Inhibition of HLA class I antigen and mRNA expression induced by Rous sarcoma virus in transformed human fibroblasts

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ABSTRACT Cells from various human nonlymphoreticular neoplasms show reduced HLA class I antigen expression. In this report, a system of human fibroblasts transformed by an avian retrovirus has been employed to investigate the mechanism of this phenomenon. Rous sarcoma virus has been used to transform in vitro human dermal fibroblasts, and clonal cell lines have been established from these cultures. In all the clones studied the integration of the provirus induced a reduction of cell-surface HLA-A, -B, -C framework antigen and β2-microglobulin expression when compared to levels for the respective parental fibroblasts. The reduction was correlated with a diminished intracellular synthesis of these molecules. Uninfected cells derived from an osteogenic sarcoma exhibited a reduced expression comparable to that of dermal diploid fibroblasts obtained from the same donor and transformed by Rous sarcoma virus. RNA gel blot analysis of total cellular RNA and of poly(A)+ cytoplasmic RNA showed a markedly decreased amount of HLA class I transcripts in the transformed cells. Southern blot study of genomic DNAs digested with several restriction endonucleases showed that the banding patterns of the HLA genes were not altered in the cells harboring the Rous sarcoma provirus. Our data are consistent with the hypothesis that the Rous sarcoma provirus that does not seem to be linked to the major histocompatibility complex class I gene superfamily may negatively control HLA gene expression.

Major histocompatibility complex (MHC) class I genes specify cell surface glycoproteins implicated in T-cell immunity (reviewed in ref. 1) as well as ill-defined nonimmunological functions (2), such as cell-to-cell contact, fibroblast adhesiveness (3, 4), and cell homing (5). Quantitative alterations of MHC class I antigen expression in neoplastic murine cells have been documented in several reports, and, in general, an inverse relationship between H-2 class I gene expression and malignancy has been observed (reviewed in ref. 6). In cells from a variety of human nonlymphoreticular tumors studied either on frozen sections from surgical specimens (7–11) or in cell lines established in culture (12–15), the attenuation or virtual absence of HLA class I antigens has also been described.

In this communication we show that clonal lines of human fibroblasts transformed by Rous sarcoma virus (RSV) had decreased cell-surface concentrations of HLA class I antigen and β2-microglobulin (β2m) when compared to the uninfected parental fibroblasts. In addition we show that infected and uninfected tumor cells derived from an osteogenic sarcoma exhibited reduced expression, which was similar to that of RSV-transformed fibroblasts from the same donor. The diminished expression of these antigens at the cell surface results from a decrease in the synthesis due, at least in part, to a reduction in the steady-state level of class I mRNA transcripts. No evidence of HLA genomic rearrangements or of hypermethylation was found in the cells harboring the provirus.

MATERIAL AND METHODS

Human Cell Lines. Clones of human diploid fibroblasts and cells isolated from sarcomas and transformed by RSV have been described (16, 17). BX is a cell line established from diploid dermal fibroblasts obtained by skin biopsy from a healthy donor. BX cells were infected and transformed with strain SR-RSV-2 (Schmidt–Ruppin RSV, subgroup B) resulting in BX(SR) cells. PAc is another cell line of diploid fibroblasts originating from a patient bearing an osteosarcoma. This cell line was also transformed by SR-RSV-2 and is designated PAc(PAs-SR). Cell line PAs is derived from the osteosarcoma of this patient. The sarcoma cells were "supertransformed" by SR-RSV-2. All the clones show evidence of integration of RSV provirus into the cellular genome and of transcription with synthesis of some of the viral proteins (17).

Immunological Assays. The following monoclonal antibodies (mAbs) were used: mAb W6/32 (18) and mAb B9.12.1 (19) react weakly with the invariant domain of HLA-A, -B, -C heavy-chain molecules and strongly with the native two-chain molecules; mAb HC-10 (20) reacts with purified heavy-chain HLA-A, -B, -C; mAb BIG6 (21) reacts with free and membrane-bound β2m.

