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The HLA Crossroad in Tumor Immunology

Ignacio Algarra, Teresa Cabrera, and Federico Garrido

ABSTRACT: It is generally accepted that human and experimental tumor cells can lose major histocompatibility complex (MHC) class I molecules. These human leukocyte antigen (HLA) losses are detected when the primary tumor breaks the basal membrane, invades the surrounding tissues, and starts to metastasize. These altered HLA class I phenotypes probably constitute the major tumor escape mechanism facing anti-tumor T-cell mediated responses. Thus, it is important to characterize these phenotypes in clinical tumor samples, analyze the mechanism(s) responsible for them, and counsel patients before and during peptide anti-cancer immunotherapy. The present paper summarizes the most relevant altered HLA class I phenotypes found in human tumor samples, indicates their frequency, and outlines the mechanisms implicated. This review also points out that the natural killer (NK) escape mechanism of HLA class I deficient cancer cells is yet to be defined. Knowledge accumulated to date reveals that HLA class I molecules are an important crossroad in tumor immunology. *Human Immunology 61, 65–73 (2000).* © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: HLA; expression; tumors; phenotypes; escape

ABBREVIATIONS

CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
HLA	human leukocyte antigen
LOH	loss of heterozygosity
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer
PCR	polymerase chain reaction
STR	short tandem repeat
TAP	peptide transformer

INTRODUCTION

The discovery of the major histocompatibility complex (MHC) and of the laws that govern tissue transplantation was possible in the early days due to the transplantation of tumor cell lines in allogeneic inbred strains of mice [1, 2]. For decades, however, a solid scientific basis for tumor immunology was lacking since the nature of tumor antigens was unknown [3]. Elucidation of the crucial role of human leukocyte antigen (HLA) molecules in antigen presentation contributed to the discovery of tumor antigens recognized by T lymphocytes [4, 5]. In addition, the description of several different routes used by tumor cells to escape immunosurveillance highlighted the importance of the immune system in controlling tumor growth [6]. Among these routes, the selection of MHC class I-deficient variants is a frequently observed mechanism in experimental and spontaneous tumors [7, 8].

It has been known since the mid 1970's that MHC class I alterations occur in mouse and human tumors [9–11]. Early reports indicated that private and public H-2 specificities of virally and chemically induced mouse tumors were absent compared with H-2 typing obtained in normal spleen or lymph node cells [12, 13]. However, it was not realized until the mid 1980's that these altered phenotypes could represent a major mechanism of tumor escape from T-cell immune responses [14, 15]. More recently, the importance of these findings has been emphasized by the discovery that the immune system has evolved a particular population of cells capable of recognizing and destroying MHC class I-deficient cells produced during viral infection or tumor development, i.e., the natural killer (NK) cells [16]. After 40 years of

From the Departamento de Analisis Clinicos, Hospital Universitario Virgen de las Nieves Universidad de Granada, Granada, Spain (T.C., F.G.), and Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaen, Jaen, Spain (I.G.).

Address reprint requests to Dr. Federico Garrido, Department Analisis Clinicos, Hospital Universitario Virgen de las Nieves, Avda.Fuerzas Armadas No. 2, 18014 Granada, Spain.

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continuous research and progress in our understanding of how the immune system controls the development and dissemination of experimental and human tumors, it is clear that HLA class I molecules are emerging as leading players in this tumor-host scenario.

This review will focus on the different altered HLA class I phenotypes found in solid human tumors and cell lines, including the mechanisms that generate each of these phenotypes; and the clinical applications of these findings in cancer patients undergoing immunotherapy with peptides.

ALTERED MHC CLASS I PHENOTYPES IN TUMORS

The use of monoclonal antibodies (mAbs) against HLA class I monomorphic, locus-specific, and allelic-specific determinants with analysis by immunohistological techniques has made it possible to define precisely in cryopreserved tissue sections different altered HLA phenotypes. These alterations seem to occur at a particular step in tumor development: when the tumor invades the surrounding tissues and starts to metastasize [7]. Sometimes results are difficult to interpret because of the heterogeneity of tumor cell populations, since HLA-positive and negative tumor cells coexist in the same primary tumor. The terms negative, heterogeneous, and positive are commonly used to define HLA clonal differences in a particular tumor tissue. In this context, it is important to remember that oncogene activation and tumor suppressor inactivation have been reported in many benign lesions that apparently show normal HLA expression [17].

