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Review

NK cell activating ligands on human malignant cells: Molecular and functional defects and potential clinical relevance

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Abstract

Malignant transformation of cells is frequently associated with HLA class I antigen downregulation or loss. This abnormality provides malignant cells with a mechanism to escape control by HLA class I antigen-restricted, tumor antigen-specific cytotoxic T lymphocytes. Surprisingly, HLA class I antigen downregulation or loss by tumor cells is not associated with control of tumor growth by natural killer (NK) cells, as it would be predicted by the "missing-self" hypothesis. Here, we discuss the role of NK cell activating ligand abnormalities as well as HLA class I molecule and ICAM-1 shedding in the lack of control of tumor growth by NK cells with emphasis on their molecular mechanisms. In addition, we discuss the impact of these abnormalities on cancer immune surveillance.

Keywords: HLA class I; HLA-G; ICAM-1; MIC; NKG2D; ULBP

Contents

1. Introduction

HLA class I antigens are frequently downregulated or not detectable in malignant lesions [1,2]. Immunohistochemical staining of malignant lesions with monoclonal antibodies (mAb) has convincingly shown that, depending on the tumor type, defects in HLA class I antigen expression range between about 16% and 50% of the malignant lesions analyzed [1,2]. The abnormal phenotypes identified include total HLA class I antigen loss or downregulation, loss of one HLA haplotype, loss or downregulation of the gene products of one HLA class I locus, selective loss or downregulation of one HLA class I allospecificity and combination of the various abnormalities [2]. These tumor phenotypes have been found to be caused by distinct

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molecular mechanisms, which have been extensively investigated in the last decade. They include structural and regulatory defects in the HLA class I subunits and antigen processing machinery components [2]. They lead to defective expression of HLA class I antigen-tumor antigen derived peptide complexes, thereby providing malignant cells with an escape strategy from cytotoxic T lymphocyte (CTL) recognition and destruction [2]. This mechanism has been invoked to explain the association between HLA class I antigen downregulation or loss and poor clinical course of the disease, which has been described in several types of malignant diseases [2]. The latter finding is somehow surprising, since the "missing-self" hypothesis, corroborated by results of *in vitro* experiments as well as of studies in animal model systems, predicts that tumor cells that have lost or downregulated HLA class I antigens become more susceptible to NK cell-mediated lysis [3,4]. Therefore, one would expect that NK cells would replace CTL in the control of tumor growth, when malignant cells escape from CTL recognition because of defects in HLA class I antigen expression.

The lack of association between HLA class I antigen defects by malignant cells and control of tumor growth by NK cells, which, with few exceptions [5–7], has been found in all the malignant diseases investigated thus far, has to be reinterpreted in light of the identification of the MHC class I antigen-recognizing NK cell inhibitory receptors (Ly49 and KIR families) [8,9] and NK cell activating receptors (NKp30, NKp44, NKp46, 2B4 and NKG2D) [10–12] and of the characterization of their role in the interactions of NK cells with target cells [10–12]. The available evidence strongly suggests that MHC class I molecule loss is not sufficient to confer sensitivity of target cells to NK cell-mediated cytotoxicity, but expression of activating ligands on target cells that signal through NK cell activating receptors is required. The ligands thus far identified include CD48 for 2B4 [10] and MHC class I chain related (MIC) molecules [13] and UL16-binding proteins (ULBP1, ULBP2, ULBP3 and ULBP4) [14,15] for NKG2D. CD48 is a membrane-bound protein ubiquitously expressed on hematopoietic cells [10], whereas MICs and ULBPs have a restricted tissue distribution and are preferentially induced by viral and bacterial infections as well as malignant transformation of cells [16]. These findings in conjunction with those of the inhibitory ligand/receptor system support the notion that NK cell activity is regulated by a balance between inhibitory and activating signals and shifting of this balance is largely dependent on the ligand availability on target cells.

Based on the characteristics of NK cell activating ligands, several alternative, but not exclusive possibilities may be envisioned to account for the limited NK cell role in the control of growth of tumors with HLA class I antigen loss or downregulation. First, NK cells may not traffic efficiently to the tumor site [17]. Second, if NK cells do infiltrate malignant lesions, they may not function properly because of lack of expression of functional NK cell activating signals by tumor cells and/or because of the presence of tumor-derived immunosuppressive molecules in the tumor microenvironment. The latter two mechanisms are discussed in this review, with emphasis on the role of abnormalities in the expression and/or function of ligands for NKG2D and secretion/shedding of NKG2D ligands and HLA class I molecules as immunosuppressive modulators during malignant transformation of cells. Specifically, first we describe the abnormal NKG2D ligand expression phenotypes and the NKG2D ligand shedding by malignant cells. Second, we discuss the scanty information about the molecular mechanisms underlying these abnormalities and the additional contribution of HLA class I antigen and intercellular adhesion molecule (ICAM)-1 shedding to the lack of NK cell-mediated lysis of tumor cells. Lastly, we discuss the role of NKG2D ligand and HLA class I antigen abnormalities in host tumor immune surveillance.