Quantitative microabsorption on live cells was performed as described (22), and ELISA was according to Douillard et al. (23). The immunoperoxidase staining for electron microscopy using cells permeabilized with 0.5% saponin (24) was described by Ohitsuki et al. (25). Immunoprecipitation of 125I]methionine-labeled cells with various mAbs was according to Dobberstein et al. (26), and the immune complexes were analyzed under reducing conditions (27). For fluorescence flow cytometry, HLA-A, -B, -C antigens were quantitated at the cell surface by measuring the reactivity with various mAbs: W6/32, B9.12.1, and HC-10. Live cell suspensions (5 × 10^5 cells) were incubated with saturating concentrations of antibodies. Immunoblots were performed on protein preparations transferred to nitrocellulose filter according to Neefjes et al. (28).

Hybridization Analysis. For RNA gel blot hybridization, total cell RNA was isolated by the guanidinium isothiocyanate method (29); cytoplasmic poly(A)+ RNA was prepared as described by Greenberg (30) and analyzed in agarose gels containing formaldehyde (30). Southern blot analysis was performed as described (31). Hybridization and washing were under stringent conditions.

The following probes were used: pSRA-2, a full-length RSV provirus (32), psrc-2, a subgenomic probe for the src gene (32), pHLA-2 specific for HLA-B locus allantoigen (33), v-myc probe (34), human c-myc probe (35), γ-actin probe

Abbreviations: MHC, major histocompatibility complex; RSV, Rous sarcoma virus; SR-RSV-2, Schmidt–Ruppin RSV of subgroup B; mAb, monoclonal antibody; EBV, Epstein–Barr virus; FAU, fluorescence arbitrary units; β2m, β2-microglobulin.
trations. The absorbed supernatants were quantitatively required to lyse 95% of the target cells O1BB, was incubated 60 min with O1BB(EBV+) (O), BX (X), PAc ( ), BX(SR) cl.A-1 (O) and cl.C-6 (M), PAs (v), and PAc(PAs-SR) cl.C-5 (A) at various concentrations. The absorbed supernatants were then tested for residual cytotoxic activity using O1BB(EBV+) lymphoblastoid cells.

(36). DNA restriction endonucleases were used according to the recommendations of the manufacturer (Boehringer Mannheim).

RESULTS

RSV-Transformed Human Fibroblasts Express Reduced Amounts of Surface HLA Class I Antigens and $\beta_2$m. Quantitative microabsorption assays using mAb W6/32 showed that the RSV-transformed cells BX(SR) cl.A-1, BX(SR) cl.C-6, and PAc(PAs-SR) cl.C-5 absorbed far less antibody than the normal fibroblasts (BX, PAc, or lymphoblastoid cells O1BB [Epstein-Barr virus (EBV+)]). The uninfected sarcoma cells PAs also were found to absorb less antibodies than the normal fibroblasts PAc cells from the same donor (Fig. 1).

The surface distribution of class I molecules was analyzed by quantitative flow cytometry on live cells labeled with mAb W6/32 (Fig. 2A). A reduction in fluorescence intensity was found in the mAb-labeled transformed cells BX(SR) cl.A-1, PAc(PAs-SR) cl.C-5, and in the noninfected sarcoma cells PAs as compared to the normal diploid parental cells. A parallel reduction in cell surface $\beta_2$m was found by using mAb B1G6 (Fig. 2B).

