The Antigen Processing Machinery of Class I Human Leukocyte Antigens: Linked Patterns of Gene Expression in Neoplastic Cells

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ABSTRACT

The ultimate outcome of an immune response (escape or surveillance) depends on a delicate balance of opposing signals delivered by activating and inhibitory immune receptors expressed by cytotoxic T lymphocytes and natural killer cells. In this light, loss and down-regulation of human leukocyte antigens (HLA) class I molecules, while important for keeping tumors below the T-cell detection levels, may incite recognition of missing self. Conversely, the maintenance of normal levels of expression (or even up-regulation) may be favorable to tumors, at least in certain cases. In this study, we took advantage of a previously characterized panel of 15 early passage tumor cell lines (mainly from melanoma and lung carcinoma lesions) enriched with class I-low phenotypes. These cells were systematically characterized by Northern and/or Western blotting (e.g., mini-transcriptome/mini-proteome analysis) for the expression of HLA-A, -B, -C, β2m, and the members of the “antigen processing machinery” of class I molecules (LMP2, LMP7, TAP1, TAP2, tapasin, calreticulin, calnexin, and ERP57). In addition, we established four pairs of cultures, each comprising melanoma cells and normal melanocytes from the same patient. We found that ~97% of the 185 tested gene products are expressed (although often weakly), and in many cases coordinately regulated in 18 of 19 tumor cell lines. Linked expression patterns could be hierarchically arranged by statistical methods and graphically described as a class I HLA “coordinome.” Deviations (both down- and up-regulation) from the coordinome expression pattern inherited from the normal, paired melanocyte counterpart, were allowed but limited in magnitude, as if melanoma cells were trying to keep a “low profile” HLA phenotype. We conclude that irreversible HLA loss is a rare event, and class I expression in tumor cells almost invariably results from reversible gene regulatory (rather than gene disruption) events.

INTRODUCTION

HLA class I molecules engage specific immune receptors expressed by CD8+ CTLs and NK cells, the two major effectors of immune lysis. Not surprisingly, the assessment of immunoevasive HLA phenotypes of transformed cells has become a major topic in tumor immunology (1–3). As known, HLA class I molecules are cell surface glycoproteins formed by three noncovalently associated subunits: a highly polymorphic heavy chain encoded on the short arm of chromosome 6 within the HLA complex, a nonpolymorphic light chain termed β2m, encoded outside the HLA complex, and a small (8–10mer) peptide antigen (4). The HLA complex encodes >1000 heavy chain alleles specified by three classical HLA-A, -B, -C loci, and several nonclassical loci (HLA-E, HLA-F, HLA-G, and so forth), along with several other proteins that are not only in physical, but also functional linkage with class I molecules (5). These include: (a) LMP2 and LMP7, two IFN-γ-inducible components of the proteasome (the cytosolic multi-protease complex that cleaves peptide antigens, including tumor antigens, from cellular proteins); (b) the TAP1 and TAP2 subunits, forming a heterodimeric membrane pump that translocates proteasome-generated peptides into the lumen of the ER; and (c) tapasin, a class I-dedicated chaperone that edits peptides and retains unassembled class I molecules in the ER (4).

The so-called antigen processing machinery (4, 5) is composed of LMPs, TAP, and tapasin, and possibly includes the thiol reductase ERP57, the “promiscuous” chaperones (e.g., chaperones mediating the folding of generic glycoproteins in addition to class I HLA) calnexin and calreticulin, and a recently described ER Aminopeptidase (6). Loss (through gene disruption) or down-regulation of HLA-A, -B, -C, β2m, and the members of the antigen processing machinery, particularly LMPs and TAP, may result in class I-defective phenotypes (4, 5, 7–11). Defects have been noted frequently in tumors and virus-infected cells, and are known to favor immune evasion by depressing antigen presentation to CTLs (1–5, 11). Some authors have claimed that gene deletion and/or mutational inactivation in the HLA region are common mechanisms of tumor immune evasion (1, 3, 12). An extensive series of tissue lesions, either poorly or not at all stained by antibodies to class I and TAP, is often cited in support of this view. However, the boundary between complete loss and down-regulation is unclear in immunohistochemical studies. Moreover, the abundance of complete loss phenotypes is in stark contrast to the limited number of established cell lines carrying gene deletions (13) and/or mutations in the HLA region. Less than 30 inactivating mutations of the genes encoding class I heavy chains (14, 15; reviewed in Ref. 3), β2m (16, 17; reviewed in Ref. 3), and TAP (15, 18) were sequenced, and only in one of these studies (17) was the percentage (12%) of tumors carrying mutated genes estimated. A high prevalence (~90%) of miscellaneous genomic losses/alterations of at least one HLA class I gene was only documented in cervical cancers (19). Despite the few sequenced mutations, high percentages (up to 90%) of tumor cell lines were found to completely lack constitutive TAP expression (20–22). No mutations were reported affecting LMP2, LMP7, tapasin, calnexin, calreticulin, and ERP57, and only a few studies (21–23) assessed the expression of some of these genes in tumors.

