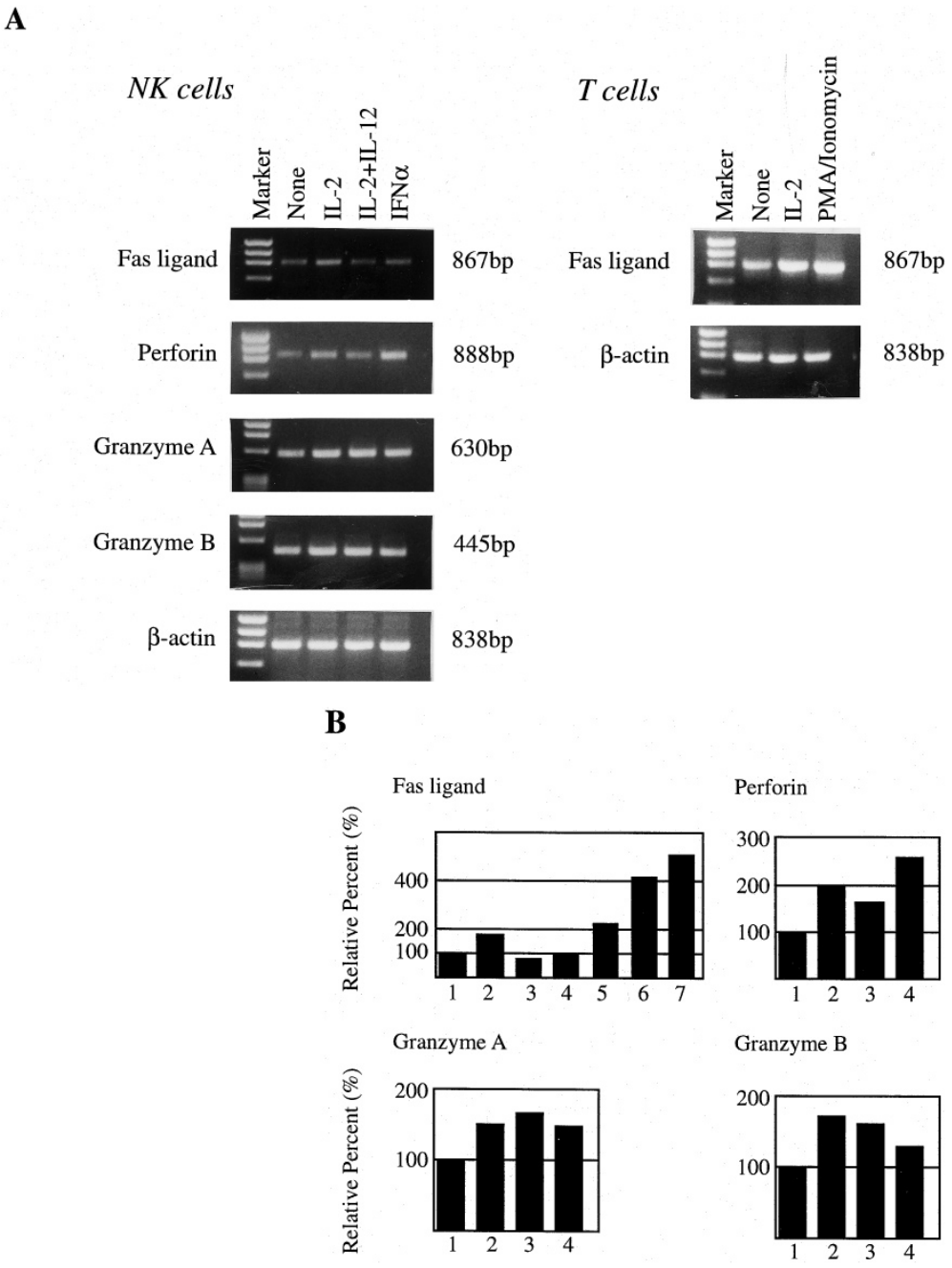

Fig. 1 A Reverse transcriptase/polymerase chain reaction (RT-PCR) for perforin, granzymes A and B and Fas ligand (*Fas-L*) in natural killer (NK) cells. Samples containing 1×10^6 cells/ml peripheral-blood-derived NK cells were incubated for 4 h in the absence or presence of interleukin-2 (*IL-2*; 0.25 μg/ml), plus IL-12 (1000 U/ml), or interferon α (IFNα; 100 U/ml). T cells were purified from human peripheral blood mononuclear cells (PBMC) using CD3-antibody-coated microbeads. T cells (1×10^6 ml) were incubated for 4 h in the absence or presence of IL-2 (0.25 μg/ml) or phorbol-myristate-acetate (*PMA*) ionomycin (10 ng/ml/200 nM). RT-PCR for perforin, granzyme A and granzyme B was performed with 5×10^4 NK cell equivalents of cDNA and 30 amplification cycles. RT-PCR for Fas-L was performed with cDNA equivalent to 5×10^4 NK cells or T cells and amplification cycles. ϕX174/*Hae*III digest was used as DNA size marker. Relative intensity of DNA bands generated from PCR. Unstimulated NK cells (*1*), IL-2 (*2*), IL-2 +IL-12 (*3*), IFNα-stimulated NK cells (*4*), $CD3^+$ T cells (*5*), IL-2 (*6*) and phorbol-myristate-acetate/ionomycin-stimulated T cells (*7*). The relative percentage was calculated on the basis of the intensity of DNA from unstimulated cells (100%)

Flow cytometry