2. Abnormal NKG2D ligand phenotypes

The lack of susceptibility to NK cell-mediated lysis of target cells with defects in MHC class I antigen expression, a phenotype frequently observed in human malignant cells, suggests that the activating signals are not transmitted into NK cells. It is our working hypothesis that this lack of transmission of activating signals is due to insufficient expression of functional NKG2D ligands on tumor cell surface. This hypothesis is supported by the results of a number of studies which have analyzed the frequency of expression of NKG2D ligands in various types of malignant lesions and by the correlation between lack of NKG2D ligand expression and HLA class I antigen loss.

2.1. Lack of NKG2D ligand expression

The expression of NKG2D ligands MICA and MICB has been examined in glioma [18], neuroblastoma [19], leukemia [20], melanoma [21,22], and carcinomas of breast, lung, colon, kidney, ovary, and prostate [23,24]. MICA-specific mAb and mAb that recognize determinants shared by MICA and MICB have been utilized in most of the published studies. To the best of our knowledge, only glioma, neuroblastoma and leukemic cells have been analyzed with MICB-specific mAb. Therefore, the data about frequency of MICB expression by malignant cells have to be interpreted with caution, since they are too limited to draw any definitive conclusions. In carcinomas, the frequency of MICA and MICB expression (or MICA/B expression) ranges from 20% (2 of 10) of the surgically removed breast carcinoma lesions to 100% of the surgically removed lung (2 of 2), renal cell (2 of 2) and prostate (2 of 2) carcinoma lesions analyzed either by flow cytometry or by immunohistochemistry [23,24]. In glioma, MICA and MICB were found to be expressed at a frequency of 100% and 75% on 12 long-term cell lines and on five short-term cultured cell lines isolated from lesions, respectively [18]. On the other hand, in leukemia, the frequency of MICA expression by long-term cell lines appears to be markedly lower than that by leukemic cells freshly isolated from patients. MICA was found to be expressed by only one (25%) of the four leukemic long-term cell lines [21], but by 11 (44%) of the leukemic preparations isolated from 25 patients [20]. The latter were also found to express MICB in seven (28%) of the samples [20]. In melanoma, MICA was found to be expressed on 14 (77%) of 18 cutaneous melanoma cell lines [22] and on 9 (75%) of 12 uveal melanoma cell lines (Table 1). Different frequen-

Table 1
MICA and HLA class I antigen expression on cultured melanoma cell lines

	HLA class I	MICA
Cutaneous[a]		
FO-1	−	+++[a]
M14	+	−
1174MEL	−	+++
Me18105	−	+++
1074MEL	−	+/−
MEL15	+	+/−
MEL.A	+	+
MEL.B	+	+/−
1259MEL	−	++
SK-MEL-33	−	++
IRNE	−	++
AUMA	−	++
1106MEL	−	−
MEL39	−	++
ME1386	−	−
Colo38	+	−
DM391	+	++
T372A	+	++
Uveal[b]		
92-1	+	+
Mel 202	+	+/−
Mel 270	+	+
Mel 285	+	+
Mel 290	+	−
OCM 1	+	−
OCM 3	+	+
OCM 8	+	+
Omm 1	+	+++
Omm 1.5	+	+
Omm 2.3	+	+
Om 431	+	−

[a] Adapted from Ref. [21].
[b] Determined by flow cytometry as described in Ref. [21].

cies of MICA expression have been found by Vetter et al. [22] and by ourselves (Table 2) in primary and metastatic, surgically removed cutaneous melanoma lesions. While Vetter et al. have reported MICA expression in 31 (77%) of 40 primary lesions and in 12 (60%) of 20 metastatic lesions [22], we have found MICA in 9 (47%) of 19 primary lesions and in 3 (20%) of 15 metastatic lesions (Table 2) derived from an Asian population. Of note in our study, the frequency of MICA expression is correlated with that of HLA class I antigen expression both in primary and in metastatic lesions (Table 2). More recently, Vetter et al. have reported that MIC was expressed in 5 (55%) of 9 primary uveal melanoma lesions and was not detectable in the 11 uveal melanoma metastases analyzed [25]. It is also worth noting that MICB is expressed on glioma and leukemic cells, but has not been detected on the surface of tumor cells of other histotypes [Unpublished observations]. In preliminary studies, we and others [19] have found that MICB is preferentially localized in the cytoplasm of melanoma and neuroblastoma cells. This phenotype probably reflects an impaired intracellular trafficking of MICB protein to the cell surface.