In contrast to the above examples, several groups (24–28), including ours (29), were unable to detect a high prevalence of complete HLA/TAP losses. In this report we argue that a high frequency of

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The abbreviations used are: HLA, human leukocyte antigens; CTL, cytotoxic T lymphocyte; NK, natural killer; β2m, β2-microglobulin; LMP, low molecular weight polypeptide; TAP, transporter associated with antigen processing; ER, endoplasmic reticulum.

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irreversible gene inactivation of the members of the antigen processing machinery is an overinterpretation of the available evidence, and alternatively propose that: (a) complete class I HLA/TAP/tapasin losses are exceedingly rare; (b) complete loss and down-regulation have different functional implications, and as such they should be kept logically distinct and treated separately; and (c) up-regulation also occurs, and in certain cases it may be advantageous to the tumor.

A strong argument supporting our view is the redundancy of immune receptors that equip cytotoxic effectors and are dedicated to the elimination of HLA-defective cells. The simultaneous expression of paired activating and inhibitory receptors for different self class I gene products, and the functional dominance of the latter (5, 30, 31), is a general mode of immune recognition of cytotoxic effectors. It ensures tolerance of normal host cells and efficient killing of cells with altered HLA phenotypes, particularly those that have lost the expression of only some HLA class I alleles or components of the class I antigen processing machinery (30). For instance, a CD8+ CTL clone bearing inhibitory receptors with an HLA class I allele specificity distinct from that of its own T-cell receptor was shown by Ikeda et al. (32) to be cytotoxic for melanoma cells that had selectively and completely lost the expression of the inhibitory class I allele. Similarly, a complete TAP loss is expected to result in the relief of inhibition signals transduced through the lectin-like CD94/NKG2A receptor expressed by NK cells (5, 30).

Recently we were able to PCR-amplify second and third exon sequences of 57 HLA-A and -B alleles from the genomic DNAs of 30 early passage tumor cell explants obtained from 30 neoplastic patients. Occasional sequencing revealed no mutation. Forty-five of these alleles in 26 class I-positive cell lines could be tested at protein level, and all of them were expressed at the cell surface, as assessed by flow cytometry. The lowest class I expression was detected in cells explanted from lesions found entirely or in part class I-negative by immunohistochemistry. This suggested a lack of sensitivity of this technique and not HLA loss in vivo. Most importantly, each allele was allowed only marginal deviations (decrease or increase) from the balanced, averaged expression of all of the other alleles in the normal WBCs of the same patient (29).

In the present study, half of the 30 original early passage cell lines (including 3 of the 4 negative cell lines) were maintained in culture until the 10th passage, and tested by semiquantitative methods, such as Northern and Western blotting, for the expression of class I heavy chains, $\beta_2$-m, LMP2, LMP7, TAP1, TAP2, tapasin, calnexin, calreticulin, and ERp57, yielding what we consider a systematic HLA class I mRNA and protein levels, and, in two cases, even increased expression in tumor cells as compared with their normal counterparts.

On the basis of these data, we propose an amended theory of immune surveillance that includes complete, irreversible class I loss as a particular case, and introduces HLA gain as an additional NK-decoy strategy. We propose that tumor cells acquire a mimetic phenotype resembling that of normal cells (a “low HLA profile”) characterized by a limited imbalance in the levels of expression of HLA class I alleles and members of the antigen processing machinery. We suggest that this low HLA profile represents a window of opportunity through which tumors effectively evade most cytotoxic effectors.

