Human Leukocyte Antigen Class I Expression on Squamous Cell Carcinoma Cells Regulates Natural Killer Cell Activity

Charles T. Lutz2 and Zoya B. Kurago

Departments of Pathology [C. T. L., Z. B. K.], Oral Pathology, Radiology, and Medicine [Z. B. K.], and the Immunology and Molecular Biology Graduate Programs [C. T. L.], University of Iowa, Iowa City, Iowa 52242

ABSTRACT

Human leukocyte antigen (HLA) class I molecules on hematopoietic cancers and melanomas inhibit attack by natural killer lymphocytes, but previous studies have not consistently demonstrated that carcinoma cells are protected by HLA class I expression. We investigated whether HLA class I molecules protect oral and pharyngeal squamous cell carcinoma cells from natural killer lymphocyte attack. Squamous cell carcinoma cell lines expressed varying levels of HLA class I, which correlated inversely with cytolyis by natural killer-enriched polyclonal lymphocytes. Cytolysis was increased by the presence of anti-HLA class I blocking monoclonal antibody (mAb). Subclones of the NK-92 human natural killer lymphoma cell line were derived by treatment with 5-aza-2’-deoxycytidine and limiting dilution cloning. NK-92 subclones expressed distinct sets of HLA class I-specific receptors. Some NK-92 subclones differentially lysed hematopoietic cells and squamous cell carcinoma cells, even in the presence of anti-HLA class I blocking mAb. This suggests that natural killer cells recognize different non-HLA ligands on hematopoietic and squamous cell carcinoma cells. In the presence of anti-HLA class I monoclonal antibody, other NK-92 subclones increased cytalysis of squamous cell carcinoma cells with moderate-to-high HLA class I levels. Anti-HLA class I mAb also increased natural killer cell attack of squamous cell carcinoma cells that were adherent to plastic. These data suggest that natural killer cell recognition of squamous cell carcinoma cells depends upon the balance of stimulatory and inhibitory ligands.

INTRODUCTION

Carcinomas account for the majority of human cancers and cancer deaths. Indirect evidence suggests that NK cells may control carcinomas. Although most tumor-infiltrating lymphocytes had relatively low natural killer cytolytic activity (1), the presence of CD16 and/or CD56 NK cells in fluids reliably indicated metastatic carcinomatous effusions (2). Among colorectal adenocarcinomas matched for grade and stage, the presence of infiltrating CD57+ NK cells predicted longer disease-free survival (3). Blood NK cell cytolytic activity generally correlated with better survival and was low in patients with advanced cancer (1). NK cells may play a role in SCCs in particular, because peripheral blood NK cytolytic activity correlated inversely with death and with regional and distant metastases but not with local recurrence from head and neck SCCs (4). This relationship was observed with poorly differentiated and moderately differentiated tumors but not with well-differentiated tumors; the latter expressed higher HLA class I levels (4).

It is not clear how NK cells recognize tumor cells. The missing self hypothesis states that stimulatory NK cell receptors recognize widely distributed ligands and that inhibitory NK cell receptors recognize MHC class I ligands (5). Stimulatory NK receptors and tumor cell ligands are still incompletely defined. Human NK cells use at least three receptor gene families to recognize HLA class I molecules. KIR molecules have two or three extracellular immunoglobulin-like domains and recognize specific groups of HLA class I alleles (6). For example, KIR3DL1 recognizes the Bw4+ group of HLA-B alleles (6–8). KIR2DL1 recognizes HLA-C alleles with amino acids N and K at residues 77 and 80, respectively, whereas KIR2DL2 and KIR2DL3 recognize HLA-C alleles with amino acids S and N at residues 77 and 80, respectively (6–9). LIR molecules have two to four extracellular immunoglobulin-like domains (10) and recognize HLA-E molecules that have bound HLA-A-, HLA-B-, and HLA-C-derived signal sequence peptides (13–15). All three receptor types have multiple members that inhibit NK cells. KIR and NKG2 family members also encode receptors that stimulate NK cells (16, 17). Individual NK cells express only a subset of available inhibitory and stimulatory receptor genes, with most NK cells expressing a predominance of inhibitory receptors (18).