Aliquots containing 2×10^5 cells were washed twice with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). For Fas-R, 1 μg specific antibody or isotype IgG was added to 50 μl cell suspension and incubated for 30 min on ice. For MHC class I, PA 2.6 ascites (1:1000), generated in our laboratory, was used. After washing twice with 1% BSA/PBS, the cells were suspended in 50 μl–100 diluted phycoerythrin-unconjugated anti-(mouse IgG), incubated for 30 min on ice and washed three times with 1% BSA/PBS. An Epics C (Courter Electronics, Hialeah, Fla.) flow cytometer was used for analysis.

Cytotoxicity

The NK-CMC activity was determined in a 4-h ^{51}Cr-release assay. NK cells were stimulated for 18 h with various stimuli. Target cells were incubated for 1 h with ZB-4 prior to the addition of the effector cells. EGTA/Mg^{2+} was added to target cells at a final concentration of 8 mM/6 mM. Samples comprising 100 μl target cells and 100 μl effector cells were mixed and subjected to the cytotoxicity assay. The percentage cyotoxicity was determined as follows: cytotoxicity (%) = 100 ×(experimental release – spontaneous release)/(maximal release spontaneous release).

Cytotoxicity by CH-11 against U937, Jurkat, CEM, and HT-29 cells was determined by the cell proliferation kit sodium 3′-(1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxyl-6-nitro)benzenesulfonic acid hydrate (TT) assay according to the manual (Boehringer Mannheim, Indianapolis, Briefly, 3×10^3 HT-29 or 1×10^4 Jurkat, U937, or