**MATERIALS AND METHODS**

**Cell Lines.** The HLA-A, -B, and -C DNA-based molecular typing (29), and the early passage (<10 passages) cell lines (7 melanomas, 5 lung carcinomas, 2 ovarian carcinomas, and 1 endometrial carcinoma) used in this study have been described previously (33). EBV immortalized B lymphoblastoid cell lines from 4 patients were generated by incubation of peripheral blood mononuclear cells with culture supernatants of the EBV-releasing B95–8 marmoset cell line, and selected in the presence of cyclosporine A (500 ng/ml). In vitro generated mutant cells were used as controls for antibody and probe specificities. The 721.221 (221 hereafter) cell line lacks the expression of all of the HLA-A, -B, and -C molecules (8). The 721.220 (220 hereafter) cell line, a gift from Dr. Peter Cresswell, lacks the expression of all of the HLA-A, -B, and -C molecules except for the HLA-C1 allele. It also lacks the expression of tapasin polypeptides of correct size, although it retains low levels of aberrant tapasin transcripts. Absence of tapasin results in low steady state levels of HLA (9). The cell line 174 × CEM.T2 (T2 hereafter) has an extensive deletion in the HLA region involving the genes encoding LMP2, LMP7, TAP1 and TAP2; T1 is the parental cell line used to generate T2 (7). All of the cell lines were cultured in RPMI 1640, supplemented with fetal bovine serum and L-glutamine (complete medium). Setting up cultures of cutaneous metastatic melanomas and normal melanocytes from the adjacent normal skin of the same patient has been described (34). Whereas melanomas were routinely grown in complete RPMI 1640, normal melanocytes were initially passaged in Ham’s F10 medium containing 10% fetal bovine serum and phorbol 12-myristate 13-acetate at 16 nm concentration. Melanocytes were then transferred to the same medium used for melanoma cells for two to three passages before the assessment of HLA expression. However, phorbol 12-myristate 13-acetate (16 nm) was maintained in the melanocyte cultures, because it does not detectably affect class I levels, but its withdrawal decreases melanocyte proliferation. The HLA-A/-B molecular typing of melanocyte/melanoma (lowercase “c” identifies melanocytes) pairs was: cMC7-MC7 (HLA-A2, 3; -B13, 35); cMS10-MS10 (HLA-A2, 24; -B35, 55); cMD12-MD12 (HLA-A3, 30; -B7, 44); and cMM17-MM17 (HLA-A2, 11; -B13, 32).

**Antibodies and Probes.** The following antibodies were used in Western blots. The murine monoclonal antibody HC-10 (a generous gift from Dr. Hidde Ploegh) is one of the most widely reacting antibodies for the detection of HLA-B and -C heavy chains. It also reacts with a limited set of HLA-A alleles (35). Namb-1 is a Western blot grade antibody to $\beta_2$-m (23). A polyclonal antibody to the COOH terminus (residues 734–748; GCYWAMVQAP-ADAPE) of TAP1 was produced in rabbits. Its specificity is identical to that of an antibody to the same sequence generously provided by Dr. John Trowsdale, who also provided a polyclonal antibody to TAP2. Both of the original antibodies have been described (36). A polyclonal antibody to the NH$_2$ terminus (residues 2–19; PAVIECFVVEDASGKGLAK) of tapasin was produced in rabbits. Its specificity is identical to that of an antibody to the same sequence generously provided by Dr. Peter Cresswell (10). A polyclonal antibody to the COOH terminus (residues 486–505; NPPV1QEEKPKKKKKAQEDL) of human ERp57 was produced in rabbits. Polyclonals to calnexin and calreticulin (Stressgen, Victoria, British Columbia, Canada), and to LMP2 and LMP7 (Affiniti, Mamhead Castle, United Kingdom) are commercially available. The following murine monoclonal antibodies were used in flow cytometry. W6/32 reacts with all of the HLA-A, -B, -C heavy chains associated with $\beta_2$-m, BBM1 and Namb-1 are to $\beta_2$-m. Allele- and locus-specific antibodies 160.30 (HLA-A3), 361–1 (A3), LT129.11 (A30), CR11.351 (A2), HA.41 (A24), BB7.1 (B7), 375.3 (B13), LGIII-220.6 (locus A), Q664 and 126.39 (locus B) were distributed through the collaborative efforts of the XII International Histocompatibility Workshop. Their use and allelic specificities, as assessed by flow cytometry, are fully referenced in a previous study of ours (29). Probes for exon 4 conserved sequences of class I heavy chain genes and for $\beta_2$-m were described (37). Full-length probes for TAP1 and TAP2, tapasin, and calreticulin were generous gifts from Drs. John Trowsdale, Peter Cresswell, Paul Lehner, Roberto Di Lauro and Lorena Perrone. Western (chemiluminescence) and Northern (isotopic) blotting techniques were extensively described in recent publications of our group (23). Densitometric scans were performed on preflashed autoradiographic films.
in the absence of the protein. These bands are nonspecific because they are also seen, below the expected Mr 48,000 tapasin band (arrow). These bands are nonspecific because they are also seen, in the absence of the Mr 48,000 band, in tapasin-defective 220 cells. For additional details about mutant cell lines see “Materials and Methods.”

RESULTS

The Lack of Class I HLA Expression Is a Rare Phenotype in Early Passage Tumor Cells. All of the early passage tumor cell lines (n = 15) were tested by Western blotting for the expression of class I heavy chains, TAP1, TAP2, tapasin, calnexin, and calreticulin (Fig. 1). A subset (n = 10) was tested for the expression of LMP2, LMP7, ERp57, and β2m (data not shown; see below). Twelve cell lines were also tested by Northern blotting (Fig. 2). Control cells included EBV-immortalized B lymphocytes from 4 of the patients from whom neoplastic cells were explanted, and a set of mutant cell lines (see “Materials and Methods”). This represents a mini-proteome/minitranscriptome systematic analysis of gene products known to be necessary for class I expression. All of the polypeptides and transcripts were detected, although at widely different levels, in all of the early passage tumor cell lines except for class I heavy chains in End 9 (Figs. 1 and 2).

