Natural killer cell lines kill autologous β2-microglobulin-deficient melanoma cells: Implications for cancer immunotherapy

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ABSTRACT Cancer vaccines used to generate specific cytotoxic T lymphocytes are not effective against tumor cells that have lost or suppressed expression of their class I major histocompatibility complex proteins. This loss is common in some cancers and particularly in metastatic lesions. We show that β2-microglobulin-deficient class I-negative melanoma variants derived from patients undergoing specific T cell therapy are lysed by heterologous as well as autologous natural killer (NK) lines and clones, but not by specific T cells. Moreover, the minor NK cell fraction but not the major T cell fraction derived from heterologous lymphokine activated killer cells kills those tumor cell lines. ICAM-1 expression by the different class I protein deficient tumors was correlated with their sensitivity to lysis by NK cells. Adoptive autologous NK therapy may be an important supplement to consider in the design of new cancer immunotherapies.

In the last decade, the field of cancer immunotherapy based on tumor-specific vaccines and antitumor-specific cytotoxic T lymphocytes (CTL) has advanced rapidly (reviewed in refs. 1–3). However, whether adoptive therapy with T cells or peptide-based vaccines to generate T cells is employed, the obstacle of major histocompatibility complex (MHC)-negative tumor variants still poses a problem for current experimental protocols in cancer therapy. Partial or complete loss of class I MHC molecules has been observed for numerous human and murine lines derived from solid tumors (reviewed in refs. 4–7). Also, frequent MHC-loss or suppression is observed in fresh tumor specimens taken from patients. For human melanomas, 16% and 58% of primary and metastatic lesions, respectively, are not stained by mAbs to monomorphic determinants of class I antigens (8, 9). The higher frequency of loss of class I antigens in metastases compared with loss in primary lesions is not unique to malignant melanoma since it has also been described in carcinomas. Of 44 human primary carcinomas and synchronous metastases from the same patients that were analyzed (10), 75% of the metastatic lesions were class I-negative compared with just 16% of the primary lesions. This difference can be interpreted as a partial success of the patient’s immune system. T cells specific for tumor-associated peptide epitopes presented by class I MHC protein, eradicated class I-positive metastasizing tumor cells, thus causing class I-negative mutant cells in the primary lesions to be selected for metastasis more frequently. In accordance, a cancer vaccine based solely on specific T cell therapy might drive the constantly changing tumor population to evolve into class I-negative secondary lesions.

In support of this hypothesis recent data have shown that, of 13 tumor lines derived from different melanoma patients, five lines lost functional β2-microglobulin (β2m) expression (11). Staining of lesion sections from which these 5 lines were derived confirmed the β2m-deficient phenotype of the tumor cells. However, archival tumor sections obtained from patients prior to immunotherapy were available for 3 patients and were found to be β2m-positive. Needless to say, the β2m-deficient lesions did not respond to further specific T cell therapy (11).

Natural killer (NK) cells have long been known to have the ability to lyse class I-negative cells in vitro (reviewed in refs. 12–14). NK cell activity is regulated by a family of NK inhibitory receptors (NKIR) expressed by NK cells. Recognition of an appropriate class I MHC specificity by a NKIR generates a lysis-inhibitory signal; each NKIR recognizes a subgroup of class I alleles (reviewed in refs. 15–18). Therefore, class I-negative tumor cells are susceptible to lysis by NK cells. In vivo, NK cell activity has been shown to drive several immune responses. NK cells in F1 MHC-heterozygous mice are involved in the rejection of marrow transplants from both homozygous parental mice, a phenomenon named “hybrid resistance” (19). Rejection of β2m-deficient marrow transplants by MHC-matched mice is also driven by NK cells (20). NK cells have a major contribution, in addition to CD8+ CTL, in the eradication of established metastases of the low class I-expressing murine melanoma B16 (21). Autologous tumor killing activity by blood lymphocytes, driven partially by NK cells, is correlated with the disease-free interval and total survival of operated cancer patients (reviewed in ref. 14). Therefore, NK cells have been long considered as an option for cancer therapy particularly for patients bearing class I-suppressed cancers. However, no studies of autologous NK lines as a potential tool for treatment of such patients have been reported.

