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

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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 cytolytic activity 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 cytolysis 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 NK1 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 with disease 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 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 lyed 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 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 adenocarcinoma cells with DMSO or with H-2D\(^{a}\) gene transfection up-regulated MHC class I expression but did not inhibit NK-mediated cytolysis (38). Likewise, IFN-\(\gamma\)-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-\(\gamma\)-treatment but not by HLA-B27 gene transfection, which equivalently induced GLC2 cell surface HLA class I expression (40).

\(\beta_2\)-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, L-glutamine, pyruvate, and folic acid; detailed formula was described (43–45). 721.221 cells transfected with HLA-B*0702 are denoted as 721.221.C27. SCC4, SCC9, SCC15, and SCC25 were grown in DMEM:Ham’s F12 media 1:1 (Life Technologies) with nonessential amino acids, sodium pyruvate, and 10% SBS (Cosmic Calf Serum; HyClone, Logan, UT). SCC4, SCC9, SCC15, and SCC25 were grown without hydrocortisone in MyeloCult H5100 media (StemCell Technologies; Vancouver, British Columbia, Canada). CAL27 and FADU cells were propagated in DMEM:Ham’s F12 media 1:1 (Life Technologies) with nonessential amino acids, sodium pyruvate, 4 \(\mu\)g/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), and 10% SBS. SCC cells were detached using 0.25% trypsin/0.1% EDTA (Life Technologies). 721.221 cells were obtained from R. DeMars (University of Wisconsin, Madison, WI). 721.221 transfrectants have been described (43–45). 721.221 cells transfected with HLA-B*0702 are denoted as B*0702.221, and so forth. Cells were grown at 37°C in 5% CO\(_2\).

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 grown without hydrocortisone in MyeloCult H5100 media and was propagated in DMEM:Ham’s F12 media 1:1 (Life Technologies) with nonessential amino acids, sodium pyruvate, 4 \(\mu\)g/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), and 10% SBS. SCC cells were detached using 0.25% trypsin/0.1% EDTA (Life Technologies). 721.221 cells were obtained from R. DeMars (University of Wisconsin, Madison, WI). 721.221 transfrectants have been described (43–45). 721.221 cells transfected with HLA-B*0702 are denoted as B*0702.221, and so forth. Cells were grown at 37°C in 5% CO\(_2\).

The specific activity of the anti-KIR mAb have been reported as follows (47): DX9 binds KIR3DL1; GL183 binds KIR2DL2, KIR2DL3, and KIR2DS2; HP-3E4 binds KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS4, and KIR2DS5; and 5.133 binds KIR3DL1, KIR3DL2, and KIR2DS4. For production of F(ab’\(\_\)2) fragments, HP-1F7 hybridoma cells were grown in serum-free medium, and IgG was digested with immobilized Ficin (Pierce Chemicals, Rockford, IL). F(ab’\(\_\)2) and F(ab’) fragments were separated from IgG and Fc fragments by immobilized protein A affinity columns. Absence of whole IgG was assessed by SDS-PAGE.

**Flow Cytometry.** Cell surface labeling and analysis by flow cytometry were performed as described (44, 48, 49). Briefly, 1–5 \(\times\) 10\(^6\) cells were incubated with saturating concentrations of primary mAb for 20–30 min on ice and washed, and when needed, a fluorochrome-labeled secondary reagent was added for an additional 20–30 min on ice. Cells were washed, resuspended in 0.1% sodium azide, 5% PBS in PBS, and analyzed on a dual laser Becton Dickinson FACS Vantage (Mountainview, CA). Logarithmic flow cytometry values were linearized as described (49). For evaluating receptor levels on NK-92 subclones, mAb binding values between 2- and 5-fold above the values of negative control antibody staining were scored as low staining. Values above and below that range were scored as ± and −, respectively.

**Cytotoxicity Assays.** Standard 5-h \(^{31}\)Cr release assays were performed as described (44). NK-92 subclones were generally tested 3–4 days after the previous feeding with rhIL-2. In mAb blocking studies, HLA class I and control mAb were added to target cells before cell dilution. DX7 (5 \(\mu\)g/ml), HP-1F7 mAb, and HP-1F7 (F(ab’\(\_\)2)) preparations (1.5 \(\mu\)g/ml) were used at concentrations that were saturating on 5 \(\times\) 10\(^5\) HLA-high 721.221 transfected cells in flow cytometry (data not shown). LUs were calculated using the von Krogh equation as described (50, 51). The MTT assay was modified from existing protocols (52, 53). Briefly, SCC cells (1 \(\times\) 10\(^6\)) per well were cultured for 20–24 h in flat-bottomed, 96-well plates in 100 \(\mu\)l of FADU/CAL27 growth medium (see above) containing gentamicin. Effector cells were added in quintuplicate in 100 \(\mu\)l for each E:T. Cells were incubated for 4 h at 37°C in 5% CO\(_2\). Wells were washed four times with Assay Media (RPMI 1640 with 5% PBS, HEPES, and gentamicin) to remove nonadherent cells and incubated with 200 \(\mu\)l of Assay Media containing 0.5 mm MTT (Sigma) for 3 h at 37°C. 75% CO\(_2\). MTT medium was aspirated, and DMSO (150 \(\mu\)l; 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 (\(A_{540}\)) was recorded (EL309 Microplate Autoreader; Bio-Tek Instruments, Winooski, VT). The percentage of reduced MTT uptake was calculated as:

\[
\% \text{ reduced MTT uptake} = \left(1 - \frac{A_{540} (\text{SCC and NK}) - A_{540} (\text{NK alone})}{A_{540} (\text{SCC alone})}\right) \times 100
\]

Reduced MTT uptake could be attributable to death, detachment, and/or metabolic inactivation of adherent target cells. MTT units were calculated exactly as for LUs in the \(^{31}\)Cr release assay.