In a previous, cited study of our group (29), 3 early passage cell lines (Mel 24, Mel 35, and End 9) had been scored class I-negative, in that class I expression (estimated by flow cytometry) did not exceed the “confidence” cutoff threshold of three times the background. However, staining slightly above background had been detected by immunohistochemistry in two tumor lesions (Mel 24 and Mel 35). The biochemical techniques used in the present study (Figs. 1 and 2) confirmed the presence of class I products in Mel 24 and Mel 35, although, as it could have been anticipated, at levels that were among the lowest detected in the present panel of early passage cell lines. This is best appreciated when the densitometric values of the Western blots (shown in Fig. 1) are sorted in decreasing order of class I expression (Fig. 3A). On the basis of these data, it appears that HLA-loss phenotypes are even more rare than estimated in our previous study, and that only one truly HLA-A-, -B-, and -C-negative cell line (End 9) remains in our original panel of 30 tumor lesions and early passage cell lines. Only in End 9 was the lack of class I molecules concordantly estimated by immunohistochemistry, flow cytometry with both locus-specific (HLA-A and -B), and allele-specific (HLA-A24 and -B52) antibodies (Ref. 29; data not shown), and biochemical techniques (this report; Figs. 1 and 2). Of interest, despite this lack of heavy chain reactivity, wild-type second and third exon sequences of the HLA-A24, -A25, -B15, and -B52 alleles could be PCR-amplified from End 9 genomic DNA (29), and a probe for conserved sequences of class I molecules revealed a normal Southern blotting pattern (data not shown). In addition, End 9 was found to express TAP1, TAP2, tapasin, and β2m (polypeptides as well as transcripts), although at the lowest levels detected in the 15 tested cell lines (Figs. 1 and 2; Fig. 3A). For instance, a particularly faint tapasin band, absent in tapasin-deficient 220 cells, could be seen in Fig. 1 (arrow). Similarly, weak β2m components were detectable after long exposures of Northern blotting filters (including that shown in Fig. 2) and immunoprecipitates from cells metabolically labeled at high specific activity (data not shown). The low expression of β2m is possibly more surprising than the low expression of TAP and tapasin, because TAP and tapasin levels are low in class I-low cell lines, whereas β2m is invariably expressed in similarly high, excess amounts in all but the End 9 cell line (Northern blotting in Fig. 2; Western blotting, data not shown).

The indicated early passage tumor cell lines (Mel 8 through End 9), EBV-immortalized B cells from 4 patients, and control mutant cell lines were lysed by the nonionic detergent NP40. Soluble extracts (100 μg/lane) were run on a 10% acrylamide SDS-PAGE slab and electroblotted. Each of two replicate filters was stained/stripped/three times with the indicated antibodies, and visualized by chemiluminescence. Selected areas of the filters are shown. Please note that the antibody used to detect tapasin reacts with two bands right above and below the expected Mr 48,000 tapasin band (arrow). These bands are nonspecific because they are also seen, in the absence of the Mr 48,000 band, in tapasin-defective 220 cells. For additional details about mutant cell lines see “Materials and Methods.”

Alternatively, chemiluminescence was digitally acquired with a Fluor-S Max Multimager (Bio-Rad, Hercules, CA).

Internet address: http://rsb.info.nih.gov/nih-image/.
These results suggest that End 9 is indeed a regulatory mutant, and the only early passage cell line whose features are compatible with a derangement in class I expression leading to a complete absence of HLA-A, -B, and -C molecules. The most likely interpretation is that End 9 underwent, in vivo, a significant down-regulation of tapasin and TAP, a drastic down-regulation of HLA-A, -B, and -C molecules. Additional studies are required to elucidate the mechanism(s) of this "quasi-coordinate" down-regulation/loss in End 9.

In summary, this set of results led us to draw the following conclusions: (a) when previous flow cytometry data are re-evaluated, and the present Western blot results are taken into account, the correlation in the HLA phenotypes between tumor lesions and early passage cell lines rises to 100%, demonstrating conclusively that all of the 30 early passage cell lines originally described by us faithfully reproduce the in vivo HLA-A, -B, and -C phenotypes of tumor lesions; (b) HLA-loss phenotypes are rare, because they can be demonstrated in only 1 of 30 early passage cell lines; (c) the levels of class I, TAP1, TAP2, and tapasin (polypeptides as well as transcripts) differed widely among cell lines, whereas, as expected, those of β2m, calnexin, calreticulin, and ERp57 were similarly high; (d) the lowest degree of variability among cell lines was seen, as expected, with the F-actin probe (Fig. 2); and (e) all of the members of the antigen processing machinery, except ERp57, appear to be coregulated, because in most cases high and low class I expression correspond to high and low expression, respectively, of most other molecules (Fig. 3A).