Compared with normal cells, many tumor cells express low MHC class I levels and are thus potentially susceptible to NK cell attack (19–23). The missing self hypothesis has received convincing experimental verification in hematopoietic cell model systems. Lymphoid tumors and bone marrow transplants that did not express host MHC class I molecules were rejected by NK cells in vivo (5, 24). MHC class I gene transfection of MHC-low hematopoietic tumors usually inhibited NK cytolysis in vitro (5, 24–27). NK cells lysed MHC class I-high hematopoietic lineage target cells only in the presence of anti-MHC class I blocking mAb (11, 18, 28, 29).

The missing self hypothesis has been tested in nonhematopoietic tumor cells. Although there were exceptions (30), most studies indicated that HLA class I molecules protect human melanoma cells from NK-mediated cytolysis (21, 31–33). NK recognition of carcinomas is less clear. In support of the missing self hypothesis, cytokine treatment coordinately regulated HLA class I levels and resistance to NK-mediated lysis in 410.4 murine mammary adenocarcinoma cells (34). Compared with confluent carcinoma cells, rapidly growing carcinoma cells expressed more HLA class I and were relatively resistant to NK-mediated cytolysis (35). A correlation was found between resistance to NK-mediated cytolysis and MHC class I expression on variants of a rat colon adenocarcinoma cell line (36). However, carcinoma MHC class I molecules appeared to have little role in resistance to NK-mediated cytolysis in most other experimental systems (36–42). There was no correlation between NK-mediated cytolysis and HLA class I expression levels on cell lines that were derived from human colorectal adenocarcinomas (36) or metastatic carcinomas to the brain (41). IFN-γ treatment of metastatic colon carcinoma cells did not alter surface HLA class I expression but inhibited NK-mediated cytolysis (41). Treatment of LINE 1 mouse
lung adenocarcinomas with DMSO or with H-2D<sup>e</sup> gene transfection up-regulated MHC class I expression but did not inhibit NK-mediated cytolysis (38). Likewise, IFN-γ-induced increases in ovarian carcinoma cell HLA class I expression did not alter sensitivity to lymphokine-activated killer-mediated lysis (39). NK-mediated lysis of HLA-negative GLC2 small cell lung carcinoma cells was inhibited by IFN-γ treatment but not by HLA-B<sup>27</sup> gene transfection, which equivalently induced GLC2 cell surface HLA class I expression (40).

β<sub>2</sub>-Microglobulin antisense oligonucleotide treatment equivalently reduced carcinoma and hematopoietic lineage tumor cell surface HLA class I expression levels (42). As a result, hematopoietic lineage tumor cells, but not carcinoma cells, were more sensitive to NK-mediated cytolysis (42).

In the present study, we tested the relevance of the missing self hypothesis to NK recognition of human oral and pharyngeal SCCs. SCC cell lines varied in their expression of cell surface HLA class I molecules, which correlated with protection from polyclonal killer cells. NK-mediated cytolysis of some SCC cells was enhanced by anti-HLA class I blocking mAb. To confirm this observation with cloned human NK cells, we developed a novel set of cloned NK tumor cells with distinct properties.