The cytofluorometric values expressed as mean fluorescent arbitrary units (FAU) observed with the various mAbs are summarized in Table 1. The amount of antigen detected with mAb W6/32 on transformed fibroblasts BX(SR) cl.A-1, cl.C-6, and cl.G-9 was 11–24% of that present in uninfected diploid fibroblasts from the same donor, BX. A similar decrease was found in the transformed fibroblasts PAc(PAs-SR) cl.A-3, cl.B-5, cl.C-5, and cl.G-9 when compared to PAc, the parental diploid cells. In the uninfected sarcoma-derived cells PAs expression of class I antigen was 24% of that in PAc, the diploid fibroblasts derived from the same donor, and, after “supertransformation” of PAs cells with RSV, the mean values of FAU were unaltered. Comparable results were obtained with other mAbs with different specificities, i.e., mAbs B9.12.1 and HC-10. A parallel reduction of $\beta_2$m in the transformed cells as compared to PAc, the parental control cells, was found using mAb B1G6. Comparison by the quantitative ELISA (23) of a fixed number (2.5 $\times$ 10^4 cells) of uninfected “parental” cells (BX, PAc, or PAs) with transformed cells [BX(SR) cl.A-1, PAs(SR) cl.A-3, or PAs(SR) cl.B-1] in serial dilutions (from 1:4 to 1:1024) by using the anti-HLA class I mAb W6/32 showed reduced (30–60%) absorbance and thus reduced antigen on the transformed cells.

Reduced Synthesis of HLA Class I and $\beta_2$m Molecules in RSV-Transformed Fibroblasts. To determine whether the reduction in the expression of HLA class I molecules at the cell surface of the transformed cells is a result of deficient synthesis of these molecules, lysates of cells biosynthetically labeled with [35S]methionine were immunoprecipitated with various monoclonal antibodies, and the immunoprecipitates were analyzed by NaDodSO4/PAGE. By using mAb W6/32 (Fig. 3), HLA protein from cell membranes of BX(SR) cl.A-1 (lane C), BX(SR) cl.C-6 (lane D), and BX(SR) cl.G-9 (lane E) is reduced when compared to that from membranes of lymphoblastoid cells O1BB(EBV+) (lane A) and uninfected BX (lane B). Lane F is BX(SR) cl.G-9 treated with an
unrelated mouse ascites fluid. Transformed cells (Fig. 3, lanes C, D, and F) contain less of the 12-kDa βm than uninfected controls (Fig. 3, lanes A and B). Similar results were obtained when we compared the clones from PAc(PAs-SR) to the normal PAc fibroblasts (data not shown).

Total cell lysates from the cell lines were also analyzed by immunoblot, using mAb HC-10, specific for HLA-A, -B, -C framework heavy-chain antigen (20). Fig. 4 shows the 44-kDa band obtained from lysates of the normal fibroblasts BX (lane A) and the reduced bands from lysates of BX(SR) cl.A-1 (lane B), BX(SR) cl.A-6 (lane C), BX(SR) cl.G-9 (lane D), uninfected PAs (lane E), PAc(PAs-SR) cl.C-5 (lane F), PAc(PAs-SR) cl.B-5 (lane G), and PAc(PAs-SR) cl.A-3 (lane H). Thus the immunoprecipitation and immunoblot studies show that the transformed fibroblasts synthesized less HLA class I heavy-chain antigen and less associated βm than the normal diploid parental cells. These results were confirmed by immunoblot analysis using another HLA class I heavy-chain specific polyclonal antibody RaHC (20) (data not shown).

The amount of precipitable class I antigen in viral-transformed PAc cells corresponds to that of the uninfected PAs cells derived from sarcoma tissue from the same patient. Indeed, no difference can be observed between PAs, the uninfected sarcoma cells, and the various PAc clones harboring the integrated provirus.

The immuno electron microscopy results on permeabilized cells also corroborate these findings. With mAbs W6/32 or HC-10, uninfected cells showed a specific immunoperoxidase staining readily observed in the endoplasmic reticulum, especially the perinuclear cisternae and in the Golgi of practically all uninfected BX fibroblasts (Fig. 5A). Such intracytoplasmic staining was only rarely observed in the transformed BX(SR) cl.A-1 cells (Fig. 5B).

