Multiple Genetic Alterations Cause Frequent and Heterogeneous Human Histocompatibility Leukocyte Antigen Class I Loss in Cervical Cancer

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Abstract

The nature and frequency of human histocompatibility leukocyte antigen (HLA) class I loss mechanisms in primary cancers are largely unknown. We used flow cytometry and molecular analyses to concurrently assess allele-specific HLA phenotypes and genotypes in subpopulations from 30 freshly isolated cervical tumor cell suspensions.

Tumor-associated HLA class I alterations were present in 90% of the lesions tested, comprising four altered pheno/genotype categories: (a) HLA-A or -B allelic loss (17%), mostly associated with gene mutations; (b) HLA haplotype loss, associated with loss of heterozygosity at 6p (50%). This category included cases with additional loss of a (third) HLA-A or -B allele due to mutation, as well as one case with an HLA class I–negative tumor cell subpopulation, caused by a β2-microglobulin gene mutation; (c) Total HLA class I antigen loss and retention of heterozygosity (ROH) at 6p (10%); and (d) B locus or HLA-A/B downregulation associated with ROH and/or allelic imbalance at 6p (10%). Normal HLA phenotypes and ROH at 6p were observed in 10% of the cases. One case could not be classified (3%).

Altered HLA class I antigen expression occurs in most cervical cancers, is diverse, and is mainly caused by genetic changes. Combined with widespread tumor heterogeneity, these changes have profound implications for natural immunity and T cell–based immunotherapy in cervical cancer.

Key words: cervix neoplasms/immunology • genes, MHC class I • DNA, neoplasm/ genetics • loss of heterozygosity • mutation

Introduction

Cancer usually develops by a multistep process, characterized by the accumulation of genetic events within the tumor cell (1). These may be associated with the activation of genes not normally expressed in somatic cells, or mutations within expressed genes. Virally induced tumors are also marked by the expression of viral genes. The resulting genotypic and phenotypic changes may provide the tumor with a selective growth advantage. The ability of T lymphocytes to recognize peptide epitopes derived from cytoplasmic, nuclear, and/or viral proteins, presented in the context of HLA class I molecules, potentially allows the immune system to respond to these changes in the transformed cell. This places HLA class I molecules at the center of the immune response to tumors. However, at the same time tumor cells may gain the ability to evade immune responses by downregulation or loss of expression of HLA class I molecules.

Abnormal HLA class I antigen expression has been observed at high frequencies in many different tumor types, including melanoma, colon, cervix, breast, lung, and laryngeal cancers, and may range from loss of a single HLA class I allele to complete loss of HLA class I antigen expression (2). To understand the exact nature and impact of HLA class I antigen loss in the course of carcinogenesis, it is crucial to elucidate the underlying mechanisms and their relative frequency in any particular tumor system. The identification of specific altered HLA class I phenotypes will be the first step to further analyze the mechanisms responsible for these HLA changes. In previous studies on the prevalence and/or mechanisms of HLA class I antigen loss in tumors, at least three major restrictions apply. First, the prevalence
of altered class I phenotypes in various primary cancers has been studied by immunohistochemistry on tissue sections (2, 3). However, the panels of anti-HLA class I antibodies (mAbs) used in such studies did not cover all the HLA-A and -B alleles represented in the patient population (3–6). Therefore, the incidence and interpretation of HLA “loss phenotypes” may be underrated. Second, criteria for interpretation and classification of staining results are not yet uniformly established (7), especially with respect to phenotypic intratumor heterogeneity. Finally, the mechanisms underlying HLA class I antigen loss have been studied mostly in cultured tumor cell lines in which molecular genetic analyses are easier to accomplish (2, 8–18). Only few such studies have confirmed results in the corresponding primary tumors (11, 19, 20). To avoid detection of defects possibly acquired in vitro, mechanistic studies should be extended to fresh primary tumor material.

Because integral analyses of complete HLA class I loss phenotypes and their underlying mechanisms in any one tumor system are still incomplete, the relative prevalence of specific HLA class I antigen loss mechanisms in any particular tumor system remains to be established.

In cervical cancer, a minimum estimate of HLA class I antigen loss in primary cervical cancers has previously been made at 73% (4), while the underlying mechanisms are poorly understood. The clinical impact of HLA class I downregulation in cervical cancer has been increasingly documented, while immunotherapeutic efforts against human papillomavirus (HPV)-infected cervical cancer cells are made at the same time (21–24). Therefore, it is mandatory to assess the exact nature and distribution of altered phenotypes in primary cervical cancer tissues. We have recently demonstrated various molecular mechanisms of HLA class I antigen loss in cervical cancer cell lines and their corresponding primary tumors (19, 20). These ranged from regulatory to structural genetic defects, including genomic deletions and mutations in class I genes. One of the latter defects, loss of heterozygosity (LOH) at chromosome 6p, containing the HLA genes in class I genes, has been shown to cause loss of complete HLA class I haplotype. LOH at 6p has been independently identified in primary cervical cancers at high frequencies, varying from 40 to 70%, without knowledge of HLA class I antigen expression in these tumors (25–29).

In this study, we have applied a methodology for the integral analysis of HLA class I antigen loss in the cervical cancer model, based on detection of all HLA-A and -B alleles by use of an extensive allele-specific anti-HLA mAb panel and multiparameter DNA flow cytometry on primary cervical cancer cell suspensions. Molecular analyses were performed on flow-sorted phenotypically distinct tumor populations from the fresh tumor samples. We determined the prevalence of particular altered HLA class I phenotypes and the related “6p microsatellite (MS) genotypes” with a focus on 6p21.3. In addition, we screened for allel-specific mutations and β2-microglobulin (β2m) mutations in tumors with allelic losses or cases with total HLA class I antigen loss, respectively. We demonstrate a successful approach to the integral analysis of HLA class I pheno- and genotype alterations in primary cervical tumors. Most importantly, we report that the majority of the observed HLA class I phenotype changes in cervical cancer cells are caused by genetic alterations at chromosome 6p21.3.