Our laboratory, in cooperation with different centers, organized the HLA and Cancer component of the XII International Histocompatibility Workshop, at which participants selected a panel of anti-HLA monoclonal antibodies (mAbs) that work in cryostatic tissue sections [18]. These antibodies provide the tools for analyzing in greater detail HLA class I expression in tumor tissues. It can now be said that invasive tumors totally or partially lose HLA antigens at a very high frequency (between 40% and 90%) [19] (Fig. 1). These figures are probably underestimates, since the panel of available mAbs that define HLA alleles and work in tissue sections is still very limited.

Different mechanisms can lead to total or partial loss of HLA expression. MHC molecules can be lost at any step required for HLA synthesis, transport, or expression on the cell surface [19, 20]. Indeed, there are reports of heavy chain and β_2-microglobulin gene mutations [21–24], alterations in regulatory factors induced by cis-trans mechanism [25], alterations in glycosylation and transport [26] and HLA gene deletions associated with loss of heterozygocity (LOH) [27].

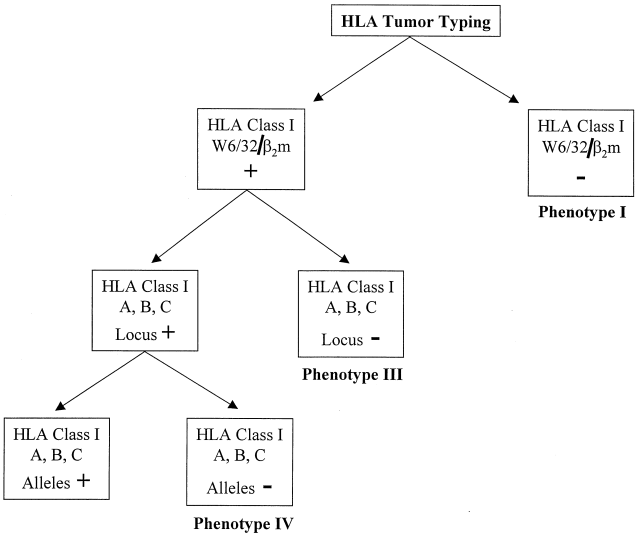

FIGURE 1 Strategy to define different HLA class I altered phenotypes using immunohistological techniques in cryopreserved tumor tissue sections and monomorphic, locus, and allelic-specific anti-HLA mAbs.

We have proposed a classification of altered class I tumor phenotypes that facilitates identification and analysis of the HLA phenotypes commonly found in tumor tissues [19]. These phenotypes include phenotype I, total HLA loss; phenotype II, HLA haplotype loss; phenotype III, HLA A and B locus loss; phenotype IV, HLA allelic loss; and phenotype V, compound phenotype. These phenotypes are frequently found in a variety of human tumor tissues derived from HLA-positive epithelia, but their exact frequency is as yet unknown (Fig. 2).

Phenotype I

Total HLA loss. This is a relatively frequent phenotype (9%–52%) readily detected in most human tumors with anti-HLA monoclonal antibodies against monomorphic determinants or against β_2-microglobulin. HLA total loss is found in melanomas (15%), head-neck (9%), and colorectal (21%) tumors [28]. In tumors derived from other tissues, e.g., the prostate and breast, these figures reach 34% and 52%, respectively. Distinct molecular mechanisms underlie this phenotype. It can be associated with a lack of synthesis or a truncated β_2-microglobulin. Any event leading to loss of $\beta_2$microglobulin production could be genetic or post-translational, resulting in a failure to form peptide-heavy chain-β2-microglobulin complexes at the cell surface. β_2-microglobulin gene alterations are a molecular finding associated with this total loss of HLA phenotype in melanoma [24], colorectal tumors [23], and lymphoma [29]. Certain cell lines