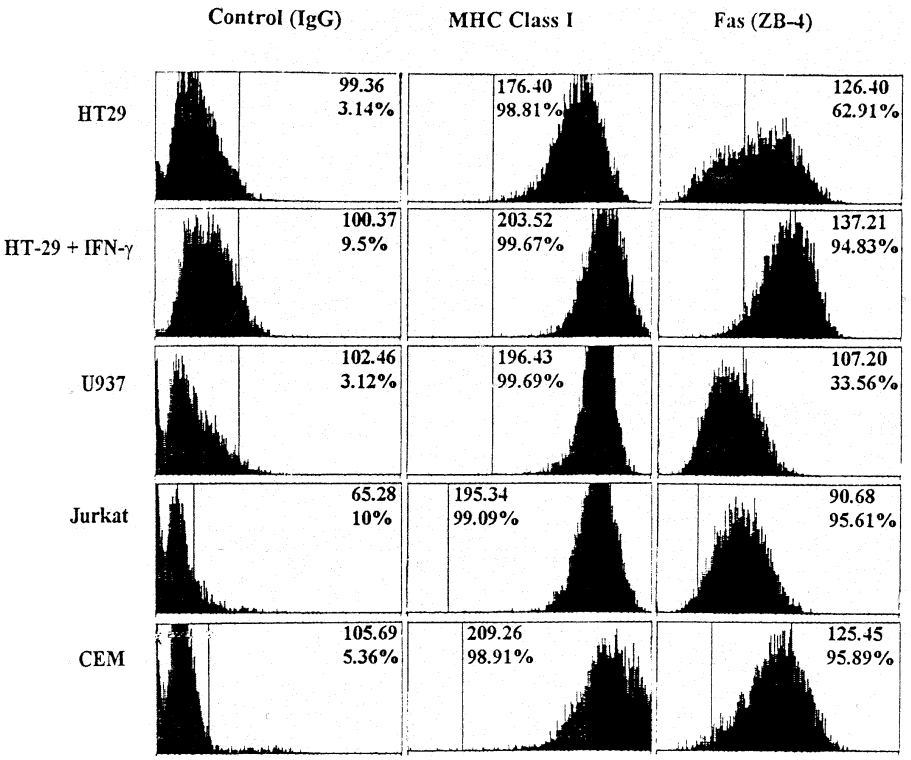

Fig. 2 Surface expression of Fas-R and MHC class I antigen on target tumor cells. Confluent HT-29 cells (5 ml) in 25-cm3 flasks were treated for 18 h with 1000 U/ml IFNγ. After detachment of the cells with trypsin/EDTA, the cells were subjected to flow-cytometry analysis. The cells were treated with normal mouse IgG or anti-Fas-R antibody (ZB-4). The numbers on the right-hand side of each panel represent the mean channel fluorescence and percentage of fluorescent cells. The data shown are representative of two experiments

CEM target cells in 100 µl were seeded in 96-well flat-bottom culture plates and treated with CH-11 for 18 h at 37 °C. A 50-µl sample of XTT was added to each well and the plates were incubated for 24 h. The absorbance A450–A650 was measured by an enzyme-linked immunosorbent assay reader.

Results

Expression of perforin, granzyme A, granzyme B, and Fas ligand mRNAs in unstimulated and stimulated NK cells

It has been well documented that perforin, granzyme A , granzyme B and Fas-L are important effector molecules in the induction of apoptosis in target cells. The expression of these molecules is regulated by various stimuli in CTL. However, the expression of these molecules in unstimulated NK cells and NK cells activated by different stimuli is not known. We therefore examined mRNA expression for perforin, granzymes A and B and Fas-L in unstimulated NK cells, derived from human peripheral blood and purified, and NK cells stimulated for 4 h with IL-2, IL-2 plus IL-12 and IFNα. Since it is difficult to purify total RNA from a small number of NK cells and $CD3^+$ T cells, we performed RT-PCR using oligo(dT)-bound magnetic beads. As shown in Fig. 1A, 30 cycles of amplification from 5×10^4 NK cells for perforin and granzymes A and B revealed a strong band, whereas a 40-cycle amplification was required to detect Fas-L mRNA from 5×10^4 NK cells. By comparison, activated T cells showed higher expression of Fas-L mRNA than IL-2-activated NK cells. The data shown in Fig. 1A on perforin, granzymes A, and B and Fas-L in NK cells are representative of ten different experiments performed with ten different blood donors. Densitometric analyses for the PCR products are shown in Fig. 1B. The level of Fas-L mRNA in NK cells was enhanced twofold by IL-2 stimulation but not by IL-2 plus IL-12 or by IFNα, and the level of Fas-L mRNA in phorbol-12-myristate-13-acetate/ionomycin-activated T cells was threefold higher than that in IL-2-activated NK cells. The mRNAs for perforin and granzymes A and B are constitutively expressed (at high levels) in unstimulated NK cells and the level of perforin mRNA is augmented by all stimuli.

Involvement of Fas-mediated cytoxicity by NK cells against HT-29 tumor target cells

The above findings demonstrating that Fas-L mRNA can be expressed in NK cells suggested that it may play a role in NK-CMC against Fas-sensitive (Fas^+) target cells. Yonehara et al. [30] reported that treatment with IFNγ sensitizes HT-29 cells to CH-11-mediated cytotoxicity. Therefore, we considered HT-29 cells useful as target cells for Fas-L-mediated cytotoxicity by NK cells. We first confirmed the findings of Yonehara et al. [30]. Figure 2 demonstrates that IFNγ treatment of HT-29 cells augments the expression of Fas-R as analyzed by flow cytometry. Treatment of HT-29 cells with the anti-Fas-R IgM antibody (CH-11) caused minimal cytotoxicity but the HT-29 cells became sensitive to CH-11 following treatment with IFNγ. The mechanism