Materials and Methods

Primary Tumor Cell Suspensions

Consecutive cases of cervical cancer tissues were obtained with informed consent from patients who were operated on according to the Wertheim hysterectomy protocol at the Department of Gynecology of the Leiden University Medical Center. Immediately after surgery, tissue for diagnostic purposes was fixed in formalin, a piece of the tumor tissue was snap-frozen in liquid isopentane and stored at −70°C, and the remaining part, ranging in weight from 0.2 to 3.0 g, was cut into 1-mm3 pieces and washed. Tumors were classified as squamous (n = 21), adenocarcinoma (n = 8), or adeno-squamous (n = 1) carcinoma based on routine pathohistological examination. Each tumor was analyzed for the presence of type-specific HPV DNA by PCR and sequencing, as described previously (30). Of the 30 tumors, 18 (60%) contained HPV16 (n = 11) or HPV18 (n = 7), 2 tumors contained HPV45 (7%), and HPV56, 59, 70, 73, and 58+67 were each found once (20%). In five tumors, no HPV was detected.

The minced tumor fragments were enzymatically digested in 5 ml/g tissue of FCS-free DMEM (GIBCO BR) medium containing 0.002% DNase and 4 mg/ml collagenase type II, or medium containing 0.002% DNase, 0.5 mg/ml collagenase type II, and 0.25% trypsin (enzymes obtained from Sigma Chemical Co.) at 37°C for 1–2 h. After washing, cell pellets were extensively pipetted using a 25-ml plastic pipette to shear cells from remaining cell lumps. Suspensions were then sieved over a 100-μm D-filter (Verseidag-Industrietextilien GmbH) and the enzymatic activity was blocked by addition of FCS to 10% concentration. After washing in FCS-containing medium on ice, the viability and viability of the resulting single cell suspension were determined using the trypan blue exclusion method. Average yields were 5 × 106 cells/g tissue. The average viability was >80%. Single cell suspensions were frozen in 90% FCS/10% DMEM in liquid nitrogen until further use.

Patient PBL/HLA Typing

Heparin blood was separately obtained from each patient, and PBLs were isolated using standard Ficoll gradient centrifugation. HLA typing on PBLs was performed by the standard National Institutes of Health microcytotoxicity technique at the Department of Immunohematology and Bloodbank of the Leiden University Medical Center.

Abs

Anti-HLA mAbs. To detect HLA class I monomorphic determinants and relevant allele specificities in each suspension, a suitable subpanel of anti-HLA mAbs, including negative isotype control mAbs, was chosen from a large panel of murine and hu-

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1Abbreviations used in this paper: a.i., allelic imbalance; APC, allophycocyanin; β2m, β2-microglobulin; HPV, human papillomavirus; LCA, leukocyte common antigen; LOH, loss of heterozygosity; MS, microsatellite; MSI, MS instability; PI, propidium iodide; ROH, retention of heterozygosity.
human anti-HLA mAbs, described in a previous study (19). Human anti-HLA mAbs (hu-mAbs) were used in flow cytometric analyses of cell suspensions and not on tissue sections, due to cross-reactivity with human immunoglobulins present in such tissues. Murine anti-HLA mAbs (mu-mAbs) were applied both in flow cytometry and immunohistochemistry. To account for differences in mAb specificities and/or affinities, several mu-mAbs were used to address the same HLA alleles. Murine and human anti-HLA mAbs were used at optimal titers as described previously (19).

A mixture of mAbs (all IgG1) designed to detect all keratins known to be differentially expressed in cervical cancers was used for staining the epithelial (tumor) cells in the suspensions (31). This mixture contained culture supernatants from clones 80, 6B10, M20, and M9 (Eurodiagnostica BV), and AE1/AE3 (Boehringer Diagnostica BV), DE-K10, OVT12/30, BA17 (DAKO A/S), KS-1A3 (Sigma Chemical Co.), and AE1/AE3 (Boehringer Diagnostica BV). All reagents were used at optimal dilutions established in titration experiments.

A mixture of mAbs was used to stain surface CD45 (leukocyte common antigen [LCA]) on cells of the leukocyte lineage. Clone V9 (IgG2b), directed against vimentin, was used for intracellular staining of nonepithelial (nontumor) cells. Both mAbs were used at optimal dilutions established in titration experiments.