**MATERIALS AND METHODS**

**Cells.** CAL27, FADU, SCC4, SCC9, SCC15, and SCC25 were derived from SCC tumors of the oral cavity or pharynx and were obtained from the American Type Culture Collection (Rockville, MD). CAL27 and FADU cells were propagated in DMEM (Life Technologies, Inc., Grand Island, NY) with nonessential amino acids, sodium pyruvate, and 10% FBS (Cosmic Calf Serum; HyClone, Logan, UT). SCC4, SCC9, SCC15, and SCC25 were grown in DMEM:Ham's F12 media 1:1 (Life Technologies) with nonessential amino acids, sodium pyruvate, 4 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), and 10% SBS. SCC cells were detached using 0.25% trypsin/1% EDTA (Life Technologies). 721.221 cells were obtained from R. DeMars (University of Wisconsin, Madison, WI). 721.221 transfectants have been described (43–45). 721.221 cells transfected with HLA-B*0702.221 are denoted as B*0702.221, and so forth. Cells were grown at 37°C in 7.5% CO<sub>2</sub>.

The K6 NK-enriched polyclonal cell line was generated and propagated as described previously (44, 45). The NK-92 human lymphoma cell line (46) was obtained from ImmuneMedicine, Inc. (Vancouver, British Columbia, Canada) and was grown without hydrocortisone in MyeloCult HS100 media (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with 100–200 IU/ml rhIL-2 (Biological Resources Branch, National Cancer Institute, Frederick, MD). Alternatively, NK-92 subclones were grown in α-MEM (Sigma) containing 10% horse serum, 10% fetal bovine serum (HyClone), 1% nonessential amino acids, 2-mercaptoethanol, and sodium pyruvate (1 μg/ml), and were cultured for 20–24 h at flat-bottomed, 96-well plates in 100 μl of FADU/CAL27 growth medium (see above) containing gentamicin. Effector cells were added in quintuplicate in 100 μl for each E:T. Cells were incubated for 4 h at 37°C in 7.5% CO<sub>2</sub>. Wells were washed four times with Assay Media (RPMI 1640 with 5% SBS, HEPES, and gentamicin) to remove nonadherent cells and incubated with 200 μl of Assay Media containing 0.5 mM MT2 (Sigma) for 3 h at 37°C. 7.5% CO<sub>2</sub>. MT2 medium was aspirated, and DMSO (150 μl; Fisher Scientific, Pittsburgh, PA) was added to solubilize the formazan crystals for 0.5 h on a shaking platform at 25°C. The absorbance at 540 nm (A<sub>540</sub>) was recorded (EL309 Microplate Autoreader; Bio-Tek Instruments, Winooski, VT). The percentage of reduced MT2 uptake was calculated as:

\[ \frac{A_{540} \text{(SCC alone)}}{A_{540} \text{(NK alone)}} \times 100 \]

Reduced MT2 uptake could be attributable to death, detachment, and/or metabolic inactivation of adherent target cells. MT2 units were calculated exactly as for LUs in the 51Cr release assay.

**Statistical Analysis.** The relationship between HLA class I level and susceptibility to K6-mediated cytotoxicity was tested by linear regression analysis, using Microsoft Excel 97 software. For target cells tested in the presence of control and anti-HLA mAb, the equality of groups after von Krog equation was tested by fitting a regression model with separate intercepts and a common slope and then testing the equality of intercepts using the t test. All statistical analyses were completed using the GLM procedure of the SAS software package (SAS Institute, 1990).

**RESULTS**

To correlate HLA class I expression with susceptibility to NK-mediated cytolysis, we tested a number of HLA-typed SCC cells (Table 1). HLA class I expression levels were determined in flow cytometry by staining with mAb HP-IF7, which binds all known HLA class I molecules (11, 28). For comparison, we stained 721.221 transfectants expressing B*0702, B*2702, or a chimeric molecule with Cw*0304 α<sub>β</sub> and α<sub>β</sub> domains and B*0702 α<sub>β</sub> domain (43). HLA class I expression by these transfectants effectively inhibited NK-
mediated cytolsis in prior studies (44, 45). SCC cell HLA class I expression levels varied 5-fold (Fig. 1), with the highest expression (SCC25) being comparable with that of B*0702.221 and B*2702.221 cells and the lowest expression (SCC9) being comparable with that of Cw*0304.221 cells.