HLA Class I and Members of the Antigen Processing Machinery Are Cooperatively Regulated. To objectively assess patterns of coordinate expression and reveal possible deviations from coordination (e.g., up- or down-regulation of individual members of the antigen processing machinery in specific cell lines), densitometric scans of the specific bands (polypeptides as well as transcripts) from the gels shown in Figs. 1 and 2 (and from additional gels) were obtained, and the relative absorbance values plotted against each other, two by two, gene product by gene product, cell line by cell line (i.e., TAP1 versus TAP2 polypeptides, Fig. 3B; TAP1 versus tapasin transcripts, Fig. 3C; TAP1 polypeptides versus TAPI transcripts, Fig. 3D, and so forth). Graphs were then constructed displaying the relationships between the different series of absorbance values for the entire set of early passage cell lines. Correlation coefficients (R²) for each series were calculated by regression analysis. Because coordi-
nation of the oligomeric subunits of a molecular complex can be expected a priori, we first verified that the closest and most consistent (across cell lines) correlations were detected in the polypeptide levels of TAP1 versus TAP2, and of LMP2 versus LMP7. The almost optimal (R² values close to 1) correlation coefficients (0.86 for TAP1 and TAP2, Fig. 3B: 0.90 for LMP2 and LMP7, data not shown) obtained from regression analysis were perfectly consistent with this assumption, and provide a precise quantitative estimate of the negligible error introduced in the measurement of polypeptide expression by our experimental procedures. The correlation coefficients (at the polypeptide level) between TAP1 on the one hand, and tapasin, LMP2, LMP7, and class I on the other, ranged from 0.80 to 0.60 (Fig. 3B), demonstrating a lesser, but significant, degree of polypeptide coordination across cell lines. TAP, tapasin, and class I transcripts were also correlated, although in some cases less strictly than the corresponding polypeptides (Fig. 3C). Somehow surprisingly, loose relationships were also found among TAP, tapasin and class I polypeptides, on the one hand, and β₂m, calnexin and calreticulin polypeptides, on the other (representative results are shown in Fig. 3B). Finally, the levels of protein and transcripts from the same gene displayed rather low correlation coefficients (Fig. 3D). A diminished concordance between mRNA and protein products from the same gene might have been anticipated, because Western and Northern blot equalization was done by equilibrating total cellular proteins and RNAs, respectively. This results in lanes of protein and RNA gels of the same cell line being loaded with different absolute numbers of cells. In addition, different mRNA species may have different transcription rates, stabilities, and translation efficiencies, resulting in different levels of polypeptide accumulation. Thus, it is conceivable that a precise stoichiometric balance of the members of the antigen processing machinery is needed at the effector protein level (where their concerted action is necessary) but not at the mRNA level.

There were four partial exceptions to the general pattern of mini-proteome coordination: the Mel 11 and Mel 20 cell lines expressed low levels of TAP polypeptides in the presence of high levels of class I molecules, whereas the Mel 24 and Mel 35 cell lines mirrored this phenotype (average or above average levels of TAP and/or tapasin associated with low class I levels; Fig. 3A). When the Mel 11, Mel 20, Mel 24, and Mel 35 outliers are not included in the regression analysis, correlation coefficients rise marginally (as expected) for TAP1 versus TAP2 (from 0.86 to 0.91) and TAP1 versus tapasin (from 0.80 to 0.82), but drastically for TAP1 versus class I (from 0.60 to 0.81), conclusively demonstrating lack of coordination in a subset of gene products and cell lines within an otherwise significantly correlated dataset.

The HLA Class I Coordinome. Linked patterns of expression detected by the mini-proteome analysis of early passage cells demonstrate that HLA class I, β₂m, and members of the antigen processing machinery are not completely free to individually undergo changes in their expression on malignant transformation. They form a hierarchically ordered array of gene products, a coordinome (Fig. 3E), in which members are allowed to deviate from one another to an extent that depends primarily on their relative positions in the coordinome layers: the closer, the lesser. ERp57 lies outside the class I coordinome, i.e., its expression is not influenced by the gene expression requirements of the class I antigen processing machinery.

The Expression of HLA Class I Molecules and Members of the Antigen Processing Machinery May Be Both Up- and Down-Regulated in Melanoma as Compared with Paired Melanocyte Cultures. From Figs. 1–3 it clearly appears that almost all of the early passage tumor cell lines (including the 4 cases for which paired immortalized B cell cultures were set up) express much lower levels of class I, TAP, and tapasin than lymphoid cells. To distinguish low, lineage-specific expression from transformation-induced down-regulation, it was mandatory to resort to pairs of normal and neoplastic cells from the same lineage and donor.