ULBP expression has been examined only in glioma [18], leukemia [20] and melanoma cells [21]. In general, ULBP molecules are expressed less frequently than MIC molecules on malignant cells. Both ULBP2 and ULBP3 are expressed on all (100%) the 12 glioma cell lines tested, while ULBP1 has been detected only on 5 (41%) of the 12 cell lines tested. Furthermore, only ULBP2 has been detected on glioma cells freshly isolated from five lesions [18]. ULBP2 is expressed in association with ULBP1 and ULBP3 in 4 (16%) of the 25 leukemic cell preparations analyzed [20], and without ULBP1 and ULBP3 in 5 (20%) of the leukemic cell preparations. In melanoma, ULBP expression has only been analyzed in cultured cell lines [21]. ULBP2 and ULBP3 were detected in 5 (27%) and 9 (50%), respectively, of the 18 cultured cutaneous melanoma cell lines tested, while ULBP1 was detected in only one (5%) of them [21]. The frequency of expression of at least one of the three ULBP molecules in malignant cells is between 18% and 100%, depending on the tumor type. Co-expression of all three ULBP molecules on one tumor cell population is rare. The frequency of ULBP4 expression in malignant cells is not clear. It is noteworthy that ULBP and MIC do not appear to be expressed in a coordinated fashion in the tumor cells examined.

In general, MICA and MICB are expressed at a higher frequency than ULBP proteins in malignant cells and lesions. Regardless, these NKG2D ligands are undetectable or weakly expressed in at least 30–60% of the examined lesions. These findings, which have to be corroborated by the results of more comprehensive studies with a larger sample size and clinical correlates, support the hypothesis that NK cells may not control tumor growth because of lack or low expression of NKG2D ligands by tumor cells.

2.2. *Molecular mechanisms underlying lack of NKG2D ligand expression in malignant cells*

2.2.1. *Structural defects*

In the analysis of seven melanoma cell lines and one untransformed melanocytic strain, we found that only the cell lines FO-1, 1074MEL and 1259MEL express MICA on their cell surface; its level was high on the cell lines FO-1 and 1259MEL and low

Table 2
MICA and HLA class I antigen expression in cutaneous melanoma lesions

Lesions	Number	HLA class I			(+) %	MICA				(+) %
		−	+/−	+[a]		−	+/−	+	++	
Primary	19	0	10	9	47	10	2	4	3	47
Metastatic	15	1	11	3	20	12	1	2	0	20
Melanocytic nevi	8	1	5	2	25	8	0	0	0	0

[a] Determined and scored by immunohistochemistry as described in Ref. [71]. The MICA-specific mAb used is BAM195 [21].

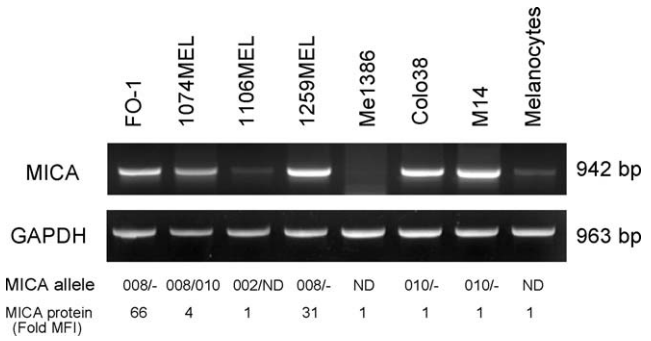

Fig. 1. MICA mRNA expression in cultured melanoma cell lines. Total RNA was isolated from the indicated seven cutaneous melanoma cell lines and one melanocytic strain and reversely transcribed to first-strand cDNA for PCR analysis using MICA gene-specific primers [21]. The GAPDH mRNA was analyzed as the internal control [21]. The predicted size of PCR products for MICA and GAPDH is 942 and 963 bp, respectively. The MICA alleles from 5 of 6 analyzed melanoma cell lines were determined by sequencing and indicated. The levels of MICA protein expression on the melanoma cells and melanocytes analyzed were determined by flow cytometry and indicated as fold MFI (fold increase in mean fluorescence intensity over the background). ND: not determined.

on the cell line 1074MEL (Fig. 1). MICA was not detectable on the melanocytic strain and on the cell lines 1106MEL, Colo38, M14 and Me1386. Analysis of MICA mRNA expression in the latter cell lines revealed two structural mechanisms leading to lack of MICA expression on their cell surface. First, in the cell line Me1386, full-length MICA mRNA was not detected and is likely to be caused by a 5′ truncation upstream of exon 3 in the MICA mRNA [Unpublished results]. Since the structure of the MICA gene remains intact in this cell line, as assessed by genomic PCR with a combination of MICA-specific primers, whether the expression of a truncated MICA mRNA transcript is caused by an alternative transcriptional start in intron 2 remains to be determined. Second, in the cell lines Colo38 and M14, only the transcripts encoded by MICA*010, a naturally existing defective allele carrying an Arg-to-Pro substitution at position 6, were detected. This substitution causes misfolding and rapid degradation of the MICA protein [26]. It is not clear whether the two cell lines are homozygous for MICA*010 or heterozygous with loss of heterozygosity or repression at the non-MICA*010 allele. The latter two abnormalities, which are frequently found in malignant cells' chromosome 6 in which the MICA gene is located [12], can account for, at least in part, the detection of only MICA*010 in tumor cells. In this regard, a natural, cancer-unrelated defective allele is revealed by a cancer-related defect in the course of malignant transformation.