Each SCC cell line encodes distinct HLA alleles (Table 1), which likely reflects distinct cell surface HLA class I expression. Therefore, we tested SCC cell cytolsis by a NK-enriched polyclonal cell line with heterogeneous HLA class I receptor expression. At the time of cytolsis assay, the K6 cell line contained 70% CD3 \(^+\) CD56 \(^+\) NK cells and 18% CD3 \(^+\) T cells, with remaining cells reflecting carryover of irradiated peripheral blood feeder cells and stimulating 721.221 B lymphoblasts. In a separate experiment, K6 cells bound mAb specific for multiple HLA class I receptors, including anti-LIR-1 (ILT2) by 38% of cells, anti-CD94 by 97% of cells, HP-3E4 anti-KIR by 71% of cells, GL183 anti-KIR by 60% of cells, 5.133 anti-KIR by 77% of cells, and DX9 anti-KIR3DL1 by 48% of cells (see “Antibodies” section of “Materials and Methods” for reported anti-KIR mAb specificity). K6 lysed HLA-A-, HLA-B-, and HLA-C-negative 721.221 cells transfected with the pHeBo vector (399 LUs) and lysed B*2702-transfected 721.221 cells less efficiently (161 LUs). K6 lysed SCC cells transfected with the pHeBo vector alone, a \(\sim\) 3.9-fold (Fig. 2). The ability of HP-1F7 to enhance K6 lysis of SCC9 cells was statistically significant (see the legend to Fig. 2). HP-1F7 mAb did not enhance K6 lysis of SCC9 cells. The ability of HP-1F7 to enhance K6 lysis roughly paralleled SCC cell surface HLA class I expression (Fig. 1).

Polyclonal NK cells express heterogeneous receptors for HLA class I molecules and HLA expression may stimulate some NK cells while inhibiting others. Therefore, we investigated the NK-92 lymphoma cell line as a source of monoclonical human NK cells. NK-92 cells lyse NK-sensitive target cells and resemble the CD3 \(^+\) CD56 \(^+\) subpopulation of peripheral blood NK lymphocytes (46). NK-92 cells contained small subpopulations with distinct KIR expression (data not shown).

**Table 1** SCC HLA class I genotype\(^a\)

<table>
<thead>
<tr>
<th>Carcinoma</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>Bw4/Bw6</th>
<th>HLA-C</th>
<th>Residues 77, 80(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC25</td>
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<td>B*08</td>
<td>Bw6</td>
<td>Cw*07</td>
<td>S, N</td>
</tr>
<tr>
<td>SCC9</td>
<td>A*01</td>
<td>B*35</td>
<td>Bw6</td>
<td>Cw*04</td>
<td>N, K</td>
</tr>
<tr>
<td>SCC4</td>
<td>A*02</td>
<td>B*07</td>
<td>Bw6</td>
<td>Cw*04</td>
<td>N, K</td>
</tr>
<tr>
<td>FADU</td>
<td>A*01</td>
<td>B*1516/1517</td>
<td>Bw4</td>
<td>Cw*07</td>
<td>S, N</td>
</tr>
<tr>
<td>CAL27</td>
<td>A*03</td>
<td>B*44</td>
<td>Bw4</td>
<td>Cw*07</td>
<td>S, N</td>
</tr>
</tbody>
</table>

\(^a\) HLA typing was performed by PCR-sequence specific priming at the Iowa City Veterans Administration Medical Center Histocompatibility Laboratory.

\(^b\) Amino acids at HLA-C residues 77 and 80. These HLA-C sequences correlate with KIR recognition (8, 9).