Four additional melanoma cell lines with an available paired melanocyte culture were then selected for mini-proteome/mini-transcriptome assessment (Figs. 4 and 5), and for flow cytometry analysis (representative results with monomorphic and allele-specific antibodies are shown in Fig. 6). At least four conclusions can be drawn from these experiments. First, whenever a member of the antigen processing machinery was detected at high levels in a cell line, most others were highly expressed. This concordant regulation is better appreciated at the polypeptide (Figs. 4 and 6) than the transcript (Fig. 5) level. Second, only in one pair (cMM17-MM17) the expression levels of most coordinome members were similar in melanocyte and melanoma cultures (Figs. 4 and 5). The remaining three pairs exemplify the two remaining, opposite modes of class I regulation: down-regulation in the melanoma culture MS10 as compared with its paired melanocyte culture cMS10; up-regulation in MD12 and (to a greater extent) in MC7 melanoma cells as compared with cMD12 and cMC7 melanocytes, respectively (Figs. 4 and 5). Differences in expression were reflected in differences in the levels of class I molecules expressed at cell surface (Fig. 6). Third, mini-proteome/mini-transcriptome experiments concordantly estimated changes in class I HLA expression within but not across melanocyte-melanoma pairs. For instance, increases in coordinome expression occurring on malignant transformation in the cMC7-MC7 pairs were concordantly detected in the Western and Northern blot experiments depicted in Figs. 4 and 5, respectively. In contrast, the levels of class I molecules expressed by cMS10 and cMC7 were estimated to be different (Fig. 4) and similar (Fig. 5) by the two techniques. This is likely because of an imperfect correlation between mRNA and polypeptide products from a given gene, as already noted (low regression values) and interpreted for the
data shown in Fig. 3D. This imperfect correlation, irrespective of the cause, does not influence the interpretation of the results. Finally, calreticulin is often expressed at slightly higher levels in melanomas than melanocytes (Fig. 4).

In summary, although the total number of melanocyte/melanoma pairs is low, from Figs. 4, 5, and 6 it can be concluded that the level of class I expression is primarily a feature of the nevomelanocytic pair, shared by normal and malignant cells. Down-regulation is only one of the possible phenotypes induced by malignant transformation. Whichever the change (if any) in the expression of class I or members of the antigen processing machinery, all or most other members will behave accordingly. Constitutive expression of LMP2 and LMP7 is seen (although in some cases at barely detectable levels) in all of the melanocytes and melanomas. LMP2 and LMP7 display the greatest difference in constitutive expression between lymphoid cells and melanocyte/melanoma pairs, possibly reflecting different lineage commitments to the constitutive usage of these IFN-γ-inducible proteasomal components.

We conclude that the differences between neoplastic cells and their normal counterparts are not too marked, as if neoplastic cells, on the average, were attempting to maintain a low profile.

DISCUSSION

The present along with a previous study of ours (29) complement approaches taken by other investigators. We took advantage of early passage (as opposed to continuous) cell lines. These cell lines were shown previously (29) to be faithful to the in vivo phenotypes of the lesions from which they had been explanted. In the present study, we confirm that these HLA phenotypes are stable for ~10 subpassages (except for minor, easily explainable discrepancies; see below) in a large subset of the original cultures. This makes it highly unlikely that the HLA phenotypes observed by us and others in early passage and continuous cell lines were the result of biased selection against mutational losses (see also our previous study for a thorough discussion).

Using this experimental model, we report that the class I HLA heavy and light chain subunits, and the members of the antigen processing machinery, can be concordantly detected by several biochemical techniques in most early passage cells, although often at low levels. Because low expression and loss can be accurately distinguished, our findings lower the divide between HLA-positive and HLA-negative tumors, and provide a reliable estimate of the relative sizes of these two tumor pools. This is not without consequence, because low HLA expression is a regulatable phenomenon, whereas loss is often thought of as irreversible. Moreover, the inclusion of a small panel of melanocyte/melanoma cell pairs allows an additional distinction to be made between down-regulation (that implies reduced expression in tumors) and low expression (that implies low absolute levels, and not necessarily a reduction).