2.2.2. Regulatory defects

Among the seven cell lines and one melanocytic strain we have investigated, the cell line 1106MEL has a markedly reduced level of steady-state MICA mRNA compared with other cell lines, but its level of expression was similar to that of melanocytes (Fig. 1). The 1106MEL melanoma cells and the melanocytes do not carry homozygous MICA*010; therefore, the low level or lack of protein expression on their cell surface is likely to result from a reduced accumulation of MICA mRNA transcripts in the cells. This finding may be in part attributed to epigenetic repression of MICA gene transcription, since the level of MICA mRNA was markedly upregulated following incubation of cells with the DNA demethylating agent 5-aza-3′-deoxycytidine, although the level of protein expression remained unchanged [Unpublished observations]. Whether the lack of transcription factors or of specific transactivators also contribute to the reduced MICA gene transcription remains to be determined.

2.3. Lack of tumor growth control by NK cells in spite of NK cell activating ligand expression

The results of the analysis of the NKG2D ligand expression in malignant lesions we have summarized in the previous section provide a mechanism for the lack of tumor growth control by NK cells in at least 30% of the lesions analyzed, since NKG2D ligands were not detectable in them. However, the expression of NKG2D ligands in about 70% of the lesions analyzed raises the question of why their growth was not controlled by NK cells, given the finding that stimulation through NKG2D overrides the inhibitory signals transmitted from HLA class I molecules [12]. This paradox may be explained by a number of alternative, but not exclusive mechanisms.

2.3.1. Shedding of NKG2D ligands

The soluble forms of NKG2D ligands have been detected in the spent medium of cultured tumor cell lines as well as in sera of patients with malignant diseases [19,20,27–31]. These soluble NKG2D ligands can potentially impair NKG2D-mediated immune function by blocking NKG2D receptors on NK cells and T cells [27,28,31]. In early studies with a small sample size, soluble MICs (sMIC) have been detected in sera from patients with malignant diseases such as leukemia [20], neuroblastoma [19], colon carcinoma [31] and prostate carcinoma [28] and have been demonstrated to downmodulate CTL and/or NK cell responses *in vitro* by downregulating the NKG2D receptor. In patients with prostate carcinoma, sMIC has also been shown to serve as a disease progression marker, with a diagnostic value similar to that of prostate specific antigen (PSA) [28]. More recently, in a study enrolling 512 individuals comprising patients with esophageal, lung, breast, gastric, liver, pancreatic, ovarian and cervical cancer, sMIC levels have been found to be significantly elevated in cancer patients compared with patients with benign diseases and healthy individuals [29]. More importantly, the elevated sMIC levels correlated significantly with cancer stage and metastasis [29]. Altogether, these findings suggest that sMIC levels not only may provide useful information in the diagnosis and staging of cancer, but may also reflect the extent of impairment of NKG2D-mediated tumor immunity.

The mechanism(s) leading to the generation of soluble NKG2D ligands in malignant cells has(ve) only been investigated by Steinle et al. and found to be associated with post-translational proteolytic cleavage [32,33]. Incubation of cells with pan matrix metalloprotease (MMP) inhibitors significantly reduced the levels of soluble MICA and ULBP2 in the culture supernatant [32,33]. Western blot analysis in conjunction with