### Table I.

| PCR   | Primer | Position (nt) | Specificity      | Sequence* |
|-------|--------|---------------|------------------|-----------|
| HLA²  |        |               |                  |           |
| Exon 2 | Aex2F  | In1 (83–100)  | HLA-A (±B/C)     | 5'-gcc cgc cgg gaa ggg tc-3' |
|       | Bex2F  | In1 (39–56)   | HLA-B            | 5'-agg cgg cag gag gaa cca-3' |
|       | Aex2R  | In2 (37–59)   | HLA-A            | 5'-ggg cgg cct ggg gat caa-3' |
|       | Bex2R  | In2 (35–58)   | HLA-B excl B5801 | 5'-gtm cgt ggg gaa gga-3' |
| Exon 3 | A² ex3F | Ex2 (250–271) | HLA-A2, 24, 34   | 5'-ggg cgg gaa gag acg gaa-3' |
|       | A³ ex3F | Ex2 (250–271) | HLA-A1, 3, 11    | 5'-ggg cga cgg gaa gaa-3' |
|       | Aex3R  | In3 (89–109)  | HLA-A            | 5'-ata ttc tgt tgt tgg ccc-3' |
|       | Bin1F  | In1 (38–58)   | HLA-B            | 5'-ggg gag mra ggg gac cgg ag-3' |
|       | Bin3R  | In3 (37–69)   | HLA-B excl B5801 | 5'-ggg cgg cct ggg gat caa-3' |
| Exon 4 | Aex4F  | In3 (575–597) | HLA-A24          | 5'-gat ggc cac atgc gct g-3' |
|       | Aex4F' | In3 (575–597) | HLA-A rest       | 5'-gat ggc cac atgc gct g-3' |
|       | Bex4F  | In3 (604–624) | HLA-B excl B5401 | 5'-gtt cca tga rat aga cca-3' |
|       | Aex4R  | Ex4 (873–891) | HLA-A excl A8001 | 5'-gac cgg cgt ggg ccc tga cgg-3' |
|       | Bex4R  | Ex4 (867–884) | HLA-B ±C         | 5'-tgtt cga gct gcc gc-3' |
| β2m   |        |               |                  |           |
| Exon 1 | β2ex1F | Ex1 (806–824) | β2m              | 5'-ggg gag gca gca ggt taa t-3' |
|       | β2ex1R | Ex1 (972–990) | β2m              | 5'-ggg gag gca gca ggt taa t-3' |
| Exon 2 | β2ex2F | Ex2 (81–102)  | β2m              | 5'-ggg gag gca gca ggt taa t-3' |
|       | β2ex2R | Ex2 (430–451) | β2m              | 5'-ggg gag gca gca ggt taa t-3' |

F, forward; R, reverse; nt, nucleotide position(s); ±, and some; k = g or t; m = c or a; r = a or g; s = c or g; w = a or t; y = c or t; ln, intron; Ex, exon. HLA class I intron sequences were described by Cereb et al. (reference 58).

*Sequentialing primers used for each reaction are the same as listed, but shortened at the 5' end for the sequence reaction at a melting temperature of 52–54°C, and labeled with T exes red. The T exes red-labeled sequencing primers used for sequencing of the HLA-B exon 3 product were described previously (reference 20).

HLA class I sequences were described by Mason and Parham (reference 59), and β2m exon sequences were taken from GenBank (acc. nos. M17986 and M17987).

The listed primers have a melting temperature of 58–60°C, except for the HLA-B exon 3 primers, with melting temperatures of 66°C and 70°C, respectively.

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20,000 single cell events were collected using a standard FACS-labeled with 1% paraformaldehyde (M erck) containing 100 μM propidium iodide (Sigma Chemical Co.) for 5 min (31) then washed with PBA. All tubes, except those containing mu-mAbs of the IgG1 isotype, were then incubated with a mixture of murine antikeratin IgG1 mAbs for 30 min. In the tubes containing mu-mAbs (of any isotype), a directly FITC-labeled antivimentin (IgG2b) mAb was added. After washing, goat F(ab')2 anti-mouse IgG1-FITC was added in this first step. Cells were washed, resuspended in 0.5 ml DNA staining solution (0.1% RNase; Calbiochem) and 100 μM propidium iodide (PI) in PBA, incubated for 30 min at 37°C, and kept at 4°C until further analysis. The exclusions in the mouse HLA mAb-containing tubes were introduced to avoid cross-reactivity caused by similar mouse isotypes; the antivimentin (all nonepithelial cells) IgG2b mAb is used to substitute for the combined use of LCA ([nonepithelial] leukocyte) directed IgG2a mAbs and antikeratin (epithelial) IgG1 mAbs. There is no anticipated cross-reactivity between IgG2a LCA- or IgG1 keratin-specific mAbs and hu-mAbs.

Flow Cytometry. For each measurement, data from 10,000–20,000 single cell events were collected using a standard FACS-Calibur flow cytometer (Becton Dickinson Immunocytometry Systems) as described previously (31). Data were analyzed using WinList 3.0 and ModFit 5.2 software (Verity Software House).

Flow Sorting and DNA Isolation.

Flow Sorting. Flow sorting was performed using a FACStar plus flow cytometer (Becton Dickinson Immunocytometry Systems). Tumor cells were sorted on keratin expression (RPE, FL2), while mutually excluding LCA- or vimentin-positive (FITC, FL1) cells. At least 500 flow-sorted antigen-positive (FITC, FL1) and antigen-negative (FL3) cells were collected. Both samples were analyzed for viability at 100°C for 10 min, and stored at 4°C.