**Statistical Analysis.** The relationship between HLA class I level and susceptibility to K6-mediated cytolysis 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 Krogh transformation 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-1F7, 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 \(\alpha_\alpha\) and \(\alpha_\alpha\) domains and B*0702 \(\alpha_\alpha\) domain (43). HLA class I expression by these transfectants effectively inhibited NK-
mediated cytolyis 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 cytolysis assay, the K6 cell line contained 70% CD3+CD56+ NK cells and 18% CD3+ 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 at varying levels of efficiency (Fig. 2). Susceptibility to K6-mediated lysis inversely correlated (R² = 0.99) with target cell HLA class I levels (Fig. 3). SCC cell susceptibility to K6-mediated cytolyis could not be explained by lack of diversity of available HLA class I genes. The relatively susceptible SCC9 cells encoded a Bw4+ HLA-B allele (Table 1), which is recognized by KIR3DL1 present on many K6 cells. SCC9 cells also contained HLA-C alleles that encode either S-N or N-K amino acids at residues 77 and 80 (Table 1). These two classes of HLA-C molecules are recognized by distinct KIR (8, 9). Although SCC9 cells had the potential to make diverse HLA class I molecules, they expressed relatively low cell surface HLA class I levels and were susceptible to cytolysis (Fig. 3). In contrast, SCC25 cells did not contain a Bw4+ HLA-B allele and lacked the ability to make one of the two classes of HLA-C molecules. Yet SCC25 cells had high cell surface HLA class I expression and were relatively resistant to K6-mediated cytolyis (Fig. 3). These observations emphasize that HLA class I expression levels must be sufficiently high to protect SCC cells from killer cells.

To further test the role of HLA class I recognition, we used blocking HP-1F7 and DX17 anti-HLA class I IgG1 mAbs. HP-1F7 and DX17 mAbs block HLA class I recognition by KIR, LIR, and CD94/NKG2 receptors (11, 28, 29, 33). IgG1 does not engage NK cell CD16 FcyRIII, and K6 cells did not bind KB61 mAb (data not shown), which detects all CD32 isoforms, including FcyRIIc (54). The presence of HP-1F7 mAb did not alter K6 lysis of pHeBo vector-transfected 721.221 cells but significantly augmented lysis of B*2702.221 cells 3.9-fold (Fig. 2). K6 lysis of SCC25 and CAL27 SCC cells was enhanced 3.2- and 4.3-fold, respectively, by the presence of HP-1F7 mAb (Fig. 2). HP-1F7 mAb enhanced K6 lysis of FADU cells by 1.7-fold. Although modest, this increase 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 lymphopa cell line as a source of monoclonal human NK cells. NK-92 cells lyse NK-sensitive target cells and resemble the CD3+16−56+ subpopulation of peripheral blood NK lymphocytes (46). NK-92 cells contained small subpopulations with distinct KIR expression (data not shown).
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 pHBe-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 pHBe-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/10^6 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-2′-deoxycytidine (“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). NK92.30.1F expressed KIR3DL1 (Table 2), which is specific for Bw4+ HLA-B alleles, including

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

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<tr>
<th>NK-92 subclone</th>
<th>GL183</th>
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<th>DX9</th>
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* Values >5× negative control mAb binding are scored as +; values <2× negative control are scored as −; intermediate values are scored as 0.
* Binds KIR2DL2, KIR2DL3, and KIR2DS2 (47).
* Binds KIR2DL1 and KIR2DS1 (47).
* Binds KIR3DL1 (47).
* NT, not tested.

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 (B27) 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) lysis 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.
NK RECOGNITION OF SQUAMOUS CELL CARCINOMA HLA CLASS I

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 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 on 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 trypsin/EDTA before use in the short-term $^{51}$Cr 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.

**Fig. 5.** Anti-HLA class I mAb increases NK92.30.1F lysis of FADU SCC cells. NK92.30.1F was tested against control B*2702.221 target cells (A) or FADU target cells (B) in $^{51}$Cr release assay. Cytolysis was tested in the presence of control anti-CD56 mAb (■) or HP-1F7 anti-HLA class I mAb (△). In the same experiment, HP-1F7 did not increase killing of control pHeBo.221 cells. Consistent results were obtained in three additional experiments using HP-1F7 or DX17 anti-HLA class I blocking mAb.

**Fig. 6.** NK lysis of CAL27 SCC cells is increased in the presence of HP-1F7 anti-HLA class I IgG or F(ab’)$_2$. NK92.30.1F (A) and NK92.30.1G (B) were tested with CAL27 target cells in $^{51}$Cr release assay in the presence of HP-1F7 IgG, HP-1F7 F(ab’)$_2$, or no mAb. In the same experiment, HP-1F7 IgG and HP-1F7 F(ab’)$_2$ had no effect on NK lysis of pHeBo.221 cells. Similar results were obtained in one additional experiment.

**Fig. 7.** Anti-HLA class I mAb increases NK92.30.1F attack of plastic-adherent FADU SCC cells in MTT assay. As described in “Materials and Methods,” reduction in MTT uptake may reflect death, detachment, or metabolic inactivation of adherent carcinoma cells. NK92.30.1F-mediated damage was tested in the presence of control anti-CD56 mAb (■) or HP-1F7 anti-HLA class I mAb (△). Consistent results were obtained in two additional experiments.
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 cytolyis, 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 cytolyis 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 recruitment of stimulatory receptors 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 cytolyis, 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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NK RECOGNITION OF SQUAMOUS CELL CARCINOMA HLA CLASS I


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

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