Complete Loss of HLA-A, -B, -C, β2m, and the Members of the Class I Antigen Processing Machinery Is a Rare Occurrence. In a previous study (29), the 15 early passage tumor cell lines characterized in this report were tested for the expression of 25 class I (HLA-A and -B) alleles and β2m. In this report, 115 additional products were tested from 9 genes (B2m, TAP1, TAP2, tapasin, LMP2, LMP7, calnexin, calreticulin, and ERp57) involved in class I-mediated antigen processing and chaperoning. In addition, 4 melanoma cell lines belonging to melanoma/melanocyte pairs were tested for the expression of 9 HLA-A and -B alleles, and of 36 independent gene products of the antigen processing machinery. As a result, 19 early passage cell lines have now been characterized in the expression of 185 gene products in total. Only 1 of these cells, the endometrial carcinoma cell line End 9, displayed no expression of the 2 class I alleles testable with the available allele-specific antibodies. Even assuming that the HLA-A, -B, -C loss of End 9 affects all of the 6 alleles (which may be an overestimate), it turns out that the expression of no more than 6 of 185 (slightly >3%) gene products was lost in 1 of 19 early passage cell lines. Because the techniques used by us may detect residual expression when only one copy of the B2m, TAP1, TAP2, tapasin, LMP2, LMP7, calnexin, calreticulin, and ERp57 genes is transcribed/translated, we cannot formally exclude functional hemizygosity at some loci. However, coordination in the levels of expression would
tend to exclude this possibility. Even in this event, no known step of the class I antigen processing machinery appeared to be irreparably damaged in 18 of 19 cell lines.

Over the past 10 years, we have tested continuous cell lines from international cell repositories (33), and reported the establishment in culture of 83 early passage tumor cell lines altogether (29, 38–40). Of the two cell lines displaying complete class I loss, the KJ29 cell line was shown to lack β₂m (37), the most common defect resulting in complete, generalized class I loss. In contrast, the End 9 cell line described herein epitomizes, to the best of our knowledge, a novel, complete, generalized class I loss. In contrast, the End 9 cell line described herein epitomizes, to the best of our knowledge, a novel, unprecedented HLA-loss phenotype. Studies are in progress to determine whether this combined loss is due to independent mutational hits acting in cis to each of the 6 class I alleles, or, more likely, to the lack/malfunction of one or more transcription regulatory factors primarily acting in trans on class I, but also affecting the expression of other members of the antigen processing machinery.

Several authors (20–22, 26, 41–43) used biochemical approaches similar to those used by us. In one case (26) the prevalence of HLA/TAP loss was low, but in all of the other studies it largely exceeded the prevalence estimated by us, to the extent that up to 90% (21) of cells expressed no detectable levels of at least one gene product (i.e., TAP, tapasin, LMP molecules, and so forth). However, in many of these studies (20, 21, 41–43) both down-regulation and loss were reversed by IFN-γ treatment, a finding that prompted the authors to exclude structural alterations in the coding genes. On the other hand, a high prevalence of genomic HLA-A, -B, and -C loss was detected and characterized by Koopman et al. (19) in cervical carcinoma explants. However, members of the antigen processing machinery were not tested in this study, and no complete, irreversible loss of TAP or tapasin was detected in another, more recent study in cervical carcinoma cell lines (22). Thus, with the possible exception of cervical cancer, which may represent a tumor histotype with an unusually high prevalence of HLA loss, reinterpretation of previous data in light of the current data would support the conclusion that most tumor cells do express class I and members of the antigen processing machinery at low levels.

Whatever the reason for the discrepancies in the literature, in the present study it is clearly impossible to enforce stringent criteria to unequivocally identify a cutoff between normal expression and down-regulation, because (a) expression levels are distributed along a continuum of values, and (b) most members of the antigen processing machinery are constrained in ranks of linked expression in most cell lines (see for instance Fig. 3A). Under these conditions, setting a down-regulation threshold for any one member would be arbitrary, as indirectly demonstrated by the misleading case of the flow cytometry-negative/biochemistry-positive cell lines Mel 24 and Mel 35 described in “Results,” which are clearly low expressers, and not HLA-defective cells.

Our findings do not conflict with most molecular studies. Poor antigen presentation by murine class I molecules transfected in human tumor cells (41) is equally compatible with a complete TAP loss and the low TAP, tapasin, and LMP levels detected by us in most early passage cell lines. Moreover, both TAP and HLA class I must be transduced in melanoma cells to restore antigen presentation (42), a finding that clearly indicates coordinated low expression and not selective loss of individual members of the antigen processing machinery (i.e., the most likely event in mutant cells).

Coordinate Expression of Class I and Members of the Antigen Processing Machinery. Mini-transcriptome/mini-proteome analysis has shown significant coordination of HLA class I and members of the antigen processing machinery in many early passage cell lines. These linked patterns of expression have led us to coin the term HLA class I coordinome (Fig. 3E) to indicate a set of collaborating gene products. The coordinome is onion-like and hierarchically organized in multiple layers. However, it must be stressed that our own data show that linkage is not indissoluble. In addition, conservation of extended motifs and spacers, such as the S/X/Y module of class I and β₂m (44), is not readily evident (our own homology search) in the promoters of the members of the class I antigen processing machinery. Thus, the coordinome must remain, at present, a useful operational concept that facilitates, in the first instance, the description of global changes in the antigen processing machinery of neoplastic cells.