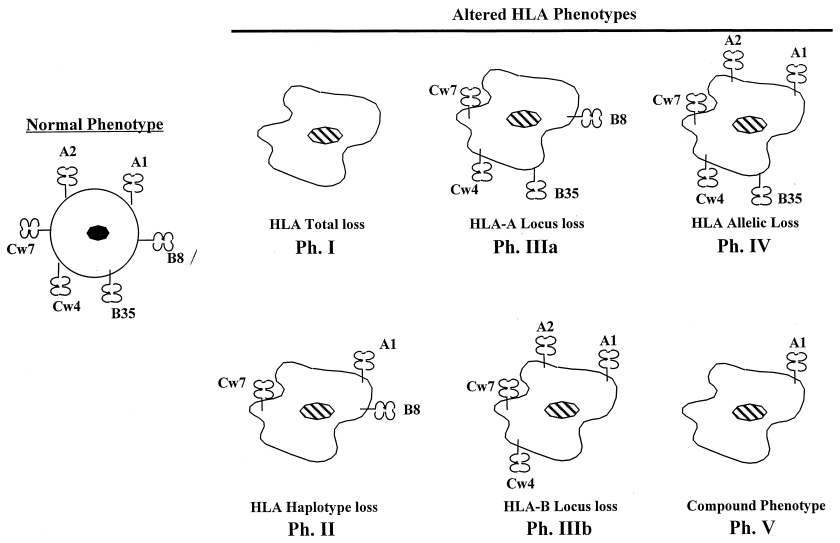

FIGURE 2 The five major HLA class I altered phenotypes (Ph.).

are representative of this phenotype; including Daudi lymphoma, FO-1 melanoma, AUMA melanoma, LoVo colon carcinoma, and other tumors [22]. In this context, we have described a new β_2-microglobulin mutation in a melanoma cell line (GR 34) originated at our laboratory. The new mutation was identified as a deletion of four bases located in the leader sequence of the β_2-microglobulin gene at codon 15–16 of exon 1. We demonstrated that the second β_2-microglobulin gene is deleted. Comparisons with β_2-microglobulin mutations in other tumor cell lines suggest a mutation hot spot in exón 1 [22]. Figure 3 summarizes the β2-microglobulin mutations already reported.

Another mechanism that causes defects in the assembly and stability of HLA class I molecules involves interference with peptides transporters (TAP) leading to failure to transport peptides from the cytoplasm to the lumen of the ER and the class I processing pathway. TAP defects can lead to a complete HLA loss phenotype and these are frequently associated with the absence of reactivity of antibodies directed against TAP proteins in HLA-negative tumor cells [26]. Tumors with structural defects in HLA genes that produce a total loss of HLA class I molecules will not recover HLA expression after cytokine treatment. However, cis-acting regulation of MHC class I genes by methylation or changes in chromatin structure have also been described in tumor cell lines, and such tumor cells may retain HLA expression responsiveness to cytokines [25]. The molecular epidemiology of the total loss tumor phenotype is unknown and could differ among tumor types. In this context, data obtained in our laboratory suggest that β2-microglobulin mutations are not responsible for the HLA total loss found in laryngeal carcinoma (Fernandez et al., unpublished data).

Phenotype II

HLA haplotype loss. Loss of an HLA haplotype has been shown in melanoma, pancreas, colon, laryngeal, and cervical cell lines [30]. The detection of microsatellite markers present in chromosome 6 and 15 using polymerase chain reaction (PCR) amplification of these short tandem repeats (STRs) has provided an easy and effective way to diagnose this altered HLA phenotype. In a pan-

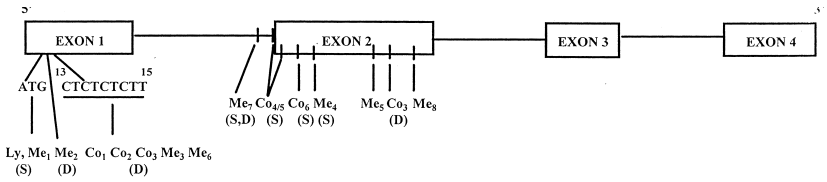

FIGURE 3 Summary of the different β2-microglobulin (B_2 m) gene mutations found in human tumors that contribute to originating HLA total loss (phenotype I). (Reprinted from Tissue Antigens 53:569, 1999.)

