SELECTIVE LOSS OF \( \beta_2 \)-MICROGLOBULIN mRNA IN HUMAN COLON CARCINOMA

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Human MHC class I antigens are composed of polymorphic HLA-A,B,C H chains noncovalently associated with an invariant L chain, \( \beta_2 \)-microglobulin (\( \beta_2m \)) (1). Cell surface expression of class I antigen requires the intracellular assembly of H and L chain (1).

CTLs can only kill tumor cells expressing a tumor-associated neo-antigen if these cells also express MHC class I antigens (2). A relationship between the loss of class I antigens and increased malignancy is suggested by some reports on class I expression in human neoplasms, including the eccrine porocarcinoma of the skin (3), B cell lymphoma (4), melanoma (5), and colon carcinoma (6).

Previous studies (6-8) have shown that colon carcinomas can lack the expression of the native HLA-A,B,C/\( \beta_2m \) complex in all tumor cells or in a proportion thereof. The question is unresolved, however, whether the basis of this defect is a coordinate loss of H chains and \( \beta_2m \), or whether the expression of one of the two subunits is selectively abrogated. We investigated the expression of uncomplexed H chain and \( \beta_2m \) in the colon carcinomas, which had been found to be deficient for the two-chain molecule in a previous screening of 200 cases (6, 7). The paper describes the exclusive loss of \( \beta_2m \) protein and mRNA by the tumor cells in all 15 carcinomas studied.

**Materials and Methods**

**Antibodies.** Mouse mAbs with the following specificities were used: W6/32 (9) and B.9.12.1 (10) against nonpolymorphic combinatorial determinants of the HLA-A,B,C/\( \beta_2m \) complex; BBM.1 (11) and B.1.G.6 (10) against free or complexed \( \beta_2m \); and HC-10 (12) against a determinant present on free HLA-B and HLA-C H chains. Additionally, a polyclonal rabbit antisemur (RaHC) recognizing free HLA-A, -B and -C H chains (12) was used. mAbs W6/32 and BBM.1, mAbs B.9.12.1 and B.1.G.6, and RaHC and mAb HC-10 were gifts of Dr. J. Johnson, Munich, Dr. F. Lemonnier, Marseille, and Dr. H. Ploegh, Amsterdam, respectively.

**Immunohistochemical Staining.** Immunohistochemical staining was performed as previously described (6). Briefly, acetone-fixed serial 5-\( \mu \)m frozen sections of selected colon carcinomas (a gift of Dr. P. Möller, Heidelberg) were incubated for 1 h with purified mouse mAbs at 10 \( \mu \)g/ml, diluted ascitic fluid in case of HC-10, or rabbit anti-H chain antisemur, respectively. The sections were subsequently incubated with rabbit anti-mouse IgG + IgM (a gift of Dr. G. Moldenhauer, Heidelberg), which was omitted in case of the rabbit antisemur, swine anti-rabbit IgG (Dako Corp., Copenhagen, Denmark), and peroxidase antiperoxidase complex (Dako Corp.) for 30 min each. Incubations were carried out in a humid chamber at room temperature followed by a 10-min wash with PBS. The binding of antibody was visual-
ized by incubating with aminoethylcarbazole/H$_2$O$_2$ substrate solution for 10 min. The sections were counterstained with hemalum and mounted.

**RNA Probes.** A 250-bp Pst I/Sac I fragment of HLA-B8 cDNA (a gift of Dr. E. Weiss, Munich) cloned into pGEM3 and a 600-bp Sac I/Hind III fragment of human $\beta_2$m cDNA (a gift of Dr. J. Burckhardt, Karlsruhe) cloned into pGEM7 were transcribed into $^{35}$S-ATP-labeled antisense RNA using the T7 promoter present in pGEM3 and pGEM7 according to the protocol recommended by the manufacturers (Promega Biotec, Heidelberg, FRG). The probes had a sp act of 10$^9$ cpm/µg input plasmid DNA (HLA-B8) and 1.2 x 10$^9$ cpm/µg ($\beta_2$m).

**In Situ Hybridization.** The hybridization was essentially carried out as described by Shivers et al. (13). Briefly, 5-µm frozen sections were placed on poly-L-lysine-coated glass slides, rapidly dried on a hot plate, and fixed for 15 min in PBS, 4% paraformaldehyde, 0.1% diethylpyrocarbonate. Pre-hybridization was done overnight at 20°C. For hybridization, sections were incubated for 65 h, at 37°C, with antisense RNA probe (2 x 10$^5$ cpm) in 30 µl hybridization buffer: 10 mM Tris (pH 7.5), 0.6 M NaCl, 1 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinyl-pyrrolidone 40, 0.02% BSA, 10% Dextran sulfate, 0.005% yeast tRNA, 0.05% herring sperm DNA, 0.005% poly(A), and 5 µM cold ribo- and deoxyribonucleotides, 0.05% sodium pyrophosphate (NaPPi), 10 mM L-methionine, 20 mM 2-ME, and 50% formamide. After a rinse in 2 x SSC, sections were digested with 20 µg/ml RNase A (Sigma Chemical Co., Munich, FRG) for 30 min, 37°C. Subsequently, there were 2 10-min washes at 20°C in 2 x SSC, 0.05% NaPPi, 1% sodium thiosulfate, 14 mM 2-ME followed by a 24-h wash (five changes) using 0.5 x SSC in the latter buffer. Sections were dehydrated, dipped in Kodak NTB2 nuclear track emulsion, and exposed for 14 d at 4°C.