In this study we demonstrate that β2m-deficient melanoma variants are lysed by different NK lines and clones expressing different NKIR, while human leukocyte antigen (HLA)-positive melanoma lines are lysed selectively based on interactions of their HLA alleles with the NKIR. Lymphokine-activated killer (LAK) cells, or T cells purified from these LAK, did not lyse well two-thirds of β2m-deficient lines or the HLA-positive melanoma line. However, the minor population of NK cells isolated from LAK efficiently lysed these lines. We further show that NK lines are potent killers of autologous β2m-negative melanomas, but not of autologous fibroblasts. Adoptive NK therapy as part of a combined cancer immunotherapy approach to cancer is discussed.

METHODS

Tumor Cell Lines. Melanoma cell lines (1074 mel, 1106 mel, 1259 mel, and 1190 mel) were established from patients 1074, 1108, 1259, and 1190, respectively (11). Tumor cells were maintained as monolayer cultures. LB33MEL.A1 and...
CD56 human serum. After 5–6 days of culture, pure preparations of PBMC:721.221, 7.5 (PBMCs) with irradiated (6,000 rad) 721.221 cells (3:1 peripheral blood lymphocyte as described (22). Briefly, NK lines heat inactivated fetal calf serum.

NK lines and clones were obtained from peripheral blood lymphocyte as described (22). Briefly, NK lines were generated by culture of peripheral blood mononuclear cells (PBMCs) with irradiated (6,000 rad) 721.221 cells (3:1 PBMC:721.221, 7.5 x 10^6 PBMC in RPMI 1640-10% AB human serum. After 5–6 days of culture, pure preparations of CD56+CD3- cells were obtained by depletion of T cells with mAbs against CD3 (T3D, ATCC) and CD4 (OKT4, ATCC) and with a low-tox rabbit complement (Cedarlane Laboratories). Purity was confirmed by flow cytometry. These NK lines were cultured in the same medium supplemented with 100 units/ml recombinant human interleukin 2 (ruhIL-2) (Boehringer Mannheim). NK clones were obtained by seeding NK lines at one or five cells per well in 96-well U-bottomed plates in RPMI 1640-10% heat inactivated fetal calf serum. Spontaneous release was determined by solubilizing target cell in 0.1 M NaOH. Maximal release was determined by incubation of the labeled target cells with medium. Assays were terminated by centrifugation at 1,000 rpm for 10 min at 4°C and 100 μl of the supernatant collected for liquid scintillation counting. Specific cpm spontaneous release = (cpm experimental well – cpm spontaneous release) x 100. Specific cpm release = (cpm experimental well – cpm maximal release – cpm spontaneous release) x 100.

RESULTS

Melanoma cell lines 1074 mel, 1106 mel, and 1259 mel are βm-deficient based on negative staining with mAb W6/32 (a reagent that detects HLA class I proteins folded with human βm and no detection of βm protein in Western blots (11). The staining profiles of these lines with mAbs against human βm (BBM.1), free HLA class I heavy chain (HC10), and HLA-C (L31) as well as with W6/32 all overlapped the staining control histogram (Fig. 1, shown for 1074 mel). Thus, 1074 mel, 1106 mel, and 1259 mel lack cell surface expression of class I heavy chain-βm complexes, βm, or free class I heavy chain. None of these mAbs stained 1074 mel tumor cells, even when the cells were preincubated with exogenous human βm to mimic conditions of free βm in human serum (data not shown). Thus, no free class I heavy chains were stabilized by exogenous βm, a phenomenon observed for some class I MHC alleles. The cell line 1190 mel is a control HLA class I positive melanoma line that stained positively with all of the mAbs (Fig. 1).