Co_1: LoVo; Co_2: HRA19; Co_3: SW48; $Co_{4/5}$: HCT15/DLD-1; Co_6: C-84

Me_1: AUMA; Me_2: FO-1; Me_3: GR-34; Me_4: IRNE; Me_5: SK-MEL-33; Me_6: Me 1386; Me_7: Me 18105; Me_8: Me 9923

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creatic adenocarcinoma, haplotype loss was demonstrated in the fresh tumor and the tumor-derived cell line, indicating that it was not due to an *in vitro* event [27]. The majority of these studies also revealed loss of heterozygosity (LOH) at other loci of chromosome 6 by the deletion of a full chromosome 6 or large genomic region. Recently, we have also shown that chromosome loss is the most frequent mechanism underlying HLA haplotype loss [31]. In most studies in solid tumors, LOH abnormalities may or may not be representative of the whole tumor. The persistence of a weak signal in one of the two alleles is normally interpreted as coming from the normal contaminating stroma that surrounds the tumor tissue. This second interpretation is potentially important when evaluating allelic losses as a random or biologically relevant event in the context of tumor progression.

A mechanism of chromosomal nondisjunction or mitotic recombination has been proposed to underlie the HLA haplotype loss [32]. Certain cell lines are representative of this phenotype, including the colon cancer cell line PC/JW [23], the pancreas cancer cell line IMIM-PC-2 [27], and melanoma FM37 [32]. Again, the frequency of this phenotype in tumors derived from different tissues is not known. We recently obtained figures of 14% to 17% in melanoma, colon, and laringeal carcinoma, and identified chromosome loss as the most frequent mechanisms for generating HLA haplotype loss [31]. These figures are probably underestimated since the heterogeneity of tumor tissues as well as the contaminating normal stroma no doubt decrease the detection level of LOH. We have proposed a protocol to standardize LOH studies using a panel of nine microsatellite markers (seven for chromosome 6 and two for chromosome 15) that includes the HLA region and the β2-microglobulin gene (Ramal et al., submitted for publication) (Fig. 4). We also have preliminary data using this panel of short tandem repeats (STRs) and microdissection of frozen tumor tissues, indicating that this procedure greatly improves LOH detection and helps to elucidate the real frequency of this mechanism in generating HLA altered phenotypes.

Phenotype III

HLA locus loss. Loss of class I locus expression has been documented in several tumors: HLA-A (3%–19%) and HLA-B (5%–19%). The mechanism of locus down regulation might be transcriptional, since HLA class I locus promoter sequences and messenger ribonucleic acid (mRNA) levels for some class I alleles in tumors differ from those in normal cells. In melanoma, lineage-specific selective HLA-B down regulation correlates with increased c-myc transcription, which interferes with HLA-B transcription at the promoter level; this is not seen in other tumor cells. In colon cancer cells, low expression of transcription factors that bind to locus-specific deoxyribonucleic acid (DNA) motifs can induce HLA-B down regulation. This type of transcriptionally mediated HLA locus specific down regulated phenotype can frequently be overidden by cytokine treatment, as in the melanoma line FM55P analyzed at our laboratory [32] (Fig. 5).