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Figure 1. Examples of altered HLA-A/B phenotypes in cervical cancer cell suspensions, detected by flow cytometry. (A) HLA-B63 allele-specific downregulation in tumor 14. I, Gates are set on the keratin-positive cells (R3, red) and on LCA-positive cells (R2, green). The diagonally shaped distribution of keratin-positive, LCA-negative cells (R 3) is caused by spectral cross-talk of the FL2 into the FL1 caused by the broad emission spectrum of the DNA stain PI (references 56, 57). II, These cells coincide with the aneuploid and diploid cell populations, respectively. However, keratin-positive cells also constitute a small peak in the diploid range (arrow). III and IV, HLA-A24 (mu-mAb A11.1M), HLA-A31 (hu-mAb D6K4F9), HLA-B27 (hu-mAb KG30A7), and HLA-B63 (hu-mAb D6K3F10) are expressed on the LCA-positive diploid cells (gated on R2). Oen histograms represent the irrelevant HLA control (mu-mAb 44B8 against HLA-A10). V and VI, HLA-A24, -A31, and -B27 are expressed on the keratin-negative aneuploid cells (gated on R 3), but HLA-B63 is not. VII, The DNA versus HLA-B63 plot shows that keratin-positive diploid cells express HLA-B63, whereas the aneuploid cells are negative compared with the irrelevant HLA control (VIII). (B) Complete loss of HLA-A26 (hu-mAb D6K4F9) and HLA-B51 (mu-mAb 116.5.28) and heterogeneous loss of HLA-A24 (mu-mAb A11.1M), HLA-B7 (mu-mAb 126.39), and total class I (mu-mAb W6/32) expression in tumor 49. I, As in A, keratin-positive cells (red) and vimentin-positive cells (green) coincide with the aneuploid and diploid cell control cell populations, respectively (II). All HLA-A/-B alleles and total HLA class I antigens are expressed on control cells (III, IV, and VIII). On tumor cells, HLA-A26 and -B51 are not expressed, as the peaks overlap the irrelevant HLA control mu-mAb GAPA3 against HLA-A3 (open histograms; black for control cells, red for tumor cells). HLA-A24 and -B7 are partly lost, as shown by the bimodal distributions in V and VI. VII, with respect to total HLA class I, three distinct tumor cell populations emerge, expressing no, intermediate, and high levels of HLA class I antigens. (C) Loss of total HLA class I antigen expression in tumor 26. In analogy to B, keratin-positive (red) and vimentin-positive (green) cells coincided with near-diploid tumor and diploid control cell populations, respectively (I). Compared with an irrelevant HLA control (mu-mAb 126.39 against HLA-Bw6) in II, no HLA class I antigens are expressed on the near-diploid tumor cell population (II). However, a small fraction of diploid keratin-positive cells is discernible that does react with the W6/32 mu-mAb.
or A3/11R (5'-cca cgt cgc agc cat aca tt-3'), both located in exon 3 at nucleotide positions 385-405 and 363-384, respectively. The amplimer sizes were ~375 (HLA-A2/24) and 360 bp (HLA-A3/11).

Amplification was performed in a total volume of 50 μl, containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.4], BSA [0.06 mg/ml], and 1.5 mM MgCl₂), 6–60 ng of DNA, all four deoxyribonucleoside triphosphates (each at 20 pmol as a mixture from Amersham Pharmacia Biotech), 50 pmol of each primer, 0.5 U Ampli-Taq polymerase (Perkin-Elmer), and distilled sterile H₂O. The touchdown PCR profile consisted of a 5-min denaturation at 96°C; five cycles of 30 s at 96°C, 30 s at 65°C (minus 1°C per cycle), and 30 s at 72°C; 30 cycles of 30 s at 96°C, 30 s at 60°C, and 30 s at 72°C; and a final 5-min extension step at 72°C.

Detection of Mutations in HLA-A/B or β2m Genes

PCR Amplification. Primers designed for separate amplification of exons 2, 3, and 4 of the HLA-A and HLA-B genes and exons 1 and 2 of the β2m gene are listed in Table I. All of these primers were 5'-labeled with Cy-5 and obtained from Isogen Bioscience BV. Amplification was performed as described in the previous section, except for slight modifications in the PCR used for amplification of HLA-B exon 3, with a touchdown profile from 70 to 65°C, 72°C elongation steps of 1 min, and final extension at 72°C for 1 min. The amplimer sizes were 420 bp (HLA-A and -B exon 2), 700 bp (HLA-A exon 3), 930 bp (HLA-B exon 2/3), 355 bp (HLA-A exon 3), 530 bp (HLA-B exon 4), 185 bp (β2m exon 1), and 370 bp (β2m exon 2).

Sequencing. Sequencing was performed as described previously (20) using the Thermo Sequenase core sequencing kit as described by the manufacturer (Amersham Pharmacia Biotech). Sequencing primers used are modified from the primers listed in Table I (see legend). All primers were purchased from Isogen Bioscience BV. In brief, 1 pmol of T exas red-labeled forward (F) or reverse (R) oligonucleotide primer was used per PCR, consisting of 25 cycles (95°C for 30 s, 60°C for 20 s, and 72°C for 20 s each) on a PTC-200 Peltier Thermal Cycler (MJ Research). Samples were directly subjected to electrophoresis on a Vistra DNA sequencer 725 (Amersham Pharmacia Biotech) at 35 W for 12 h, and subsequently analyzed with the Assign v5.0 software package (Amersham Pharmacia Biotech).

Results

Detection of Altered HLA-A and -B Phenotypes in Cervical Cancer Cell Suspensions

Flow Cytometry. 30 cervical tumor cell suspensions were analyzed by multiparameter DNA flow cytometry to determine allele-specific HLA-A and -B expression. Allele-specific HLA class I antigen expression of tumor cells was measured in the keratin-positive fractions, and on normal cells in the keratin-negative, LCA/vimentin-positive fractions (Fig. 1).

Tumor-associated HLA-A/B phenotype alterations were initially identified in 26 (87%) of the 30 tumors (see Fig. 4). Various altered HLA class I phenotypes emerged, which varied from total or partial loss of a single HLA-A or -B allele (Fig. 1 A), total or partial loss of two HLA-B alleles or an HLA-A and -B allele (Fig. 2 A), and loss of three alleles (two HLA-A and one HLA-B or vice versa, in three cases, to loss of all four HLA-A/B alleles in six cases. The latter category comprised three tumors with (partial) retention of W6/32 reactivity (total HLA-A/B loss, Fig. 1 B) and three tumors that completely lacked W6/32 mAb reactivity (total HLA class I loss, Fig. 1 C). In four cases, the constitutive HLA-A/B phenotype was expressed on the tumor cells.

The examples shown in Fig. 1, A–C, and Fig. 2 A illustrate the use of keratin mAbs is critical to the identification of tumor cells within an overall DNA diploid cell suspension (Fig. 2 A), whereas it clearly facilitates this identification in DNA aneuploid or near-diploid tumors (Fig. 1, A–C).