PNGase F deglycosylation treatment showed that the molecular weight of soluble MICA in the culture supernatant was similar to that of the molecule consisting of only the three ectodomains, which is approximately 33 kDa [32]. However, this molecular weight does not match the one (25 kDa) calculated by prediction taking into account the localization of a candidate MMP cleavage site (usually the C–N bond between Gly and a hydrophobic residue) [34] between Gly^{220} and Val^{221} in the α3 domain of MICA. Whether this discrepancy reflects the presence of an alternative MMP cleavage site or implies an alternative proteolytic mechanism is not known. In addition, only MMP inhibitors with broad specificities have been utilized in the above-mentioned experiments; therefore, it is not clear which MMPs, presumably the membrane type MMPs (MT-MMPs), are responsible for the described proteolytic activity. A similar puzzle seems to exist in the study of ULBP2 shedding. ULBP2, like ULBP1 and ULBP3, are GPI (glycosylated phosphatidylinositol)-anchored membrane glycoproteins, which can be cleaved off at the GPI anchor site by phosphatidylinositol phospholipase C (PI-PLC) [35]. The nascent GPI-anchored protein polypeptide chain, or the "unprocessed" protein, contains a GPI anchor signal peptide at the carboxyl terminus which is cleaved and replaced with a GPI moiety attached to the endoplasmic reticulum (ER) membrane [36]. This transamidation reaction is thought to be catalyzed by an ER enzyme complex which recognizes a three-residue motif in the anchor signal [37]. The motif, by its N to C positions, is designated as ω, ω + 1 and ω + 2, where ω stands for the residue to which GPI is attached [37]. The amino acid preferences at ω and ω + 2 thus far characterized include Ser > Asn > Asp > Gly, Ala, Lys at the ω site, and Ala > Gly > Ser, Thr, Val at the ω + 2 site [37,38]. Using this rule, we have located the ω, ω + 1 and ω + 2 positions in ULBP2, which are Ser^{118}, Ser^{119}, Gly^{120}, seven residues downstream the reported GPI anchoring site for ULBP2. According to this information, the processed ULBP2 should be shorter than its unprocessed counterpart by thirty residues. Nevertheless, this prediction is not supported by the similar electrophoretic mobility of the PI-PLC-treated, processed ULBP2 expressed on the cell surface and unprocessed protein in the cell lysate as detected by SDS-PAGE and Western blotting [33]. It should be noted that the only ULBP2 protein species that can be detected in the cell lysate by Western blotting is the unprocessed one, since following processing, the protein is covalently linked to the plasma membrane and cannot be solublized by non-ionic detergents. Regardless, in the same experiment a lower molecular weight (by ∼5 kDa) band was detected in the spent medium, suggesting that soluble ULBP2 was cleaved at a site at least 40 residues upstream the GPI anchor site. However, this observation is not paralleled by the predicted MMP cleavage site in ULBP2, which is just five residues (Gly^{112}, Ala^{113}) N terminal to the GPI anchor site. In this regard, the role of MMPs in the generation of soluble ULBP2 as well as of the above-mentioned soluble MICA is not yet clearly defined. This controversy may be clarified by utilizing more specific knock-down of MMP activities such as siRNA technology, etc., as well as by employing more detailed biochemical analyses.

2.3.2. Expression of non-functional NKG2D ligands

An additional mechanism for the lack of tumor growth control by NK cells in spite of the expression of NK cell activating ligands is represented by the expression of NKG2D ligands with a low affinity to the cognate receptor. This possibility, although not yet recognized, is supported by the *in vitro* differential affinity to the NKG2D receptor of different MICA allelic variants as the recombinant, soluble form [39]. It is noteworthy that thus far this phenotype cannot be identified at the protein level since the antibody reagents recognizing individual polymorphic MICA molecules are not yet available. This limitation may be in part overcome by MICA geno-typing.

The functional consequence of MICA polymorphism is indicated by its association with various inflammatory or autoimmune diseases [40,41]. This association may directly or indirectly result from the differential affinity of different MICA alleles to their common cognate receptor NKG2D. In malignant diseases, this finding implies that expression of MICA does not necessarily result in triggering NK cell cytotoxicity of tumor cells, if the expressed ligand is not functional. It is noteworthy that although there are at least 56 allelic variants of MICA, they differ only in a few residues dimorphically. All of them are distributed quite evenly in the three ectodomains of the molecule [40]. The role of the dimorphic residues in the MICA α1 and α2 domains, with which the NKG2D receptor interacts, is likely to be indirect, since the crystal structures of MICA*001 by itself and in complex with the NKG2D receptor [42,43] has shown that none of these residues is located in the MICA–NKG2D interface. The latter involves mainly the two helical bundles of MICA. The possible allosteric contribution of a few residues located in the non-helical, loop regions to the stability of the MICA–NKG2D complex has been investigated by us. Using site-directed mutagenesis, we have found that a substitution of Val for Leu at the dimorphic position 122 (L122V) but not that of Ser for Gly at position 175 (G175S) of MICA expressed on transfected cells drastically reduces its binding to NKG2D and significantly lowers its ability to trigger NK cell-mediated cytotoxicity [Unpublished observations]. This finding may be attributed to the insufficient van der Waals contacts between the Val side chain to the α2 helix such that the proposed allosteric effect is diminished, resulting in a less stable MICA–NKG2D complex.