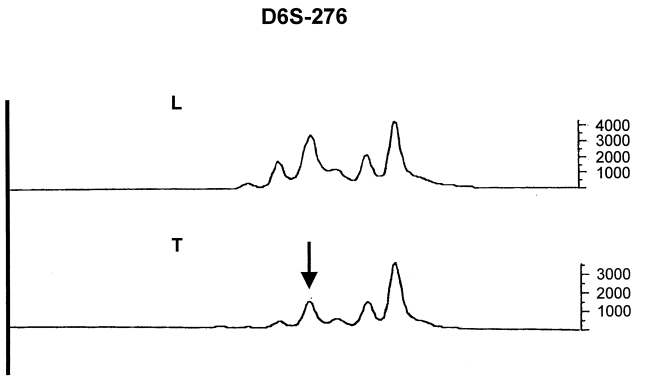

FIGURE 4 Example of a colorectal carcinoma with LOH associated with HLA haplotype loss (phenotype II). D6S-276 microsatellite PCR amplification on tumor (T) and autologous lymphocyte (L) DNA showed a clear reduction in the signal obtained in one allele (arrow) compared with the control in PBLs.

Phenotype IV

HLA allelic loss. It is difficult to define the precise frequency of tumors that show loss of expression of only one HLA allele because of deficits in the repertoire of allele specific antibodies. The use of a wide set of mAbs against individual HLA alleles shows that selective allele down regulation, not categorized as phenotypes I–III, is observed in many tumors (15%–51%) [33, 34]. Such allelic loss might result from point mutations, partial deletions of HLA class I genes, or as a consequence of chromosomal breakage or somatic recombination. These types of mechanism are not overridden by cytokine treatment. The colon carcinoma line LS411 and melanoma lines LB33 and 624-MEL are representative of phenotype IV. An example of an HLA allelic loss is shown in Fig. 6.

Phenotype V

Compound phenotype. Some tumors display complex HLA class I phenotypes that do not fit into categories I–IV. This may reflect multiple events, producing compound phenotypes, such as a tumor that retains expression of a single HLA class I allele product and that results from immunoselective events during the natural history of the

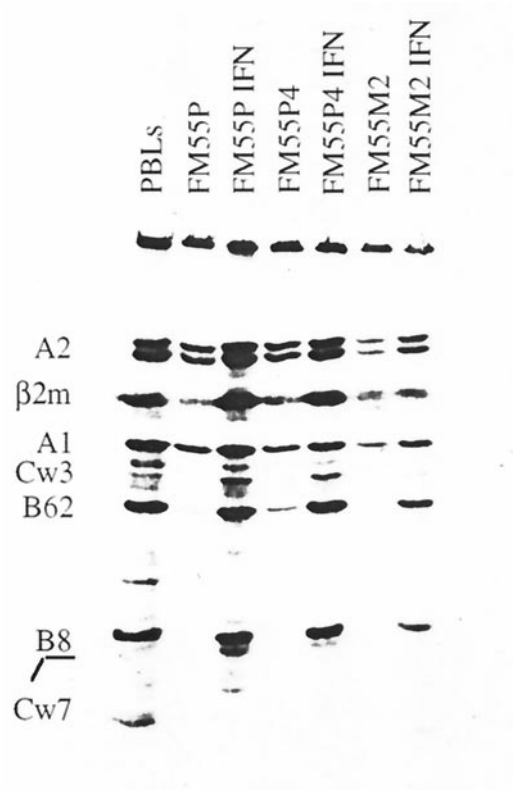

FIGURE 5 Example of HLA B locus down regulation (phenotype IIIb) in a melanoma cell line. Isoelectric focusing of FM55p, p4, and M2 melanoma cell lines showed absence of HLA B locus products compared with the expression in autologous PBLs. Expression is recovered after IFN-γ treatment.