Flow Cytometry vs Immunohistochemistry. To illustrate the identification of tumor cells, stromal, and or infiltrating immune cells within the original histological context, as well as to independently analyze the expression of HLA-A/B alleles and monomorphic HLA class I (W6/32), frozen sections of each tumor were stained by immunohistochemistry. Some allelic specificities could not be detected by the anti-HLA-directed mu-mAbs used on tissue sections. These could only be analyzed on the corresponding suspension by flow cytometry using anti-HLA-directed hu-mAbs. The example in Fig. 2 depicts a tumor with loss of HLA-A2 and HLA-B51 expression in the tumor cell fraction, as measured by flow cytometry (Fig. 2 A). Immunohistochemistry on consecutive frozen sections of this tumor illustrates the same loss of HLA-A2 and HLA-B51 expression within tumor cell areas as concomitantly identified with antikeratin mAbs (Fig. 2 B). Positive staining of these HLA antigens was observed on LCA-positive stromal cells and tumor-infiltrating leukocytes. This illustrates the advantage of flow cytometry and sorting over microdissection of tumor cells from a frozen tumor section in the presence of contaminating normal infiltrating leukocytes.

Since several alleles were undetected, resulting from the lack of allele-specific mu-mAbs available for frozen sections, the assignment of each of these tumors to a particular loss phenotype category was not possible by immunohistochemistry. Apart from these differences, immunohistochemical and flow cytometric detection of allele-specific HLA-A/B or total HLA class I antigen expression was incongruous in several cases. These discrepancies were mostly such that while heterogeneous (positive and negative) expression was observed by flow cytometry, negative expression was seen by immunohistochemistry. In some other cases, positive expression as detected by flow cytometry was detected as heterogeneous or negative expression by immunohistochemistry. Both observations underline the high sensitivity of flow cytometry compared with immunohistochemistry.

Altered HLA Class I Phenotypes in Relation to Chromosome 6p MS G genotype

To determine the status of heterozygosity at chromosome 6p, in particular in the HLA region at 6p21.3, MS analysis was performed on DNA isolates from flow-sorted HLA-phenotyped tumor cell subpopulations. Where possible, tumor cell fractions that displayed heterogeneous, i.e., positive and negative, expression of HLA alleles were separately sorted.
An integral example of the analyses performed to determine HLA class I phenotype and 6p MS genotype is shown in Fig. 2. Fig. 2 C shows a representative MS measurement, performed on the cell fractions sorted from a tumor cell suspension that displayed loss of HLA-A2 and HLA-B51 expression (Fig. 2 A). The allelic ratio for MS marker D6S265 in DNA from autologous PBLs was the same as observed in DNA from the flow-sorted vimentin-positive internal control cells. One of the D6S265 alleles was completely lost in DNA obtained from flow-sorted keratin-positive tumor cells (Fig. 2 C), representing true loss of heterozygosity (LOH), apparently involving both HLA-A2 and -B51 loss.

Other examples illustrating allelic imbalance (a.i.), LOH, and retention of heterozygosity (ROH) are shown in Fig. 3. For clarity, the chosen examples match the tumors depicted in Fig. 1, A–C. Fig. 3 A is representative for a.i. observed in DNA from flow-sorted aneuploid tumor cells that displayed loss of HLA-B15 expression (Fig. 1 A). Fig. 3 B represents the MS analysis for tumor 49 shown in Fig. 1 B, which displayed heterogeneous and complex loss of all HLA-A and -B alleles and HLA class I antigen reactivity. All sorted tumor cell fractions of this tumor, including separately sorted HLA-A24 and HLA-B7 positive and negative cells and the overall keratin-positive (HLA-A26 and -B51 negative) cell population, show the same LOH. This implies that not HLA-A24 and -B7, but rather HLA-A26 and -B51, are involved in the observed LOH. Fig. 3 C shows a representative MS measurement for the flow-sorted fractions of tumor 26 with loss of total HLA class I antigen expression, shown in Fig. 1 C. In the flow-sorted tumor cell fraction, the same allelic ratio is found as in control cells, demonstrating ROH.

The analyzed data for the complete panel of MS markers used on DNA from each flow-sorted tumor cell fraction, together with a description of the corresponding HLA-A/B phenotype, are presented in Fig. 4. On the basis of these combined results, four major altered phenotype categories emerged. First, loss of a single HLA-A or -B allele with variable patterns of allelic retention, imbalance, and LOH at 6p was found in five tumors (17%; Fig. 4 A). An example from this category is shown in Fig. 1 A and Fig. 3.
A. Second, loss of an HLA-A/B haplotype, in conjunction with LOH at 6p for at least 3 markers, was initially identified in 14 tumors (47%; Fig. 4 B). Examples from this category are shown in Fig. 1 B, Fig. 2, and Fig. 3 B. In three of the tumors in this category (tumors 4, 15, and 30), an additional (third) HLA-A or -B allele was lost. In two other tumors (49 and 17) in this category, all four HLA-A and -B alleles were lost. In tumor 17, HLA class I antigens, as measured by W6/32 mAb, were still expressed, but in tumor 49, a proportion of tumor cells was unreactive with the W6/32 mAb (Fig. 1 B). The question as to which HLA-A and -B alleles belonged to which haplotype (haplotype assignment) involved in LOH in these cases is addressed in the next section. Third, loss of expression of all class I antigens, as detected by W6/32 mAb, with concurrent retention of 6p heterozygosity was observed in three cases (10%; Fig. 4 C). A fourth case of total HLA class I antigen loss was found in a fraction of tumor 49 (Fig. 1 B and Fig. 4 B), as described above. In tumor 26, some HLA class I antigen expression was detected on a small percentage of diploid cells in the keratin-positive fraction (Fig. 1 C). For one marker (TNF-a), the aneuploid tumor cell fractions of tumor 34 did not show ROH, but an aberrant pattern of LOH and additional alleles, similar to the tumor illustrated in Fig. 5. Finally, in three tumors (10%), loss of all HLA-A and -B allelic expression despite W6/32 reactivity, or downregulation of B locus expression, together with allelic retention and/or imbalance at 6p, was noticed (Fig. 4 D).