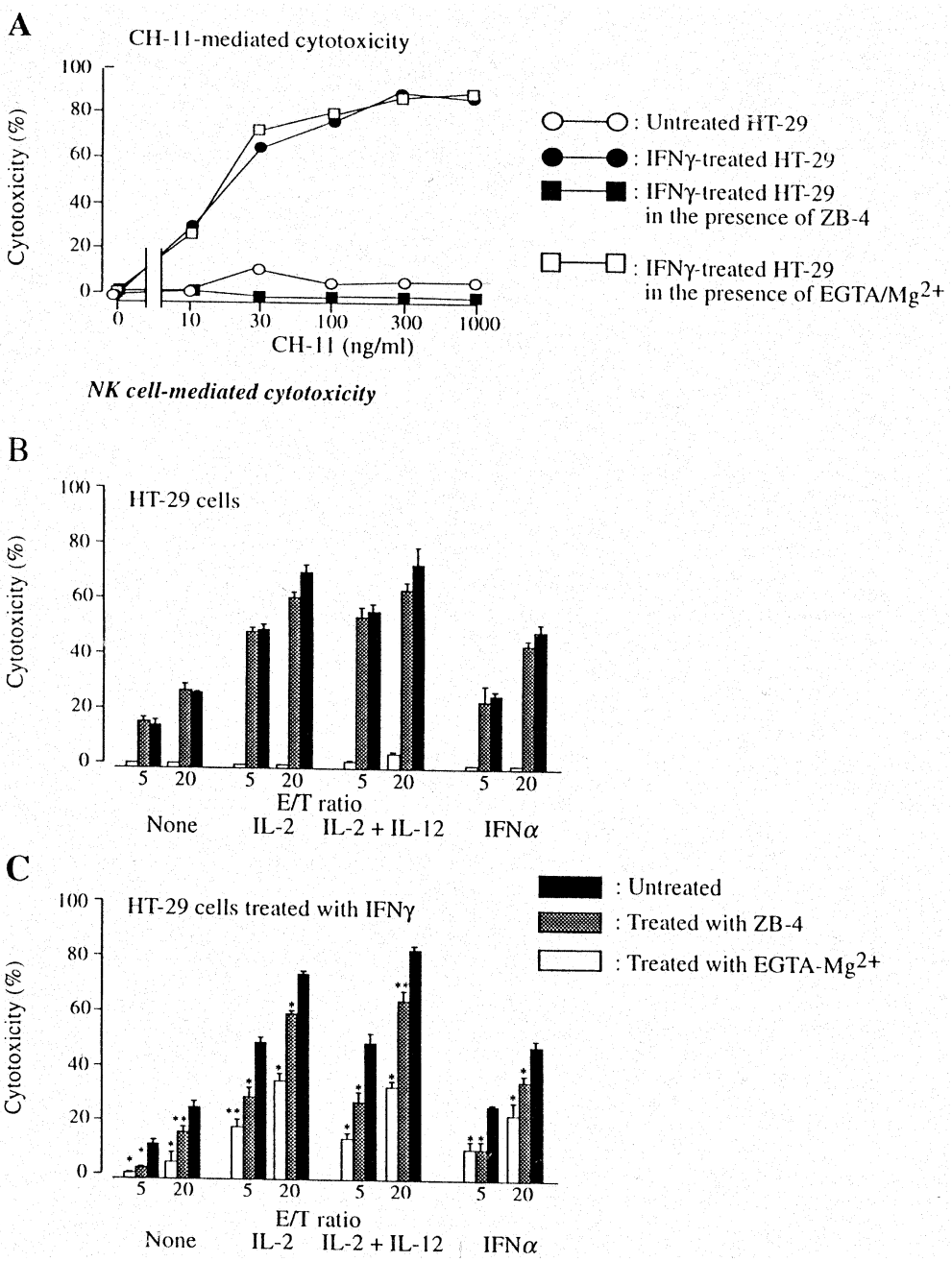

Fig. 3A–C CH-11, and NK-cell-mediated cytotoxicity against HT-29 cells. Confluent HT-29 cells (5 ml) in 25-cm3 flasks were treated for 18 h with or without 1000 U/ml IFNγ. **A** Effect of IFNγ on CH-11-induced cytotoxicity against HT-29 cells. 3×10^3 of HT-29 cells were cultured for 18 h with or without CH-11. For CH-11-induced-cytotoxicity, HT-29 cells were incubated for 1 h in the presence of 2 μg/ml ZB-4 before addition of CH-11. EGTA/Mg^{2+} was added at a final concentration of 4 mM / 3 mM. Cytotoxicity was determined using the XTT assay. Effects of EGTA/Mg^{2+} and ZB-4 on NK-CMC against untreated HT-29 cells (**B**) and IFNγ-treated HT-29 cells (**C**). EGTA/Mg^{2+} was added to target cells at a final concentration of 4 mM / 3 mM. HT-29 cells were pretreated with 2 μg/ml ZB-4. The inhibition of NK-CMC against IFNγ-treated HT-29 cells by ZB-4 or EGTA/Mg^{2+} was significant. (**$P<0.04$, *$P<0.01$). The data are reported as means ± SD of triplicate samples. The data in the figures are representative of three experiments

of IFNγ-mediated sensitization is still unclear. The CH-11-induced cytotoxicity was inhibited by the addition of neutralizing anti-Fas-R antibody (ZB-4) and was not inhibited by the addition of EGTA/Mg^{2+} (Fig. 3A).