2.3.3. Deregulation of NKG2D ligand expression

At least 50% of tumor cell lines have abundant expression of NKG2D ligands, in particular MICA. This finding suggests that malignant transformation plays some role in the induction of MICA expression on tumor cells. This possibility is supported by recent studies that have shown that activation of DNA damage response through ATM/ATR kinases [44], activation of NF-κB [45] and BCR/ABL [46], and binding of the adenovirus serotype 5 E1A to the transcriptional coadaptor protein p300 [47] may be responsible for the induction and maintenance of NKG2D ligand expression on mouse malignant cells or MICA expression on human malignant cells. Although these studies have provided new insight into the mechanisms regulating NKG2D expression in cells, a number of questions remain to be addressed. First,

only a limited number of tumor cell types have been examined. In the study where DNA damage pathways were investigated [44], only two artificially transformed mouse ovarian epithelial tumor cell lines were used; no equivalent human tumor cells were utilized. Similarly, only HeLa cells and chronic myelogenous leukemic cells were investigated in the studies where NF-κB and BCR/ABL were examined, respectively [45,46]. In this regard, it is not clear whether the reported findings can apply to other tumor cell lines. Second, the molecular pathways with regard to how these molecules induce NKG2D ligand expression have not been confirmed by results obtained utilizing other techniques such as chromatin immunoprecipitation assays. Nevertheless, an interesting but less discussed finding described in the DNA damage response study is the lower responsiveness of tumor cell lines to genotoxic stress than of their normal counterparts. In preliminary studies, we have found that following UV-C exposure MICA is induced on the surface of human melanocytes, a finding that parallels the results of other studies with mouse fibroblasts [44], but is markedly downregulated on human tumor cell lines [Unpublished observations]. The latter finding, in conjunction with intereferon-dependent downregulation of NKG2D ligand H60 on mouse tumor cells [48] and of MICA on the human breast carcinoma cell line MDA-MB-231 [Unpublished results], as well as with AP1/junB-dependent downmodulation of RAE-1ε gene transcription in mice [49], suggests that tumor cells exhibit a distinct mode of NKG2D ligand regulation in response to stimuli or insults in their microenvironment. This type of regulation may provide them with a mechanism to escape from NKG2D-mediated immune elimination when a particular stimulus is present.

2.3.4. Shedding of classical and non-classical HLA antigens and of ICAM-1

Like NKG2D ligands, soluble forms of classical and non-classical HLA class I molecules (HLA-G) and of ICAM-1 are present in the spent medium of cultured tumor cell lines and in sera from healthy donors and from patients with malignant diseases [50,51]. These molecules utilize a different mechanism, namely, exosome secretion [52], to decrease the efficiency of host NK cell control of tumor growth even when classical HLA class I molecules are markedly downregulated and NK cell activating ligands are expressed on tumor cells. Exosomes are nanometer-sized, vesicle-like plasma membrane dislodged from tumor cells embedded with membrane-bound molecules [52] which may include HLA class I molecules and/or ICAM-1. In this regard, the shed molecules maintain their native length and possibly the native conformation when detected as soluble forms. The mechanism(s) underlying the disintegration of plasma membrane in malignant cells is not yet clear.

Soluble classical HLA class I molecules and HLA-G molecules as well as β_2-microglobulin-free HLA class I heavy chains have been shown to induce apoptosis of NK cells as well as of activated T cells by interacting with CD8 and triggering the release of Fas ligand [50,53–55]. On the other hand, soluble ICAM-1 is able to interact with the cognate LFA-1 receptor on NK cells [56] and is thought to block the function of LFA-1 as a bridging molecule in the immunological synapse formed between NK cells and their target tumor cells [57]. As a result, the capacity of NK cells to destroy their target cells is decreased. It is noteworthy that when membrane-bound HLA-G and MICA are co-expressed on tumor cells, the effect of activating signals transmitted through MICA–NKG2D interaction appears to be tuned down since the sensitivity of target cells to NK cell-mediated lysis is markedly decreased [58]. Whether this phenomenon is attributable to the overriding capacity of the HLA-G-recognizing ILT-2/4 receptor signaling over the NKG2D receptor signaling is unknown.