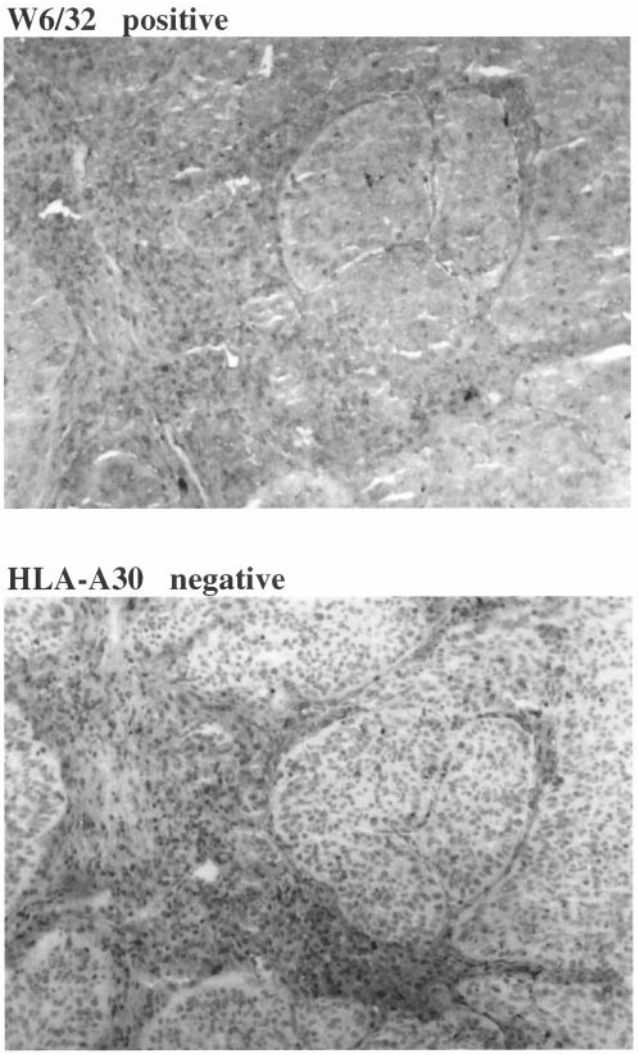

FIGURE 6 HLA class I allelic loss (phenotype IV). Pattern of reactivity of anti-HLA class I mAbs defining monomorphic and allelic HLA class I determinants in a cryopreserved laringeal tumor. This tumor did not react with anti-HLA A30 mAb.

cancer. Recently, an HLA phenotype that is the result of HLA-B locus down regulation (phenotype III) and HLA haplotype loss (phenotype II) has been identified in a tumor cell line in our laboratory [32] (Fig. 7). The alteration was found in two melanoma cell lines generated from two patients; one was derived from an *in vivo* lesion (FM37), and the other obtained after *in vitro* immunoselection (R22.2). The R22.2 cell line was isolated from FM55P, a cell line derived from a primary melanoma after *in vitro* treatment with a heterologous HLA-A2-restricted cytotoxic- T- lymphocyte (CTL) clone. Simple tandem-repeat polymorphism markers spanning chromosome 6 showed that DNA from the two samples (FM37 and R22.2) showed LOH. In both cases, homozygosity was observed on 6p, which maps the HLA region. The final result was the appearance of a cell line expressing a single HLA class I allele (HLA-A3 and HLA-A1, respectively) (Fig. 8). Such a phenotype has been described in another melanoma cell line selected by CTLs *in vivo* [35]. In this case, the melanoma line MEL.B had lost expression of all class I molecules except for HLA-A24. By stimulating autologous lymphocytes with MEL.B, it was possible to obtain an HLA-A24-restricted CTL clone that lysed these cells. These CTLs, active against tumor cells showing partial HLA loss, may constitute an intermediate line of antitumor defense between the CTLs and the NK cells, and may recognize HLA loss variants [36, 20].

Additional support comes from evidence of an increased incidence of HLA class I down regulation in cervical carcinoma lymph node metastases compared with the primary cancers [26].

All these observations are consistent with the selection of MHC class I defective variants during tumor progression, which may influence the clinical outcome [37]. The level of HLA class I expression can also influence the presentation and immunogenicity of CTL epitopes and

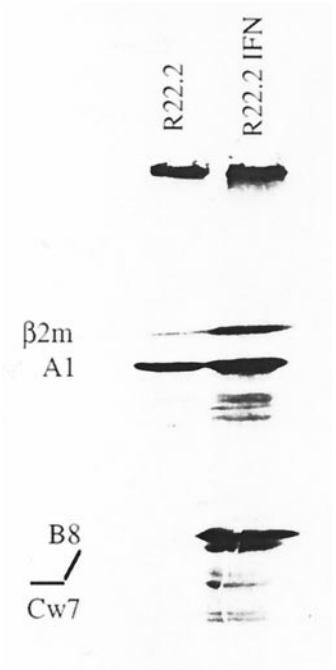

FIGURE 7 Isoelectric focusing of a melanoma cell line (R22.2) expressing a single HLA class I allele (phenotype V). This cell line expresses only HLA-A1 antigen. After IFN-γ treatment it expresses one HLA haplotype.