The role of the Fas pathway in NK-CMC by activated NK cells was examined against that of untreated or IFNγ-treated HT-29 cells. Purified NK cells were stimulated for 18 h with IL-2, IL-2 plus IL-12, or IFNα and thereafter the cultures were assessed for cytotoxicity against HT-29 cells. Figure 3B demonstrates that untreated HT-29 cells were relatively sensitive to NK-CMC and all stimuli augmented NK-CMC. The cytotoxic activity against untreated HT-29 cells was completely blocked by the addition of EGTA/Mg^{2+}, and ZB-4 did not affect the NK-CMC reaction. These data suggest that NK-CMC against untreated HT-29 cells is mediated primarily by a Ca^{2+}-dependent mechanism of cytotoxicity.

We then examined NK-CMC against IFNγ-treated HT-29 cells. The addition of EGTA/Mg^{2+} reduced by 80% the cytotoxicity mediated by unstimulated NK cells and by 60% the cytotoxicity mediated by NK cells stimulated with IL-2, IL-2 plus IL-12 or IFNα. It is noteworthy that the addition of ZB-4 reduced cytotoxicity by 25%–35% (E:T 20:1) and by more than 50% (E:T 5:1) with both unstimulated and stimulated NK cells (Fig. 3C). These data demonstrate that NK-CMC against IFNγ-treated HT-29 cells utilizes both the perforin/granzymes and the Fas pathways. Further, in comparison with unstimulated NK cells, the

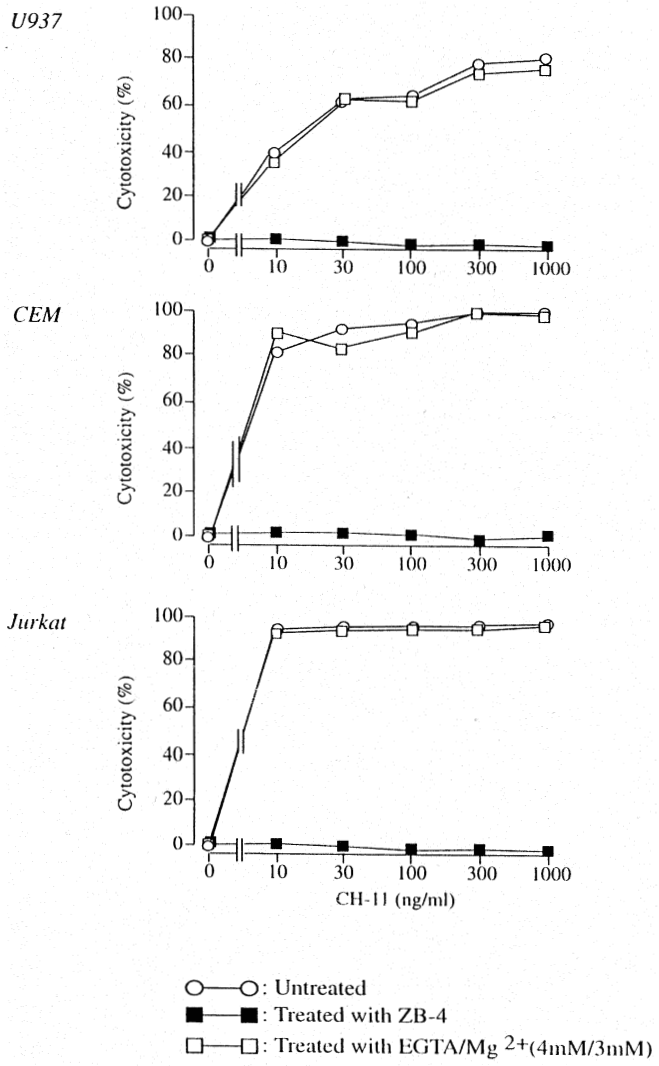

Fig. 4 CH-11-mediated cytotoxicity against U937, CEM and Jurkat cells. 1×10^4 tumor cells were cultured for 18 h with or without CH-11. Tumor cells were treated with ZB-4 each at a final concentration of 0.5 μg/ml ZB-4 (U937) and 2 μg/ml ZB-4 (CEM and Jurkat cells). The data are reported as means ± SD of triplicate samples. The data are representative of three experiments

susceptibility of target cells to Fas-mediated NK-CMC was not altered by activated NK cells.