\(^c\) Determined by mAb binding in flow cytometry, as described (48).
Fig. 2. Anti-HLA class I mAb increases K6 lysis of B*2702-transfected 721.221 cells and some SCC cells. K6 was tested at the E:T indicated for lysis of control B*2702- or pHeBo-transfected 721.221 target cells or SCC target cells. The 51Cr release assay was carried out in the presence of control anti-CD56 mAb (●) or HP-1F7 anti-HLA class I mAb (○). For the two mAbs, the differences between intercepts were not statistically significant for pHeBo-transfected 721.221 (P = 0.54) and SCC9 (P = 0.33) target cells. Cytotoxicity differences in the presence of the two mAbs were significant for the other target cells: B*2702-transfected 721.221 (P = 0.006), CAL27 (P = 0.0002), SCC25 (P = 0.0008), and FADU (P = 0.005). Similar results were obtained in four additional experiments with HP-1F7 or DX17 anti-HLA class I blocking mAb.

Fig. 3. SCC cell HLA class I expression level correlates with susceptibility to K6 lysis. The cell surface HLA class I expression as determined by HP-1F7 mAb median fluorescence intensity (Fig. 1) is plotted against the number of LUs/10⁶ produced by K6 cells in 51Cr release assay (Fig. 2). The point representing each SCC cell tested is indicated.

To obtain lymphoma cells with distinct phenotypes, NK-92 clones were isolated at limiting dilution. Additional subclones were isolated after short-term treatment of NK-92 clones with 5-aza-dCytidine (“Materials and Methods”). NK-92 subclones had distinct surface KIR expression (Table 2) and differentially lysed hematopoietic and carcinoma target cells (see below).

Representative NK-92 subclones were tested with transfected 721.221 target cells and with SCC target cells that expressed HLA class I molecules at relatively high (CAL27) or low (SCC9) levels (Fig. 1). NK92.35 lysed both hematopoietic and SCC target cells well, whereas NK92.10 lysed hematopoietic cells well and SCC cells poorly (Fig. 4). The addition of HP-1F7 mAb did not significantly augment cytolysis by either NK92.35 or NK92.10 (Fig. 4). The failure of NK92.10 to lyse SCC9, CAL27 (Fig. 4), and other SCC cells (data not shown), even in the presence of anti-HLA class I blocking mAb, may have been attributable to the inability of NK92.10 stimulatory receptors to recognize SCC ligands. DX9⁺ NK92.26.5 lysed hematopoietic B*2702.221 cells modestly, and cytolyis was greatly increased by the addition of HP-1F7 mAb. HLA class I mAb blockade modestly increased B*0702.221 cell cytolyis, perhaps by preventing engagement of inhibitory LIR-1 or CD94 receptors on NK92.26.5. NK92.30.1G poorly lysed CAL27, and cytolyis was significantly increased by the presence of anti-HLA class I blocking mAb (Fig. 4). Similar effects were seen with B*2702-transfected hematopoietic 721.221 cells. HP-1F7 anti-HLA class I mAb had modest effects on cytolyis of SCC9 and no effect on cytolyis of B*0702-transfected 721.221 cells (Fig. 4). Testing additional NK-92 subclones, we found that HLA class I on SCC cells inhibited cytolyis by some NK-92 subclones but not by others (data not shown). NK92.30.1F lysis of B*2702.221 cells was greatly enhanced by the presence of HP-1F7 mAb (Fig. 5).

Table 2. NK-92 subclone binding to anti-NK receptor mAb

<table>
<thead>
<tr>
<th>NK-92 subclone</th>
<th>GL183</th>
<th>EB6</th>
<th>DX9</th>
<th>LIR-1</th>
<th>CD94</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK92.10</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NK92.26</td>
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<td>+</td>
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<td>NK92.26.5</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>NK92.30.1F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NK92.30.1G</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>NK92.35</td>
<td>NT</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Values >5× negative control mAb binding are scored as +; values <2× negative control are scored as −; intermediate values are scored as . Values are given as means ± SEM of triplicate wells. Subclone NK92.35 binds KIR3DL1 (47). Subclone NK92.30.1G binds KIR2DL1 and KIR2DS1 (47). Subclone NK92.30.1F binds KIR2DL2, KIR2DL3, and KIR2DS2 (47). Subclone NK92.30.1E binds KIR2DL1 (47). Subclone NK92.30.1D binds KIR2DL1 (47). Subclone NK92.30.1C binds KIR2DL1 (47). Subclone NK92.30.1B binds KIR2DL1 (47). Subclone NK92.30.1A binds KIR2DL1 (47).