FIGURE 8 The compound HLA phenotype V is shown in two examples: melanoma cell lines FM55pR22.2 and FM37. (Reprinted from Int J Cancer 75:317, 1998).

the modulation of NK cell responses: tumor phenotypes that fail to upregulate their HLA expression in response to cytokines may affect clinical progression.

Altered HLA Phenotypes Associated with T and NK Cell Escape

The absence of a particular MHC class I molecule is, no doubt, a clear way for a tumor cell to escape a specific T-cell-mediated anti-tumor response against a tumor antigen restricted by that particular MHC molecule. However, HLA class I deficient tumor cells are theoretically capable of activating some NK cell subpopulations when the inhibitory signal provided by that class I molecule disappears [19]. Some important questions emerging from these findings have not yet been answered. In particular, why is a tumor cell with an HLA total loss (phenotype I) not destroyed by NK cells if there is no inhibitory signal to interact with NK inhibitory receptors (KIRs)? It has recently been proposed that some non-classic HLA class I molecules, such as HLA G, E, etc., may be aberrantly expressed in some tumor cells negative for HLA class I (A, B, and C) [38]. This tumor phenotype would theoretically provide a T and NK cell escape, since it avoids antigen presentation but retains the signal that is inhibitory to the KIRs. Such a phenotype is currently found in the syncytiotrophoblast of the placenta [39] to protect the mother tissues against an allogeneic immune response from the fetus. However, we could not corroborate this hypothesis from a study of a broad panel of human solid tumors and tumor cell lines for HLA G expression [40]. Only the U937 myelomonocytic cell line was positive for HLA G after (interferon) IFN-γ treatment [40]. The rest of the tissues and cell lines were negative for HLA G expression, although messages for different HLA G isoforms were detected.

Another open possibility is the existence of selective HLA class I losses on some tumor cells that will lose the antigen presenting capacity but retain the expression of some other classic class I molecules, such as HLA C, which interact with the KIRs to maintain the inhibitory signal to NK cells. We have identified such a HLA tumor phenotype present in a minority of colon and breast carcinomas [33, 34] (Fig. 9). There is no doubt that several NK escape mechanisms must exist to avoid NK killing of HLA class I deficient tumor cells, but such mechanisms need to be clearly identified.

Clinical Application: Monitoring HLA Antigens in Patients Undergoing T-Cell Based Immunotherapy

The HLA abnormalities which have been identified in a large variety of malignancies are likely to have a negative impact on the outcome of peptide based immunotherapy, since they provide cancer cells with a mechanism to escape from T-cell recognition. Thus, expression and functional integrity of the antigen processing machinery

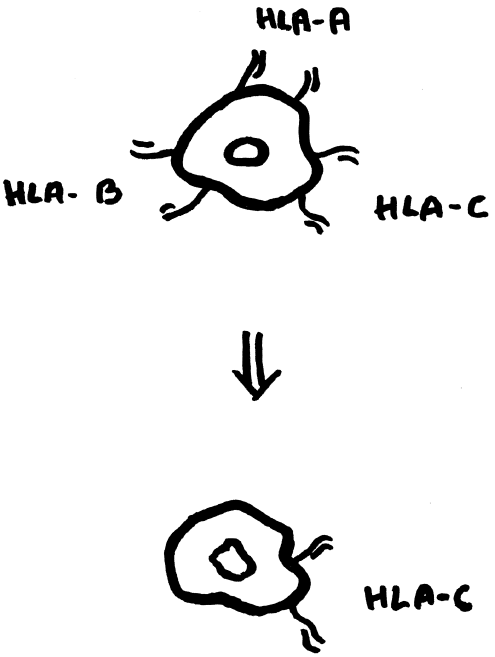

FIGURE 9 This particular altered HLA class I phenotype expresses only HLA C locus products. It is found in a minority of colorectal, laryngeal, and breast carcinomas (2.5%, 3% and 9.5%, respectively). It is hypothesized that these tumor cells could escape T and NK cell cytotoxicity.