Lysis of Different Melanoma Lines by NK Lines and Clones. NK lines and clones were generated from healthy donors. Although NK lines contain heterologous NK cells, they can display HLA-allele specific inhibition of lysis. This specificity of NK lines is defined functionally; NK1-specific lines are inhibited by target cells expressing any HLA-C allele with Asn-77 and Lys-80 (e.g., Cw2, w4, w5, w6, recognized by NKIR1), whereas NK2-specific lines are inhibited by the expression of HLA-C alleles with Ser-77 and Asn-80 (e.g., Cw1, w3, w7, w8, recognized by NKIR2). Thus, both NK1- and NK2-specific lines will efficiently lyse 721.221 cells (a class I-negative human B cell line). However, NK1 lines will lyse Cw7-transfected 721.221 cells (721.221/Cw7) but not 721.221/Cw6 cells, and vice versa for NK2 lines (22). NK clones are defined based on their NKIR expression as analyzed by flow cytometry; four different NK clone types (NKIR1 'NKIR2', NKIR1 'NKIR2', NKIR1 'NKIR2', and NKIR1 'NKIR2') were employed.

The βm-deficient melanoma lines 1074 mel, 1106 mel, and 1259 mel were lysed by all of the different NK clone types used in this study as well as by both NK1 and NK2 lines (Figs. 2, 3, and data not shown). The NK2 line and NKIR2 positive NK clones, but not the NK1 line and NKIR2 negative NK clones, were inhibited by the HLA-C positive melanoma line 1190 mel. In accordance, 1190 mel was found to express HLA-Cw7 and Cw8...
(determined by HLA typing), both ligands for NKIR2. Moreover, mAb to NKIR2, but not mAb to NKIR1, permitted lysis of 1190 mel by the NKIR2 positive NK clones (data not shown). Note however that 1259 mel and 1190 mel were lysed to a greater extent than 1074 mel and 1106 mel (Figs. 2 and 3). Patient 1074 is HLA-A2.1 positive, and thus \( \beta_2m \)-transfected 1074 mel cells were lysed by HLA-A2.1-restricted anti-melanoma tumor infiltrating lymphocytes (11). Such a tumor infiltrating lymphocytes line, 1200TIL, did not lyse the \( \beta_2m \)-deficient 1074 cells or any of the other melanomas (data not shown). Thus, sensitivity of these melanoma lines to lysis by heterologous NK cells follows the established rules for NK recognition and lysis (15–18).

**Fig. 2.** Cytotoxicity of different NK clones against melanoma lines. NK clones were generated as described. NK clones are classified based on their NKIR1 and NKIR2 expression as determined by flow cytometry using the mAb EB6 (NKIR1) and GL183 (NKIR2) (22). Different NK clones with the same classification manifested similar lysis patterns, and each lysis point is the average lysis of two NK clones from the same group. Clones were reacted with the indicated [\(^{35}\)S]methionine-labeled target cells for 5 h. Data obtained at an E/T of 1/1 are shown. The range was under 5% of the mean of triplicates in this and all following experiments (see Figs. 3, 4B, 5, and 6).

**Fig. 3.** Cytotoxicity of NK lines against melanoma lines. NK lines were generated as described. NK1-specific and NK2-specific lines were obtained from donors OM and MM, respectively. Effectors were reacted with the indicated [\(^{35}\)S]methionine-labeled target cells for 5 h and the data obtained at an E/T of 10/1 are shown. The results shown in Figs. 3 and 5 are from the same experiment.

**NK Lysis Sensitivity of Melanoma Lines Is Correlated to Their ICAM-1 Expression.** The melanoma line LB33MEL.A1 was derived from patient LB33 after surgery in 1988. LB33MEL.B1 was derived from a metastatic lesion removed from the same patient in 1990. LB33MEL.A1 cells express HLA-A24, A28, B13, B44, Cw6, and Cw7 while LB33MEL.B1 cells express only the HLA-A24 allele (23). The HLA-Cw6 and Cw7 proteins expressed by LB33MEL.A1 should inhibit lysis by NK1 and NK2 lines, respectively. On the other hand, the HLA-C negative LB33MEL.B1 cells should be lysed by both NK lines. However, both LB33MEL.A1 and .B1 were poorly lysed by the NK1 and NK2 lines shown in Fig. 3. A NKIR for which HLA-A24 is a
ligand could be functionally expressed by these two NK lines, therefore inhibiting lysis of LB33MEL.B1. However, an alternative explanation is available.