Fig. 4. Anti-HLA class I mAb has a variable effect on NK-92 subclone lysis of SCC cells. The percentage of specific lysis at an E:T of 5 is shown for the NK-92 subclone indicated on the left. 51Cr release assay was performed in the presence of control anti-CD56 mAb (hatched column) or HP-1F7 anti-HLA class I mAb (open column with thick borders). Target cells are CAL27 and SCC9 SCC cells and control B*2702-transfected 721.221 cells (B7) and B*0702-transfected 721.221 cells (B7). In some cases, lysis in the presence of anti-CD56 is slightly less than (NK92.35 versus B*2702-transfected 721.221) or equal to (NK92.35 versus B*0702-transfected 721.221) in the presence of HP-1F7. These data are representative of several experiments that included the NK-92 subclones and target cells in different combinations.
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...with trypsin/EDTA before use in the short-term 51Cr release assay. To address concerns that trypsin/EDTA detachment might have altered SCC ligand expression levels and disposition, we measured NK92.30.1F-mediated attack on FADU cells that had been allowed to attach and grow overnight in flat-bottomed plastic wells. NK92.30.1F cells were incubated with adherent FADU cells for 4 h. Then nonadherent NK and SCC cells were removed by washing, and the presence of adherent, metabolically active cells was measured by MTT uptake. A similar MTT assay has been used by others to measure NK toxicity toward carcinoma target cells (53). NK92.30.1F toxicity to FADU cells was increased in the presence of HP-1F7 anti-HLA class I mAb (Fig. 7). Expressed in terms of MTT units, analogous to LUs, NK92.30.1F was 7.1-fold more toxic to FADU SCC cells in the presence of HP-1F7 mAb than in the presence of anti-CD56 mAb. Thus, NK cells recognize HLA class I molecules on SCC target cells that are anchored to surfaces.

DISCUSSION

The missing self hypothesis states that target cell MHC class I expression prohibits NK cell activation and NK-mediated cytolysis (5). The missing self hypothesis has been verified in the majority of experimental systems using hematopoietic lineage tumor target cells (5, 24–27). In addition, melanoma cells were protected from NK-mediated cytolysis when MHC class I levels were sufficiently high (32). Results with carcinomas have been mixed, with most studies finding that MHC class I provided little or no protection from NK-mediated cytolysis (36–42). This result has important implications for cancer immunotherapy because carcinomas are more prevalent than any other type of cancer. The missing self hypothesis had not been tested in SCC, which is the predominant cancer of the lip, oral cavity, esophagus, larynx, cervix, vagina, and external genitalia, and is an important cancer of skin, lung, and other sites. We show here that some human NK cells were inhibited by SCC cell HLA class I molecules. The degree of protection correlated with the SCC class I expression level.

Our findings are consistent with clinical observations. Schantz and Ordonez (4) found that high peripheral blood NK activity correlated with fewer metastases and improved survival in patients with moderately to poorly differentiated head and neck SCC but not in patients with well-differentiated SCC. HLA class I expression was relatively high in well-differentiated SCC and relatively low on moderately to poorly differentiated SCC (4). It is tempting to speculate that HLA class I expression protected the well-differentiated SCC cells from NK attack in vivo. This suggests that treatment of SCC patients with...
agents that enhance NK cell activity might eliminate additional metastatic SCC cells, particularly if the tumors express little or no HLA class I. Furthermore, it might be expected that IFN-γ, which often increases HLA class I expression and resistance to NK-mediated cytolysis, will have little or no clinical value for certain tumors. This reasoning suggests that agents that promote NK activity but not HLA class I expression would enhance NK surveillance of SCC metastases. Several chemokines activate NK cells and are under investigation (55).