3. Role of NKG2D ligand and HLA antigen abnormalities in tumor immune surveillance

NK cells have long been postulated to be the first line of defense in infectious and malignant diseases; the discovery of the NKG2D system has provided a molecular basis for this role of NK cells. Because of their characteristics, NKG2D ligands, when induced, are likely to shift the "immune balance" toward activation, thereby initiating host immune surveillance against newly arising tumor cells [59,60]. Following activation, NK cells or NKG2D-bearing immune cells can also secrete cytokines to amplify the initial immune response in a paracrine fashion. Subsequently, the antigen processing and presentation capacity is enhanced in antigen presenting cells, resulting in the activation of the adaptive arm of the immune system which targets HLA class I-tumor antigen derived peptide complexes on tumor cells. During this process, tumor cells which have defects in the expression and/or function of NKG2D ligands and HLA molecules can escape from immune surveillance and/or elimination and become a dominant population at the tumor site. While the evidence for the role of HLA class I antigen abnormalities in tumor immune evasion has been well documented in the last decade [1,2], the investigation of the role of NKG2D ligand defects in tumor escape is still at an early stage. MICA loss has been found to be associated with resistance to NK cell-mediated lysis of two human melanoma cell lines isolated from recurrent metastases in spite of HLA class I antigen loss [21]. In mice, the mock-transfected, but not the Rae-1-transfected, RMA lymphoma and B16 melanoma cells form tumors in syngeneic hosts and depletion of NK cells in mice abolishes the control of Rae-1-transfected tumor cell growth [61,62]. Most recently, the role of the NKG2D system in controlling *de novo* tumorigenesis has been convincingly demonstrated in a methylcholanthrene (MCA)-induced sarcoma mouse model [63]. In this model, neutralization of NKG2D markedly enhances the sensitivity of wild-type C57BL/6 and BALB/c mice to MCA-induced fibrosarcoma [63]. The sarcomas emerged from the wild-type mice have no or variable Rae-1 expression, as opposed to the universal expression of this NKG2D ligand in sarcoma tumors grown in the perforin-deficient mice [63]. All of these experimental data have provided compelling evidence for the causal role of lack of NKG2D ligand induction in tumor cell escape *in vivo*. If so, the abnormal "lack of expression" phenotype characterized in a panel of cultured human tumor cell lines and malignant lesions we have described may not simply be a random by-product of tumor genomic instability.

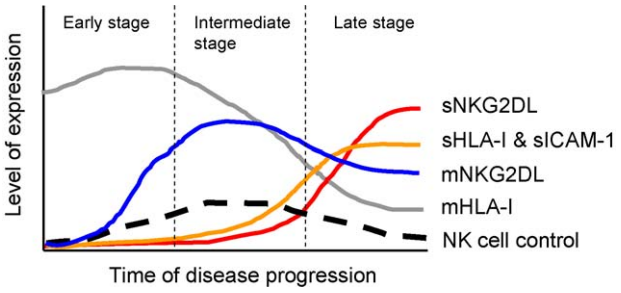

Fig. 2. A hypothetical model for the temporal changes in the levels of membrane-bound NKG2D ligands (mNKG2DL), membrane-bound HLA class I molecules (mHLA-I), soluble NKG2D ligands (sNKG2DL), soluble HLA class I molecules (sHLA-I), and soluble ICAM-1 (sICAM-1) during tumor progression and their relationship with the extent of NK cell-mediated control of tumor growth.

The role of NKG2D ligand shedding in tumor cell escape *in vivo* has not been investigated in a causal fashion, although there is some *in vitro* evidence for its immunosuppressive effect [27,28,31]. Nevertheless, two studies have demonstrated that sustained membrane-bound NKG2D ligand expression *in vivo*, either systemically [64] or locally [65], actually impairs NKG2D-mediated immune control of tumor growth by constantly downregulating the NKG2D receptor. These findings suggest, but do not prove, that the "ligand shedding" phenotype we have described for MICA, ULBP and perhaps soluble HLA class I molecules and ICAM-1 in tumor cells may have a similar downmodulating effect on the immune cells bearing the corresponding receptor *in vivo*. These results in conjunction with those of "lack of expression" imply that the NKG2D ligand-receptor system benefits tumor immune surveillance or elimination only within a specific time frame. NKG2D receptor downregulation may serve as a default negative feedback mechanism to turn off the otherwise uncontrolled immune response, a phenomenon paralleled by the T cell receptor downregulation following engagement with MHC-peptide complexes [66]. Once the induced ligand expression returns to its normal latent state, the receptor would no longer be downregulated and resumes to its constitutive level. It is likely that this default system is blocked in the case of chronic NKG2D stimulation such as in malignant diseases. On the other hand, thus far there is no information regarding the role of the described two additional abnormal phenotypes, i.e. "polymorphism" and "low responsiveness to stress" in tumor cell escape *in vivo*.

Based on the available information, we propose a kinetic model for the combined influence of membrane-bound and soluble forms of NKG2D ligands, classical and non-classical HLA class I molecules and ICAM-1 on the ability of NK cells to control tumor growth *in vivo* (Fig. 2). At an early stage of tumor development, while HLA class I molecules remain expressed on malignant cells, NKG2D ligands are beginning to be induced, as a result of the activation of the intrinsic DNA damage response pathways. The soluble forms of NKG2D ligands and HLA class I molecules are either not detectable or expressed at low levels, because the MMP and exosome secretion have not been activated. The net change at this stage is a shift in the "immune balance" toward activation; NK cells are activated to some extent and immune surveillance is initiated. At an intermediate stage, due to a more extensive DNA damage and genomic instability, NKG2D ligands are markedly elevated, concomitantly with HLA class I molecule downregulation. Soluble forms of the membrane-bound ligands are produced because of the activation of MMP and exosome secretion systems. As a result, NK cells are activated to a great extent in conjunction with the activation of adaptive immune responses, leading to immune elimination and emergence of escape variants. At a late stage, the tumor site is dominated by escape variants expressing low levels of HLA class I molecules and medium to low levels of NKG2D ligands on the plasma membrane, but shedding and/or secreting large amounts of soluble NKG2D ligands, HLA class I molecules and/or ICAM-1. The net influence in this period is sustained NKG2D receptor downregulation, resulting in impaired NK cell control of tumor growth.