Metastatic or invasive melanoma cells can express high levels of ICAM-1 (CD54) (24). However, tumor cells expressing higher levels of ICAM-1 are better targets for specific CTL (25). Is the different sensitivity of the above melanoma lines to lysis by NK cell also correlated with their ICAM-1 expression? Indeed, the most sensitive lines 1259 mel and 1106 mel were ICAM-1high, as was the NK-sensitive line 721.221 (Fig. 4A). The less sensitive 1074 mel and 1106 mel were ICAM-1dim as were the LB33MEL.A1 and -.B1 lines. To further test the correlation, target tumor cells were incubated with anti-ICAM-1 mAb, washed, and exposed to different NK lines (Fig. 4B). The mAb treatment reduced the lysis of the ICAM-1high 1259 mel and 721.221 cells but not the lysis of the ICAM-1dim LB33MEL.B1 cells. The extent of lysis by NK lines of anti-ICAM-1-treated 1259 mel and LB33MEL.B1 cells was similar. Thus, the low level of lysis by the HLA-C-negative LB33MEL.B1 melanoma line by different NK lines is probably related to its low level of ICAM-1 expression.

Lysis of Melanoma Lines by LAK, T-LAK, and NK-LAK Cells. LAK cells were described as antitumor effectors generated by IL-2 treatment that can lyse nonspecifically transformed cells regardless of MHC expression (26). The sensitivity of the melanoma lines to lysis by LAK and NK lines were therefore compared. LAK cells, generated from a healthy donor, did not lyse 1074 mel and lysed to a very low extent 1106 mel and the HLA positive 1190 mel (Fig. 5). Yet, 1259 mel was lysed by LAK cells to the same extent as the LAK-sensitive Daudi cell line (a prototypic b2m-negative human B cell line, Fig. 5). LAK cells contain both CD3+ and CD56+ cells (27). T lymphocytes, but not NK cells, express the CD3 complex; NK cells as well as a subpopulation of T cells express the CD56 determinant (28). Two-color staining of LAK cells with anti-CD3 and anti-CD56 revealed that just 10% of the cells manifested the NK phenotype CD56+CD3-, while the other cells were CD3+ T cells. CD3+CD56+ T lymphocytes constituted only 3% of the cells. To analyze the lytic activity of lysed NK and T components, LAK were depleted with either anti-CD3 (and anti-CD4) or with anti-CD56+ to get NK-LAK (CD56+CD3--) or T-LAK (CD3+CD56-), respectively. The purity of these populations was confirmed by flow cytometry (data not shown). T-LAK showed a lysis pattern similar to parental LAK cells (Fig. 5) while NK-LAK lysed efficiently both the b2m-deficient lines and the HLA positive line 1190 mel, a lysis pattern similar to that of the NKIR1 “NKIR2” NK clones (Fig. 2).

Lysis of Autologous Tumor Cells and Primary Fibroblasts by NK Lines. Peripheral blood NK activity could be significantly reduced in cancer patients, due to either a reduction in circulating NK cells or to a much higher proportion of NK cells with suppressed lytic activity (14, 29). Thus, the potency of NK lines derived from patients 1,074 and 1,106 bearing b2m-deficient tumor lesions was examined. The 1074NK and 1106NK lines, derived from frozen PBMCs, showed a normal pattern of growth and a potent lysis ability. They lysed both autologous and heterologous b2m-deficient melanoma lines (Fig. 6 and data not shown). Yet, autologous primary fibroblasts were not lysed by these NK lines (Fig. 6).