A critical question with clinical relevance is whether SCC HLA class I molecules inhibit other NK functions. It is likely that NK migration and cytokine secretion are relevant to NK attack on SCC cells. Under some circumstances, hematopoietic cells differentially trigger NK-mediated cytolysis and IFN-γ secretion (45). It will be important to investigate whether SCC cells differentially stimulate various NK effector functions and whether HLA class I expression equally inhibits all NK functions in vitro and in vivo.

Several factors may account for the lack of protection by HLA class I molecules on some SCC cells reported here and by carcinoma cells in other experiment systems (36–42): (a) HLA class I levels must be sufficiently high to afford protection. This was shown in our study of SCC cells and in other studies of hematopoietic lineage cells (26) and of melanomas (32); (b) the tumor cells must express HLA class I alleles that interact with the specific inhibitory receptors that are expressed on the NK cells being tested; and (c) target cells must express appropriate levels of stimulatory ligands. Our study shows that some NK-92 subclones differentially lyse hematopoietic and SCC target cells independently of HLA class I recognition. This suggests that hematopoietic and SCC cells differ in stimulatory ligand expression. A number of stimulatory NK receptors and ligands have been identified, but the roles of these candidates is not clear in physiological settings (6, 7, 56). Although not all stimulatory ligands are known, it is likely that target cells with insufficient stimulatory ligand expression will not activate NK cells, even when interactions between inhibitory NK receptors and MHC class I ligands are prohibited (7).

In contrast, high target cell stimulatory ligand expression may strongly activate NK cells, potentially overriding abundant inhibitory NK receptor/MHC class I interactions. NK cell activation that was induced by mAb cross-linking of a single type of stimulatory receptor was prohibited by co-cross-linking inhibitory KIR3DL1 receptors. However, KIR3DL1 cross-linking did not prohibit NK activation that was induced by mAb engagement of multiple stimulatory receptor types (7). Presumably, NK cells respond similarly to natural ligands. Thus, the apparent failure of the missing self hypothesis to apply to NK cell recognition of carcinoma cells in some experimental systems may be attributable to insufficient expression of specific MHC class I molecules or to inappropriately high or low expression of stimulatory ligands.

Hematopoietic lineage cells grow in suspension in vitro, whereas carcinoma cells adhere to plastic. For use in the classic 51Cr release assay, carcinoma cells are detached from plastic, most often with the aid of trypsin to cleave adhesion proteins. On confluent adherent cells, expression of plasma membrane proteins and lipids differs on apical and basolateral surfaces (57). Even in the absence of cell-cell contact, apical proteins do not localize to the attached face of the cell membrane (57). Therefore, it is possible that the disposition of stimulatory ligands may not be equal on adherent and detached carcinoma cells. In addition, trypsin used to detach cells may cleave protein ligands from the carcinoma cell surface. NK receptors that recognize carcinoma cells are beginning to be identified (58, 59), but the carcinoma ligands that stimulate NK cells remain largely uncharacterized. We were concerned that the trypsin/EDTA treatment used to dissociate SCC cells from tissue culture plastic surfaces may have changed the balance of stimulatory and inhibitory ligands. Therefore, we adopted the MTT assay, which allowed us to test SCC cells that had been allowed to attach to plastic and re-express trypsin-cleaved molecules. As in the 51Cr release assay, HP-1F7 blocking anti-HLA class I mAb increased NK-mediated damage, indicating that HLA class I expression on adherent SCC cells inhibited NK attack.

A full understanding of how NK cells control SCC requires characterization of the NK receptors and HLA ligands that inhibit NK cytolysis, migration, and cytokine secretion in clinical settings. A pressing need is to identify the SCC ligands and NK receptors that stimulate NK effector functions and that potentially override inhibitory signaling by HLA class I ligands.

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