Four tumors (13%) expressed their constitutive HLA class I phenotype, three of which showed complete retention at 6p (Fig. 4 E). The 6p LOH found in tumor 13 probably reflected loss of one of the HLA-A/B haplotypes for which this patient was homozygous. In retrospect, this tumor was therefore assigned to the second category (haplotype loss). The resulting percentages of tumors assigned to both categories are carried through in Fig. 6 (see Discussion).

Tumor 57 did not fit any of the preceding categories (Fig. 4 F). At the phenotype level, this tumor had homogeneously lost expression of HLA-A1 and -B8. However, this was not concurrent with extended LOH at 6p, as shown for other
tumors with an HLA-A and -B (haplotype) loss (Fig. 4 B). The MS analysis for this tumor demonstrated an alternate pattern of MS instability (MSI), homozygosity, ROH, "shifted LOH," and again ROH for consecutive MS markers spanning the HLA class I gene region (Fig. 5).

MSA analysis and/or PCR-based haplotype assignments

In tumors 4, 15, 30, 49, and 17 (Fig. 4 B) it was not directly clear which HLA-A/-B haplotype was associated with the observed LOH. In tumor 15, heterogeneity of HLA-A24 expression was observed within the (HLA-A74/-B62-negative) tumor cell fraction. Since an identical LOH pattern (Fig. 4 B) was observed in both of the separately flow-sorted HLA-A24-positive and -negative cell fractions, this LOH was therefore assigned to the HLA-A74/-B62 haplotype.

Tumors 4 and 30 were both heterozygous for the HLA-Bw4/Bw6 "broad" specificities. To determine which of the two HLA-B alleles was implicated in the genomic loss at 6p21.3, a Bw4- and Bw6-specific PCR was performed on the normal and tumor cells. In tumor 4, HLA-Bw4 (HLA-B44) sequences were amplified in the tumor and normal DNA, whereas the Bw6-specific PCR was negative in the tumor DNA only (data not shown). Therefore, the observed LOH at 6p in this tumor included the HLA-A2 and -B8 haplotype. Similarly, in tumor 30, HLA-Bw6 (HLA-B7) sequences were amplified in the tumor and normal DNA, whereas the Bw4-specific PCR was negative in the tumor DNA only (data not shown). Thus, the HLA-A33 and -B44 alleles were implicated in the haplotype LOH.

As discussed earlier, the LOH in tumor 49 involved the HLA-A26/-B51 haplotype (Fig. 1 B and Fig. 3 B). For tu-
mor 17, which was homozygous for HLA-Bw4, the Bw4/Bw6 PCR was not relevant for assigning the haplotype affected by LOH. Since all alleles were lost in this particular case, elucidation of the haplotype implicated in LOH has been abandoned.

Tumors 46 and 47 were initially assigned to the first category (allelic loss), since at the phenotypic level, only one HLA-B allele was downregulated. However, the extensive LOH observed across 6p for all informative MS markers prompted further inquiry into suspected loss of an HLA-A and -B haplotype by LOH in both cases. Since both tumors were of the HLA-A2, -A11 type, different PCRs for these alleles were performed on normal and tumor DNA from these tumor cell suspensions. In fractions of both tumors 46 and 47, the HLA-A11-specific PCR was negative and the HLA-A2-specific PCR was positive, whereas both HLA-A-specific PCRs were positive on DNA from the corresponding normal cells (data not shown). Based on these results, both cases were assigned to the haplotype LOH loss category, leaving the observed HLA-A11 expression as unexplained “rest expression.”

Mutation Analyses

To analyze whether mutations in HLA class I genes had caused allelic loss (tumors 6, 14, 24, 50, and 58 in Fig. 4A), third allele loss (tumors 4, 15, and 30 in Fig. 4B), or whether mutations in β2m genes had caused loss of total HLA class I antigen expression as defined by the lack of W6/32 reactivity (tumor 49 in Fig. 4B, tumors 26, 34, and 59 in Fig. 4C), the relevant cases were analyzed for the presence of mutations. The detected mutations are shown in Table II.

Sequencing. PCR amplimers of HLA-A or -B exons 2-4 or β2m exon 1-2 from tumor and normal DNA were sequenced. In the allelic loss category, tumor 6 was found to contain a C to T substitution at codon 180 in exon 3 of the HLA-A2 gene, generating a stop codon (Table II). In tumor no. 14, a deletion of ~230 nucleotides containing parts of intron 2 and exon 3, was found in the HLA-B63 (B15) gene. In DNA from tumor 58, the HLA-A24 sequence was not detected in any of the screened exons. This appears to be caused by partial loss of chromosome 6p heterozygously observed in tumor 58 (Fig. 4A). For tumors 24 and 50 in this category, the tumor and wild-type sequences in the screened HLA-A or -B exon 2-4 products were identical. For tumor 24, the exon 3 amplimer was not available, because of the lack of material.

In the “haplotype LOH + 1 allele” category, a 16-nucleotide deletion and insertion of a G were found at codon 121 in the HLA-B7 exon 3 sequence of tumor 30. The HLA-A24 mutations in tumor 15 of this category are described under PCR-based mutation detection below. In tumor 4, the HLA-B44 sequences detected in the exon 2-4 amplimers were all wild-type.

In the “total class I loss” category (Fig. 4C), no β2m mutations were detected in the exon 1 and 2 sequences analyzed. However, in tumor 49 in the “haplotype LOH + 2 allele” category, which contained a fraction of cells that did not react with W6/32, a two-nucleotide deletion was found in exon 1 of the β2m gene (Table II). This deletion would result in frameshift, as observed in the tumor DNA, leading to a completely altered amino acid translation.