Lack of involvement of the Fas pathway in the cytotoxicity mediated by NK cells against Fas-sensitive tumor target cells

We examined the role of the Fas pathway in NK-CMC against tumor cells that are sensitive to anti-Fas-R IgM antibody (CH-11). Three cell lines were used, namely U937, CEM and Jurkat cells, and all three expressed surface Fas (Fig. 2). Figure 4 shows the sensitivity of all three cell lines to CH-11 and complete blocking of cytotoxicity by ZB-4, and there was no inhibitory effect following the addition of EGTA/Mg^{2+}. The sensitivity of three cell lines to NK-CMC by unstimulated NK and stimulated NK cells was determined. In all cases, the cells were sensitive to NK-CMC and cytotoxicity was augmented following treatment of NK cells with IL-2, IL-2 plus IL-12, and IFNα (Fig. 5). Treatment with ZB-4 had no detectable inhibitory activity against any of the three cell lines. NK-CMC against U937 was completely inhibited in the presence of EGTA/Mg^{2+} whereas a residual cytotoxic activity against CEM and Jurkat was observed in the presence of EGTA/Mg^{2+}. Montel et al. [17] demonstrated that there remained residual NK-CMC against Jurkat cells in the absence of Ca^{2+} and that neutralizing anti-Fas-R antibody completely inhibited this residual cytotoxicity. We also tested the effect of ZB-4 in the absence of Ca^{2+} in NK-CMC against Jurkat cells and the data show that ZB-4 had no effect on NK-CMC in the presence of EGTA/Mg^{2+} (Fig. 6). These findings suggest that NK-CMC against U937 is Ca^{2+}-dependent and that, against CEM and Jurkat cells, it is predominantly mediated through a Ca^{2+}-dependent pathway.

Discussion

This study demonstrates that human PBMC-derived NK cells can mediate cytotoxic activity by both the perforin/granzyme pathway and the Fas pathway. While the perforin/granzyme pathway appears to be the predominant one, certain target cells are killed by the Fas pathway. Compared to T lymphocytes, the Fas-L mRNA is minimally expressed on resting NK cells and is up-regulated by IL-2; however, its level in NK cells was much lower than that in activated T cells. In contrast, the perforin, and granzyme A and B mRNAs are constitutively expressed at high levels in resting NK cells as compared to Fas-L mRNA. Although IL-2, IL-2 plus IL-12, and IFNα treatment of NK cells augmented the cytotoxic function, these cytokines did not augment selectively either the perforin/granzyme pathway or the Fas pathway.

While the Fas pathway is well established in CTL-mediated cytotoxicity, its role in NK cells has been contradictory. Well-documented evidence showed that NK-like tumor cells can use the Fas pathway to kill Fas-R transformants. However, in vivo NK cells isolated from the perforin-knock-out mouse could not kill Fas^+ YAC-1 cells. In contrast, in another study, significant cytotoxicity through the Fas pathway was observed when murine thymocytes were used as target cells [2]. The present study provides evidence for the role of the Fas pathway in target cell lysis by freshly isolated human NK cells. The Fas pathway is operative under certain conditions that are dependent on the nature of the target cells used.

Montel et al. [19] reported the up-regulation of Fas-L mRNA expression in phorbol-12-myristate-13-acetate/ionomycin-stimulated NK cells derived from PBMC. Our study demonstrates that Fas-L mRNA is expressed in freshly derived NK cells. Our study also reveals that IL-2 was the only cytokine used that up-regulated the level of Fas-L mRNA in NK cells, although its level is low