Reduced Levels of HLA Class I RNA in Whole Cells and of Poly(A)* RNA in Cytoplasmic Fractions of RSV-Transformed Human Fibroblasts. Since in transformed cells the reduction of cell-surface HLA class I antigen correlated with a significant reduction of immunoprecipitable peptide chains, the

![Fig. 3. NaDodSO4/PAGE analysis of normal and RSV-transformed cells metabolically labeled with 100 μCi of [35S]methionine (800 Ci/mmol; 1 Ci = 37 GBq) for 10 hr. Membrane preparations were incubated with mAb W6/32, and aliquots of precipitates containing equal amounts of acid-precipitable radioactivity were analyzed. Lymphoblastoid cells, O1BB(EBV*) (lane A); uninfected fibroblasts, BX (lane B); BX(SR) cl.A-1 (lane C); BX(SR) cl.C-6 (lane D); BX(SR) cl.G-9 (lane E); BX(SR) cl.G-9 with immunologically unrelated ascites fluid (lane F) are shown.](image)

![Fig. 4. Immunoblot analysis. Equivalent amounts of proteins from control and transformed cells (4 × 10⁶ cells) were separated by NaDodSO4/PAGE, and, after transfer, the filters were incubated with mAb HC-10, diluted 1:100, followed by peroxidase-conjugated anti-mouse immunoglobulin, diluted 1:2000. Total cell lysates from BX (lane A), BX(SR) cl.A-1 (lane B), BX(SR) cl.C-6 (lane C), BX(SR) cl.G-9 (lane D), PAs (lane E), PAc(PAs-SR) cl.C-5 (lane F), PAc(PAs-SR) cl.B-5 (lane G), and PAs(SR) cl.A-3 (lane H) are shown.](image)
level of HLA class I mRNA was investigated. Total cellular RNA from uninfected and transformed lines were analyzed by RNA gel blots with the probe for HLA-B locus allantigen (33). The band corresponding to the 1.8-kilobase (kb) transcript from uninfected BX fibroblasts is shown in Fig. 6A (lane J). The bands corresponding to the RNAs of the transformed or the uninfected PAs cells are either decreased or barely detectable (lanes A–I). The poly(A)⁺ RNA prepared from cytoplasmic fractions hybridized with the same probe for HLA class I is illustrated in Fig. 6B. A band corresponding to the 1.8-kb transcript can be observed in the control cells BX (lane J) but not in the transformed cells under the conditions of exposure (lanes A–I), and they are weakly demonstrable after prolonged exposure of the filters.

The bands obtained with the γ-actin probe as control are shown in Fig. 6C. No increase in transcription of v-myc or human MYC genes was observed in the transformed cells (data not shown). The reduced levels of class I HLA heavy-chain and β₂m transcripts in whole cells and in cytoplasmic fractions of transformed cells thus seem to result, at least in part, from impaired transcription.

To test whether this transcriptional inactivation might be due to an insertional mutation following the integration of RSV provirus within or near the HLA gene complex, DNA obtained from the various cell lines was digested with the restriction enzymes EcoRI, EcoRV, or BamHI, and the digests were hybridized with the HLA class I probe. The patterns of the EcoRI-digested DNA fragments from the RSV-transformed cells were similar to those of the uninfected cells (Fig. 7). Comparable results were obtained with EcoRV and BamHI restriction enzymes (data not shown). Thus it appears unlikely that a class I HLA genomic rearrangement is induced by viral integration. Moreover, blots of DNA from the various cell lines digested with Msp I or Hpa II were hybridized to the HLA class I probe. No evidence of hypermethylation was observed in the HLA genome of the transformed cells as compared to control cells (data not shown).

**DISCUSSION**

The present work was undertaken in an attempt to explore the mechanisms of HLA class I antigen suppression in human

![Fig. 5](image-url)  
**Immunoelectron microscopic localization of HLA class I molecules with mAb HC-10 followed by peroxidase-conjugated goat anti-mouse IgG on cells permeabilized with saponin. (A) Normal diploid fibroblasts BX. (B) BX(SR) cl.A-1. (×1000.)**