PCR-based Mutation Detection. The loss of HLA-A24 sequences in tumor 58 was independently confirmed by allele-specific PCR using the HLA-A24-specific primers described in Table I and the Materials and Methods. A 375-bp HLA-A24-specific product, as observed in DNA from PBLs from the same patient, was not detected in tumor-derived DNA. Tumor cell suspension 15 (in the “haplotype LOH + 1 allele” category) corresponded to a cell line and primary tumor previously reported to contain point mutations in the HLA-A24 gene at codons 89 and 279 of exon 2 and 5, respectively, both resulting in stop codons (20). Therefore, we confirmed these independently by PCR amplification using primers specific for the mutation and wild-type sequences as described (20). Both mutations were detected in DNA from HLA-A24-negative fraction of the tumor cell suspension, whereas DNA from the HLA-A24-positive fraction was amplified only in the wild-type-specific reaction (data not shown).

Discussion

This study demonstrates a high frequency of HLA class I alterations in cervical cancer, characterized by extensive pheno- and genotypical inter- and intratumor heterogeneity. In contrast with previous studies of cervical and other cancers (4-6), the multiparameter DNA flow cytometry approach presented in this paper enabled us to concurrently analyze HLA class I pheno- and genotype alterations directly in fresh cervical tumor cell suspensions. A major ad-
vantage of the present method of tumor cell separation from its tissue context is the possibility for detailed molecular genetic analyses of distinct cell populations in tumors that display phenotypic intratumor heterogeneity, as well as to avoid contamination by normal cell infiltration.

We showed that among the 90% of the cervical cancers with altered HLA class I phenotypes, almost 70% were caused by multiple genetic alterations at chromosome 6p (Fig. 4, Fig. 6, and Table II). Loss of at least one HLA haplotype was caused by tumor-specific LOH at chromosome 6p in 50% of the cases. In one third of these cases, additional allelic losses were demonstrated, most of which were proven to be due to mutations in HLA class I or β2m genes. Moreover, in more than half of the single allelic loss cases HLA class I gene mutations were found. The loss phenotypes observed in the remaining tumors included HLA-A/B or HLA-B locus loss, and total HLA class I loss.

Thus far, some of the observed HLA class I phenotypes and associated genotypes have been separately described, primarily in cell lines of a few different tumor types. HLA haplotype losses caused by LOH at chromosome 6p have been demonstrated in cell lines from melanoma, colon, pancreas, and laryngeal and cervical cancers (8, 10, 11, 19, 34), few of which were confirmed in the corresponding primary cancers. These data prove that LOH at chromosome 6p21.3, which occurs at high frequencies in cervical cancer (25–28, 35, 36), indeed represents an important and common mechanism by which HLA genes and their products are abolished.

In five of the eight cases with (additional) allelic loss, mutations were demonstrated, varying from point mutations to larger deletions in the relevant HLA-A or -B genes (Table II). In a previous report, we described a four-nucleotide insertion in exon 2 of the HLA-B15 gene leading to loss of HLA-B15 expression in a cervical cancer cell line and its primary tumor (20). Mutations underlying allelic loss have also been characterized in a few melanoma cell lines (12, 13, 15) and a colon cancer cell line (10). These vary from a point mutation in intron 2 (15), partial deletion of the 5' end of the HLA class I gene (13), to partial chromosomal deletions or rearrangements (10, 12). However, the presence of these defects in the original tissues was not investigated in these studies.

Because little information was available on mutations in HLA class I genes in tumors, we initially chose to screen for mutations in those HLA class I regions that seemed prone for (germline) mutations in individuals with HLA “null” or “blank” alleles (37–39). These are the exons 2, 3, and 4, en-

| Tumor | Sequence Observed at position (nt/codon) | Type of mutation |
|-------|----------------------------------------|-----------------|
| 6     | HLA-A2                                 | Exon 3 (nt 610/codon 180) Single base substitution → stop codon |
| 14    | HLA-B63                               | From intron 2 (nt 141 or 146) to exon 3 (nt 485 or 490/codon 138 or 140) 230-nt deletion |
| 58    | HLA-A24 (not detected in tumor sample) | Exons 2-4 Partial chromosome loss |
| 15    | HLA-A24i (mutations described in a cell line derived from this tumor) | Exon 2 (nt 337/codon 89) Single base substitution → stop codon |
| 30    | HLA-B7                                 | Exon 5 (nt 907/codon 279) Single base substitution → stop codon |
| 49    | β2m                                    | Exon 1 (nt 924–925 [T C: underlined] or 925–926 [C T: bold]) 2-nt deletion → frameshift |

* Genomic regions screened on normal and tumor DNA for HLA-A and HLA-B were exons 2-4; those for β2m were exons 1 and 2.
† Nucleotide (nt) positions in exons were counted starting from the first nucleotide in exon 1, excluding introns (cDNA sequence). Intron sequences were described by Cerè et al. (reference 58). β2m sequence data are available from EMBL/GenBank/DDBJ under accession nos. M17986 and M17987.
‡ MS analysis results (see Fig. 4 A) for this tumor indicate inclusion of the HLA-A24 gene in partial chromosomal loss in the HLA region. In addition, HLA-A24-specific PCR was negative on tumor DNA (see text).
§ Mutations were originally described in a cell line derived from this tumor (reference 19), and were confirmed in the tumor cell population used in the present study by PCR (see text).
coding the extracellular α1, α2, and α3 domains, respectively. The upstream regulatory regions, exons 1 (leader peptide) and 5 (transmembrane domain), and the introns of the relevant HLA class I genes were not analyzed, but it is now increasingly clear that these may harbor mutations that lead to downregulation or loss of cell surface expression (15, 20). This was also substantiated by the involvement of intron 2 in a mutation identified in the present study (Table II). Further investigations are in progress to clarify the mechanisms underlying the as-yet unexplained allelic losses.