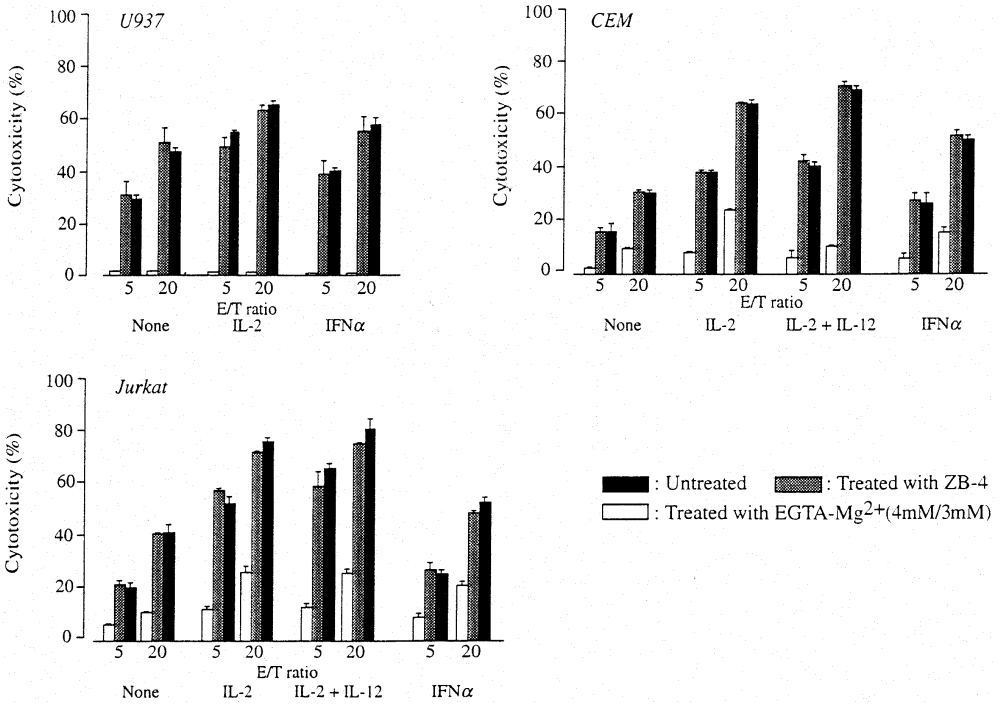

Fig. 5 NK-cell-mediated cytotoxicity (*CMC*) against U937 and Jurkat cells. NK-CMC was determined by 4 h ^{51}Cr-prelease assay. Tumor cells were treated with ZB-4 each at a final concentration of 1 μg/ml ZB-4 (U937) and 4 μg/ml ZB-4 (CEM and Jurkat cells). EGTA/Mg^{2+} was added to target cells at a final concentration of 4 mM / 3 mM. The data are reported as means ± SD of triplicate samples. The data are representative of three experiments

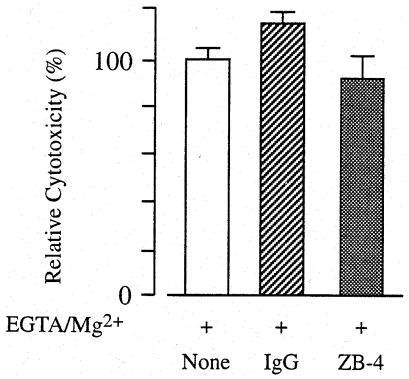

Fig. 6 Effects of ZB-4 on NK-cell-mediated cytotoxicity against Jurkat cells in the presence of EGTA/Mg^{2+} (4 mM / 3 mM). NK cells were incubated for 18 h in the presence of IL-2 (0.25 μg/ml). The target cells were treated for 1 h with ZB-4 (8 μg/ml) or isotype mouse IgG (8 μg/ml) prior to addition of the effector cells. EGTA/Mg^{2+} was added to the target cells at a final concentration of 8 mM / 6 mM and an equal volume of effector cells was added. The E/T ratio was 20:1. The percentage reduction was calculated on the basis of cytotoxicity against cells that had not been treated with ZB-4. The data are reported as means ± SD of triplicate samples. The data are representative of wo experiments

compared to that found in activated T cells. IL-2 plus IL-12 did not change the expression of Fas-L mRNA as compared to treatment with IL-2 alone. Therefore, the expression of Fas-L mRNA by NK cells is consistent with the potential functional role of the Fas-L in triggering cell death in Fas^+ target cells.

When human thymus was organ-cultured in the presence of superantigens, superantigen-reactive clones were detected through the Fas pathway [30]. ZB-4 inhibited this reaction demonstrating that this antibody neutralizes the Fas-L-mediated cytotoxic pathway. The requirement of Ca^{2+} in Fas-L-mediated killing has been examined by many investigators. Several reports [1, 17, 24) indicated that Fas-mediated apoptosis is Ca^{2+}-independent and our findings also demonstrate that CH-11-mediated killing was not inhibited in the presence of EGTA/Mg^{2+} (Fig. 3, 4). Recently, Lowin et al. [18] reported that EGTA/Mg^{2+} inhibited Fas-mediated killing by primary T cells. They suggested that T-cell-receptor-mediated up-regulation of surface Fas-L expression is essential in the antigen-specific Fas killing pathway and that Ca^{2+} is essential in T-cell-receptor-mediated signaling. In NK cells, it is not known whether surface Fas-L expression on NK cells is increased following interaction with target cells.

In this study, we determined the involvement of a functional Fas pathway in NK cells by various means, namely by anti-Fas-R (IgG) neutralizing antibody [28] and by the use of the Ca^{2+} chelator, EGTA/Mg^{2+}. In our studies, we demonstrate that IFNγ-treated HT-29 cells are susceptible to lysis by NK cells via both the Fas and the perforin/granzyme pathways. In contrast, other Fas^+ cell lines, like U937, CEM and Jurkat cells, were killed by NK cells predominantly via a calcium-dependent pathway. These studies clearly indicate that the target cell make-up may dictate whether perforin/granzymes, the Fas pathway or both pathways are utilized by NK cells in cytotoxicity.