![Fig. 6](image-url)  
**RNA gel blot analysis. (A) Total RNA isolated from transformed and uninfected cells hybridized with HLA class I probe. Equal amounts (6 μg) of RNA were applied to each lane. To the left, sizes in kb of denatured DNA standards; the unlabeled markers correspond to 23S and 16S RNA standards. (B) Poly(A)⁺ RNA from 100 μg of cytoplasmic RNA hybridized with the HLA probe. (C) The filter in B rehybridized with the γ-actin probe. Lanes: A, PAc(PAs-SR) cl.A-1; B, PAc(PAs-SR) cl.A-3; C, PAs(SR) cl.B-1; D, PAs; E, PAs(PR) cl.E-3; F, PAs(PR) cl.G-8; G, BX(SR) cl.G-9; H, BX(SR) cl.C-6; I, BX(SR) cl.A-1; J, uninfected BX.**

![Fig. 7](image-url)  
**Southern blot of EcoRI-digested genomic DNA from transformed and uninfected cells, hybridized with HLA class I probe (A), γ-actin probe (B), and the human c-myc probe (C). Lanes: A, PAc(PAs-SR) cl.G-9; B, PAc(PAs-SR) cl.C-5; C, PAc(PAs-SR) cl.A-3; D, uninfected PAc; E, PAs(PR) cl.G-8; F, PAs(PR) cl.H-3; G, PAs(PR) cl.E-3; H, PAs(SR) cl.B-1; I, PAs(SR) cl.A-3; J, uninfected PAs; K, uninfected BX; L, BX(SR) cl.A-1; M, BX(SR) cl.C-6; N, uninfected BX.**
fibroblasts transformed by an oncogenic virus. Our observations are in agreement with those obtained in rat (37) and mouse (38) cells transformed by adenovirus type 12, where a decrease of MHC class I antigens and reduction of the corresponding mRNA were found. In mouse cells, the apparent absence of H-2 class I antigen seems to result from posttranscriptional controlling events (39).

In human lung small-cell carcinoma lines (15) a deficit of HLA-A,-B,-C antigens and of $\beta_2$m has been observed and was also related to a decrease of HLA class I mRNA synthesis. In human chorioncarcinoma (13) and neuroblastoma cells (14) decreased levels of HLA class I antigens were detected without reduction of $\beta_2$m. Such cell lines from naturally occurring tumors may not comprise the initial stages of malignant transformation, since they may have been subjected to immunoselection during tumor progression in vivo. Moreover the “normal” cells used as controls were not the progenitor cells (13, 14), and there is variation in the expression of HLA genes in cells from various tissues (40).

The possibility was considered that in RSV-transformed human cells the reactivity of HLA molecules might be attenuated by the protein tyrosine kinase present in the transformed cells. Indeed, it has been shown that purified HLA class I molecules can be phosphorylated in vitro by the purified chicken pp60$^\text{v-src}$ (41). However, in the BX(SR) cells metabolically labeled with $^{32}$P$_i$ phosphorylation in the tyrosine residues of class I molecules was not found (J. Strominger, personal communication).

Data from the literature (12–15) and from this work suggest that MHC class I antigen expression in neoplastic cells might be modulated by a variety of mechanisms probably depending on the nature of the transforming agent and more so on the type of cell of origin. In RSV-transformed human fibroblasts the decreased expression of HLA class I antigen seems, at least in part, to be the consequence of a down-regulation at the transcriptional level. However, in the absence of appropriate analysis, such as a nuclear run-on assay of the rate of transcription initiation, it cannot be excluded that the decreased expression of HLA class I antigens may result from nuclear posttranscriptional events, especially polyadenylation, or splicing.

The results presented here are not the consequence of inactivation of the HLA genes function by insertion mutation, since we have not found integration of the RSV provirus within the HLA class I gene complex. Furthermore, preliminary results of an in situ hybridization study have so far failed to show a localization of the RSV provirus on chromosome 6, the site of the HLA genes (A. Govaerts, B.T., and G.F.R., personal communication). In conclusion, the reduced expression of HLA class I genes in RSV-transformed human cells may result from a negative controlling factor acting on the transcriptional level, coded by a gene not linked to the MHC, which is possibly the RSV provirus.