Paired Neoplastic and Normal Counterparts: Down-Regulation versus Low Expression, Coordination versus Selective Loss. Having concluded that current criteria for scoring HLA losses are not applicable to our collection of early passage cell lines, we decided to revisit the concept of down-regulation. It is the actual rationale of assimilating low expression to down-regulation that lends itself to substantial criticism. One may argue that, unless proven otherwise, a lower than average expression in a given tumor cell is not a result of down-regulation at the time of malignant transformation, but rather a physiologically low class I expression typical of nonlymphoid tissues. In other words, at least some differentiated normal cells may express very low levels of class I molecules, and certain tumor cells that clonally derive from these may inherit low expression, either directly from normal, differentiated cells, or from a common progenitor. To test this hypothesis, we decided to adopt the most stringent criteria of comparison of the class I phenotypes of normal and malignant cells: we compared normal and neoplastic counterparts from the same donor. An experiment fulfilling all of these requirements would be extremely difficult in most tumors of epithelial origin, for practical and ethical reasons. Therefore, we took advantage of methods to establish melanocytes in culture from the normal skin of healthy subjects and applied them to obtain normal melanocytes from the uninvolved skin of melanoma-bearing patients. At least one previous study described similarly low levels of class I molecules in melanocytes and melanoma cells, although from different patients and donors (3).

Using melanocyte-melanoma pairs, we report that both the absolute and the relative expression levels (the “blend”) of the individual members of the coordinome are transmitted as a legacy from melanocytes (or their progenitors) to melanoma cells, and that neoplastic transformation/progression may induce a significant imbalance without completely subverting predetermined expression levels and blends. Possibly, extensive quantitative and qualitative alterations are avoided by tumor cells because they may be perceived as an “altered self” by the immune system. A determination of the prevalence of these deviations and their allowed ranges awaits future study.

Theories of Immune Evasion: Low Profile or Strikeout. The cited study by Ikeda et al. (32), irrefutably demonstrating a series of irreversible class I losses during the progression of metastatic melanoma, is often called into question to support the view that melanoma cells losing HLA evade the immune system. However, whereas there is agreement that HLA loss may have favored escape from conventional CTLs in the beginning, it is difficult to rationalize two facts: (a) appropriate cytotoxic effectors with inhibitory receptors for a missing class I allele could be isolated and characterized in their cytotoxicity for the HLA-defective tumor variant on recurrence; and (b) the patient was unquestionably an unusually long survivor. Thus, once the interpretation of this report is freed from preconceived ideas, it is evident that irreversible gene inactivation was not a successful mechanism of tumor escape. On the contrary, it is clear that this kind of HLA loss was counterproductive for the tumor.

We propose that moderate down-regulation, without complete loss, may be a winning strategy for both tumors and viruses. Lentviruses, much like tumors, have to entertain long-term interactions with the host. In the case of viruses, complete evasion is not optimal because
rapid killing of the host does not favor transmission (45). In the case of tumors, a complete escape through complete loss of a member of the antigen processing machinery is worthless, because later on this may activate classes of cytotoxic effectors with inhibitory receptors, resulting in tumor rejection. Tumors (somewhere like viruses) just need time. In the case of tumors, time favors the accumulation of critical mutations (outside the HLA region, if one accepts our view) and/or derangements in the expression of cancer-causing genes, which will eventually provide them with an uncontrollable invasive potential. Therefore, we propose that early stage tumors, like virus-infected cells, manage to survive the immune attack by maneuvering HLA expression levels within a rather narrow, optimal window, and modify the expression of the individual HLA alleles, LMP, TAP, and tapasin molecules without deviating too much from the balance inherited from their normal cellular counterparts. In other words, they try to keep what is perceived by the immune system as a low profile. The low, predetermined HLA-A, -B, and -C expression of normal non-neoplastic cells is certainly another major factor that, maintained in tumor cells, plays per se in favor of the tumor and against CTL recognition.

Recently (46), an Icelandic group found that a subset of breast carcinoma lesions with “mixed” (heterogeneous) HLA class I phenotypes had a significantly higher probability of disease recurrence than patients with either HLA class I-positive or -negative tumors, particularly after >5 years. This subset with intermediate levels of expression may have considerable analogies with low-profile tumors in our classification. On the basis of these data, it would appear that a low HLA profile is the best escape phenotype. Furthermore, a Dutch group found that colon carcinomas unreactive for HLA-A have a better prognosis than those that reacted (47). Thus, the concept that HLA loss per se is detrimental to tumor immune surveillance needs additional validation before it can gain conclusive acceptance.

The distinction between complete, irreversible loss and down-regulation (or low expression) is not academic, but bears practical implications. Our results show that, contrary to the predictions of HLA-loss theories, there may be ample scope for class I/TAP/tapasin up-regulation in patients undergoing immunotherapy with specific peptide antigens.

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