One of four cases of total HLA class I antigen downregulation, as measured by complete lack of W6/32 reactivity, was explained by a combination of haplotype loss by LOH at 6p and mutation in exon 1 of the β2m gene. We restricted our screening of β2m mutations to exons 1 and 2, in which mutations underlying total HLA class I antigen loss were previously identified in melanoma and colon cancer cell lines and primary tumors (14, 17, 18, 40–43). Yet, mutations may also occur in sequences outside the regions screened (42, 43), which is the subject of further studies.

The high frequency of phenotype alterations established in our study exceeds the variable estimations previously made, ranging from ~30 to 80% (4, 22, 44-46). This variation was probably mostly due to the lack of complete allelic coverage by the panels of antibodies used. We detected all HLA-A and -B alleles represented in the patient population through the use of an extensive panel of HLA-directed mAbs, which allowed for classification of the 30 tumors into distinct HLA loss phenotypes. By demonstrating extensive LOH at 6p underlying haplotype loss in 15 cases (3 that harbor additional mutations in β2m or HLA class I genes) and allele-specific mutation in 3 cases, we have proven a genetic basis for 67% of the observed altered HLA class I phenotypes. As the yet unsolved cases in the haplotype and/or allelic loss categories as well as the total HLA class I antigen losses may harbor mutations outside the regions screened, this extent may be even higher.

The remaining tumors with altered phenotypes include two cases of HLA-B locus-specific downregulation and one case of HLA-A/B loss with retention of W6/32 reactivity. Further investigations are needed to elucidate the basis for these losses. HLA-B locus-specific downregulation has also been observed in a cervical cancer cell line and melanoma cell lines and is thought to be caused by defects at the transcriptional level (16, 19, 47). Reduced expression of transporter associated with antigen processing (TAP)-1/2 at the protein or RNA level has been described as a mechanism underlying reduced or total loss of HLA class I antigen expression in several tumors (48). In our series, the possibility of disturbed TAP function remains to be explored.

Heterogeneity, with regard to HLA class I antigen expression and DNA content, was often observed within individual tumors, and offers an outstanding opportunity to study HLA class I antigen losses during tumor progression, as reflected by aneuploidization. Taking into consideration that aneuploid tumor cells are preceded by diploid precursor cells, the observation of HLA losses in diploid cell fractions of largely aneuploid tumors (Fig. 4, tumors 6, 10, 25, 36, and 56) in this study suggests that loss of HLA class I antigens occurs early during tumor development. Tumors 10, 25, and 36 in the haplotype loss category (Fig. 4 B) show LOH and haplotype loss in such diploid cells. Similarly, in the allelic loss category, the HLA-A2 loss in tumor 6 is already present in the diploid cells (Fig. 4 A), suggesting that the mutation underlying this loss (Table II) may have occurred early during tumor progression, at least before manifestation of the largely aneuploid tumor population. On the other hand, HLA class I antigen loss was also found in conjunction with (tumors 12, 14, 23, and 26), or after (tumors 15, 28, 34, 49, and 50) aneuploidization (Fig. 4). These observations were not confined to any particular HLA loss category. Thus, HLA class I antigen loss seems independent of increased genomic instability, as reflected by aneuploidy.
The exact onset of HLA class I antigen loss in the course of cervical carcinogenesis is not clear. Loss of class I antigen expression has previously been indicated in premalignant cervical intraepithelial neoplasia lesions (23, 49), without investigation of the underlying mechanisms. Conversely, LOH at 6p has been demonstrated in cervical intraepithelial neoplasia lesions (20, 50, 51) with concomitant loss of HLA-A/B haplotype expression demonstrated in one report (20). Ongoing analyses of LOH patterns and/or mutations in the premalignant and/or metastatic lesions of the tumors studied here may provide information regarding the time of occurrence of the genetic changes underlying HLA class I antigen loss.

Our data did not point to a relation between HPV types and specific loss mechanisms. It remains unknown whether the pheno- and genotypical changes in HLA class I are related to the presence of HPV and/or HPV type. If HPV drives the cell to lose presentation capacity by HLA class I antigen loss, then a difference could exist between HPV-induced and non-HPV-related tumors. As virtually all cervical cancers contain HPV DNA, this cannot be assessed within this tumor type. Similar HLA changes have been identified in non-HPV-related tumors such as melanoma and colon. It would be interesting to compare the occurrence and predominance of the various different phenotypes and their underlying mechanisms among different tumors. However, a complete inventory of HLA loss phenotypes and underlying mechanisms in other primary tumors, comparable to the one presented here, is needed before any further conclusions can be drawn.

The multiplicity of molecular mechanisms leading to altered HLA expression and phenotypic heterogeneity within a given tumor is suggestive of selection of immunologically privileged variants during its multistep evolution (2). We previously showed in HLA-A24-positive and -negative variant cell lines derived from the heterogeneous tumor 15, that HLA-A24 expression was crucial for recognition by CTLs present within the autologous bulk tumor-infiltrating leukocyte culture (20). Intratumor heterogeneity probably constitutes a major hurdle for effective immune surveillance by T and/or NK cells. The limited success of clinical immunotherapeutic efforts made so far against immunogenic tumors such as melanoma and cervical cancer may be partly explained by varied expression of tumor antigens and the immune selection that occurs after immunization (52–55). Certainly, the HLA class I tumor phenotype remains a very important parameter in relation to the anticipated patient-specific immune responses in any future investigation of natural immune responses or T cell-based immunotherapy in cervical or other types of cancer.

We conclude that the loss of HLA class I antigen expression in cervical cancer is widespread, heterogeneous, and predominantly results from irreversible genetic changes at chromosome 6p21.3, which may seriously affect natural immunity and/or therapeutic immune intervention.

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