DISCUSSION

The central issue arising from this study is the need for a supplement of NK activity in antitumor immunotherapeutic protocols, particularly in those vaccine protocols inducing specific antitumor T cell reactivity and in patients bearing HLA class I-negative lesions. Since the primary tumor is usually removed surgically, these vaccines mostly aim to induce immune reactivity against metastatic lesions. However, class I expression is suppressed partially or completely in a high percentage of secondary lesions (8, 10). Whether the class I-negative tumor cells evolved because of immunological or nonimmunological necessity, the immunological outcome is the same; CTLs directed against tumor peptide-class I MHC complexes will not lyse these tumor cells. Five of thirteen melanoma patients studied for class I expression of their cancer had class I-negative lesions due to the loss of b2m expression (11). Further specific T cell therapy did not affect these lesions. Class I-negative melanoma lines derived from the lesions were not lysed by different tumor specific CTLs, unless endogenous class I expression was reconstituted via transient transfection of b2m-encoding gene (11). Incubation with exogenous interferon-γ did not restore the endogenous class I expression of these melanoma cells. Therefore, secretion of interferon-γ...
from tumor specific T cells or other sources in a mixture of class I-positive and -negative tumor cells could not cause these β2m-deficient tumor cells to be lysed by the T cells. Conversely, those lines were sensitive to lysis by diverse heterologous NK lines and clones expressing different NKIR (Figs. 2, 3). On the other hand, a control class I positive melanoma line was lysed selectively, based on recognition of its class I alleles by the appropriate NKIR on the NK cells (Figs. 2, 3). Thus, these melanoma lines display in vitro the pattern of sensitivity to NK lysis shown by B cell lines and do not manifest additional non-MHC based NK inhibitory mechanisms. In addition, a correlation between susceptibility of the different melanomas to lysis by primary NK lines and their ICAM-1 expression was observed (Fig. 4). Similarly, ICAM-1 expression on murine target cells was a requirement for their lysis by the human NK-leukemia YT2C2 (30). The observation that class I MHC deficiency of tumor cells is a necessary but not sufficient condition for efficient lysis by primary NK lines is a probable obstacle for successful treatment of such tumor cells with adoptive NK therapy. Additional studies are needed to examine the importance of cell surface expression of ICAM-1

Fig. 5. Cytotoxicity of LAK and LAK subgroups against melanoma lines. LAK and LAK subgroups (NK-LAK and T-LAK) were generated as described. LAK population contained 10% CD56+CD3− NK-LAK and 87% CD56−CD3+ T-LAK cells. Effectors were reacted with the indicated [35S]methionine-labeled target cells for 5 h and the data obtained at an E/T of 10/1 are shown. The results shown in Figs. 3 and 5 are from the same experiment.

Fig. 6. Cytotoxicity of NK lines against autologous β2m-deficient melanoma lines and autologous primary fibroblasts. NK lines were generated from frozen PBMC of patients 1074 and 1106 (1074NK line and 1106 NK line, respectively) as described in Materials and Methods. Primary fibroblasts of patient 1074 (1074fibro) were generated from adherent PBMC, cultured in complete medium containing RPMI medium 1640 and 10% heat inactivated fetal calf serum. Primary fibroblasts of patient 1106 (1106fibro) were generated from firmly adherent cells obtained from harvest of subcutaneous lesion and cultured as before. Effectors were reacted with the indicated [35S]methionine-labeled target cells for 5 h and the data obtained at an E/T of 10/1 are shown.
and/or other adhesion molecules for the efficient lysis of HLA class I-suppressed tumor cells by primary NK lines.

Previous experimental protocols aimed to strengthen the non-specific antitumor activity of cancer patients involved adoptive transfer of LAK cells with infusion of IL-2 (31, 32). LAK cells, specific antitumor activity of cancer patients involved adoptive class I-suppressed tumor cells by primary NK lines.

90–95% CD3

NK cells generated from different healthy human donors contain (described in Methods, PBMC harvested from one buffy coat) adoptive autologous NK therapy.

dose or high dose rIL-2 infusions are not an optimal substitute for in vitro observed. However, these cells need further incubation with IL-2 is used, then the small selective up-modulation of NK is still included this rIL-2 infusion regimen. If a low dose rIL-2 protocol induce life-threatening toxicities; thus, current experimental pro-