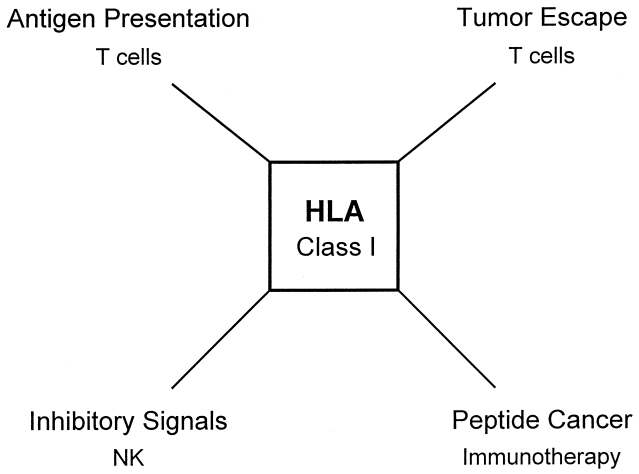

FIGURE 10 HLA class I molecules are crucial structures for antigen presentation to T lymphocytes and the physiological ligands of NK receptors. They disappear from the tumor cell surface to escape T cell recognition. Therefore, they must be monitored in cancer patients undergoing peptide immunotherapies.

and HLA class I antigen cell surface expression in malignant lesions could represent an important criterion for selecting patients to enter trials of T-cell based immunotherapy. The HLA analysis of frozen sections of these malignant lesions and the corresponding tumor-derived cell lines will be crucial to decide whether a cancer patient may or may not enter a particular clinical trial.

We have recently shown that tumor cells from two melanoma patients immunized with HLA-A1 restricted MAGE-encoded peptides did not bear HLA class I molecules on their surface. The molecular lesions responsible for the altered HLA class I phenotypes were two different β2-microglobulin mutations: one that abolished the initiation codon and the other in the second exon that produced a premature stop codon and, thus, a truncated β2-m protein. In addition, LOH in chromosome 15 indicated the absence of the second β2-m allele in the tumors of both patients [21]. The combination of β2-microglobulin gene mutation in one allele and loss of heterozigosity in the other could be an important mechanism leading to phenotype I (HLA total loss).

CONCLUSIONS

How can our knowledge in HLA and tumor antigens be applied to cancer patients in clinical HLA laboratories? The identification of HLA class I alteration in tumors with the precise definition of the mechanism responsible for them are, doubtless, powerful data to consider when designing a particular vaccination strategy based on HLA mediated T-cell immunotherapy. Indeed, regressions of some melanoma lesions were reported when patients were immunized with peptides encoded by different cellular genes and presented by a particular HLA class I molecule [41]. However, no correlation was established between responders/non-responders and HLA expression, due to the lack of HLA tumor studies in these patients. In this context, we have shown that two nonresponder patients to HLA-A1 MAGE-3 derived peptides did not express any HLA class I molecule at the tumor cell surface [21]. Two different β2-microglobulin gene mutations associated with gene deletions on the other chromosome fifteen were responsible for these HLA alterations. We think that these laboratory data should be mandatory before and during peptide immunotherapy to offer these patients a real hope of response. The HLA crossroad first encountered in organ transplantation now appears in the setting of cancer immunotherapy (Fig. 10). Cooperation between surgeons, oncologists, immunologists, molecular biologists, and pathologists working together in a multidisciplinary team will be required to undertake the difficult task of establishing real T-cell based cancer vaccination and immunotherapy.

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