If proven correct, the model we have proposed may explain the currently documented paradoxical findings regarding the relationship between NKG2D ligand expression and tumor immune escape.

4. Conclusion

In the last 5 years, we have witnessed an explosion of investigations about the NKG2D ligand-receptor system at the atomic, molecular, cellular and whole body levels. The impressive amount of information published in the literature in a relatively short period of time greatly highlights the importance of this newly defined biological system in terms of basic understanding and clinical application. While most of the studies are focused on the induction and/or activation mechanisms of the NKG2D system, in the context of tumor immunity it is important to determine why this system does not function properly in most of the human malignant diseases. The data we have summarized suggest two mechanisms for the lack of control of tumor growth by NK cells which have no defects in trafficking to malignant lesions. One is represented by the lack of induction of NK cell activating ligands in malignant lesions. The other one is represented by functional defects in NK cell activating ligands expressed by tumor cells, such as shedding, low responsiveness to stress and perhaps polymorphism.

In general, both phenotypes are not unique of NK cell activating ligands, since they have already been amply documented for HLA class I antigens and tumor antigens expressed by tumor cells. It is noteworthy that unlike HLA class I antigens, which are constitutively expressed on most, although not all normal nucleated cells [67], NKG2D ligands are induced in normal tissues by certain stressful stimuli. In this regard, "lack of induction" is preferable to "downregulation" to describe an abnormal NKG2D ligand phenotype in malignant cells, which has been documented in approximately 30% of the malignant lesions analyzed. In conjunction with "shedding" and "low responsiveness to stress", the "lack of induction" phenotype can be combined with HLA class I abnormalities and ICAM-1 shedding within one tumor cell population. The net influence of this combination is impaired innate and adaptive immune responses to tumor antigens.

As far as the molecular mechanisms underlying abnormalities in NK cell activating ligand expression and function are concerned, only scanty information is available as we have described in this article; some of the proposed mechanisms remain controversial and need clarification. Nevertheless, the available information suggests that they share several characteristics with those underlying HLA class I antigen and tumor antigen abnormalities, but display also distinct features. As described for the latter two antigenic systems [2], genetic and epigenetic mechanisms as well as regulatory abnormalities also underlie defective expression and/or function of NK cell activating ligands. On the other hand, allelic instability has been described only for the latter markers.

One might ask what leads to the generation of lesions with defects in NK cell activating ligand expression and/or function. A number of *in vitro* and *in vivo* studies [2] have convincingly shown that lesions with HLA class I antigen or tumor antigen loss are generated because immune selective pressure favors the outgrowth of tumor cells which have acquired a T cell-resistant phenotype. Similarly, according to the limited information in the literature, immune selection also leads to the generation of lesions with a defective NK cell activating ligand phenotype. The immune selective pressure imposed by NK cells may operate at an earlier stage in the course of the disease than that imposed by CTL. This possibility, together with the well documented role of T cell selective pressure in the generation of malignant lesions with HLA class I antigen and tumor antigen abnormalities [2], invites one to exercise caution when applying NKG2D-depedent immunotherapy of cancer. In this regard, in addition to targeting the NKG2D system using cell-based strategies, soluble molecules such as a bispecific fusion protein containing a membrane-bound-tumor antigen-specific antibody and a soluble form of MICA has been shown to enhance the sensitivity of tumor cells to NKG2D-mediated immune elimination, regardless of the functional status of the endogenous MICA in tumor cells [68,69]. Moreover, because of their preferential induction on tumor cells versus normal cells, NKG2D ligands by themselves can serve as tumor antigens. In this regard, tumor cells opsonized with MICA-specific antibodies can sensitize dendritic cells, leading to an efficient cross-priming of tumor antigen-specific T cells [70]. This finding suggests that antibodies targeting MICA *in vivo* can elicit T cell immunity against tumors of diverse embryological origin. Lastly, to counteract the immunosuppressive effect of soluble NKG2D ligands at a late stage of a malignant disease, one may perform a plasma purging procedure to absorb soluble NKG2D ligands circulating in blood, utilizing a NKG2D ligand antibody affinity column. This approach, which eliminates the negative modulators in the circulation, may greatly enhance the efficacy of other therapeutic modalities by shifting the net "negative" influence to a net "positive" one.

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