The underlying mechanism by which a preferential trigger of the perforin or Fas or both pathways is regulated is not clear. The Fas-L/Fas-R interaction is certainly important but clearly it is not sufficient to trigger the Fas

system. All three lines, U937, CEM and Jurkat cells, express high and levels of Fas-R (Fig. 2) and are sensitive to CH-11 (Fig. 4), and neutralizing anti-Fas antibody did not affect NK-CMC against these target cells. In contrast, HT-29 cells, which also expresses Fas-R (Fig. 2), are not sensitive to CH-11 unless treated with IFNγ. Untreated HT-29 cells are killed by NK cells exclusively by a calcium-dependent pathway, suggesting the perforin/granzyme pathway, but, when treated with IFNγ, HT-29 are killed by both the perforin/granzyme and the Fas pathways. It appears that activation by IFNγ, aside from sensitizing the cells to CH-11-induced cytotoxicity, must have modulated the target cells so that they can trigger the Fas pathway.

We examined whether activation of NK cells by various stimuli like IL-2, IL-12, and IFNα might regulate the cytotoxic pathway mediated by the NK cells. The findings demonstrate that these various stimuli did not significantly affect the cytotoxic profile of the NK cells, suggesting that the effector cells might be passive but ready to be triggered for either cytotoxic pathway depending on the target cells. Clearly it is important to identify cytokine/growth factors that can trigger selectively one mechanism over another in the clinical setting. In some cases, the perforin/granzyme pathway may be the one of choice as it results in an inflammatory response. Further, it is possible that certain target cells, like tumor cells, are not susceptible to killing by the perforin/granzyme pathway but can be killed by the Fas pathway. In this case, the regulation of the NK cells to utilize the Fas pathway may be beneficial.

The Fas pathway was not involved in NK-CMC against U937 and untreated HT-29 cells. ZB-4 did not reduce NK-CMC against CEM and Jurkat cells; however, residual cytotoxicity remained that was not blocked by EGTA/Mg^{2+}. The significance of this residual activity is not clear. One possibility is that the amount of ZB-4 used was insufficient to block Fas-mediated cytotoxicity. This is unlikely, since an excess of ZB-4 (4 μg/ml) was used and cell death path induced by 300 ng/ml CH-11 is completely blocked by 1 μg/ml of ZB-4, and soluble Fas-L has a 2.5-fold higher specific activity than CH-11 [15]. A second possibility is that other molecules on CEM or Jurkat cells, like CD28, that are involved in recognition, may be required for killing via the Fas pathway [19]. A third possibility is that effector molecules other than perforin/granzymes and Fas-L may be involved in NK-CMC. Recently, Braun et al. [5] demonstrated that there was still residual cytotoxicity mediated by T cells derived from both perforin- and Fas-L-deficient mice, suggesting the existence of another novel mechanism of cytotoxicity that is involved in induction of apoptosis in target cells.

Published reports indicate that the Fas pathway does not play an important role in NKCMC. Bradley et al. [4] reported that NK cells from Fas-L-defective mice (*gld*) did not show impairment of NK-CMC by unstimulated NK cells or NK cells stimulated with IL-2. Furthermore, the granule exocytosis pathway is disrupted in cells from patients with Chédiak-Higashi syndrome. Although Chédiak-Higashi-derived CTL still exhibit some cytotoxicity, Chédiak-Higashi NK cells are virtually devoid of cytotoxic activity [8]. Furthermore, we observed that IL-2 induces Fas-R expression on NK cells and CH-11 can induce apoptosis in IL-2-activated NK cells (data not shown). However, NK cells stimulated with IL-2 alone do not undergo apoptosis. Therefore, it appears that the Fas-L expressed on IL-2-activated NK cells is insufficient to induce autologous killing in NK cells. However, the involvement of the Fas pathway in the regulation of the immune response has been well documented. Eischen et al. [6] demonstrated that anti-CD16 antibody induces Fas-L mRNA expression in cloned NK cells established from PBMC, and that anti-CD16-antibody-stimulated NK clones, but not unstimulated NK cells, kill both Fas-R transformants and NK cells themselves via this Fas pathway. These data suggest that there exists another role for Fas-L expression in NK cells, and that Fas-L may function to suppress excess killing activity by NK cells. In conclusion, this study demonstrates that NK cells can kill certain target cells via the Fas pathway, and activation of NK-CMC by IL-2, IL-12, or IFNα does not selectively regulate the perforin/granzyme or Fas pathway of cytotoxicity.

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