**Results and Discussion**

**Immunohistological Stainings.** In an indirect immunoperoxidase technique on serial frozen sections, we used antibodies recognizing (a) the HLA-A,B,C H chain when complexed with $\beta_2$m; (b) free or complexed $\beta_2$m; or (c) free class I H chains, respectively (Table I). Using the antibodies W6/32 and B.9.12.1, which recognize HLA class I H chains only in the presence of $\beta_2$m, the tumor cells of the 15 colon carcinomas studied were negative, while non-neoplastic stromal cells intersecting the tumor islands were markedly stained (Fig. 1 A). Normal colon epithelium occasionally present adjacent to the tumor masses was always strongly positive for the two-chain complex (not shown). Stainings with antibodies BBM.1 and B.1.G.6 specific for $\beta_2$m paralleled the stainings with antibodies directed to the two-chain complex (Fig. 1 B), in-

| Table I | Immunohistological Stainings and In Situ Hybridizations |
|---------|---------------------------------------------------------|
| Colon carcinoma | Cell type | Immunohistology | In situ hybridization |
| mAb W6/32 and mAb B.9.12.1 (H chain/$\beta_2m$ complex) | mAb BBM.1 and mAb B.1.G.6 ($\beta_2m$) | mAb HC-10 and RaHC (free class I H chain) |
| No. 1-15 | Tumor cells | - | - | + |
| Stroma cells; normal epithelium | | + | + | + |
| No. 10, 13, and 15 | Tumor cells | - | - | + |
| Stroma cells; normal epithelium | | + | + | + |
Figure 1. Immunoperoxidase stainings of serial frozen tissue sections of a colon adenocarcinoma (case No. 15); counterstain of nuclei. (A) mAb W6/32 recognizing the class I H chain/β2m complex strongly stains the cells in the stroma intersecting the tumor while the pseudoglandular structures formed by the tumor cells (T) are entirely negative. (B) The staining for β2m with mAb BBM.1 is superimposable to the staining with W6/32, indicating the absence of free or bound β2m from the tumor cell population. (C) The tumor cell cytoplasm is strongly stained with mAb HC-10, indicating the presence of great amounts of free class I H chains. The stromal cells are more weakly labeled than the tumor cells. × 100.

dicating that in none of the cases detectable amounts of free β2m were present in the tumor cells. In contrast, antibodies to free H chains labeled the tumor cells intracellularly in all 15 cases (Fig. 1 C). Cellular components of the stroma and normal epithelial cells were also clearly stained for free H chains. The label of tumor cells, however, was stronger, which is suggestive for an accumulation of free H chains in the β2m-deficient tumor cells.
**In Situ Hybridizations.** In situ hybridizations were performed with three of these tumors in order to address the question of whether β2m mRNA was expressed. ³⁵S-labeled antisense RNA probes specific for β2m and HLA H chain were used in the hybridization procedure on formalin-fixed frozen sections. Both probes hybridized to non-neoplastic stromal cells that serve as an internal positive control for the reaction. In all three cases, the H chain probe strongly hybridized to the tumor cells, whereas the β2m probe labeled the tumor cells only at background levels (Fig. 2). As expected, normal colon epithelium was labeled with both β2m and H chain probes (not shown). By in situ hybridization, however, it cannot be decided whether this lack of β2m mRNA is due to down regulation of transcription or to the instability of the transcripts.

Our in situ analysis of colon carcinomas has to be discussed in the context of previous reports on the abrogation of MHC class I antigen expression by neoplastic cells in vitro. The Burkitt lymphoma cell line Daudi, which fails to express the H chain/β2m complex on the cell surface due to the complete absence of β2m protein, expresses β2m mRNA (14). This mRNA is, however, not functional due to a mutated initiation codon (15). The mutant mouse thymoma cell line R1E lacks β2m due to genetic defects in both β2m genes. Low levels of H-2K/D have been detected at the cell surface of R1E cells after transfection of the β2m gene (16, 17). In human small cell lung cancer (18) and melanoma cell lines (19), a coordinate low level of H chain and β2m transcription and translation has been found, which could be considerably increased by IFN treatment. The mouse teratocarcinoma cell line F9 has been found

![Figure 2](image-url)
to have a transcriptional block of both H chain and β2m mRNAs but transcription was achieved when differentiation was induced with retinoic acid (20). The choriocarcinoma cell line JaR (21) and certain neuroblastoma cell lines (22) provide examples for tumor cells that contain β2m in the absence of detectable HLA H chains.

Hence, a selective loss of β2m expression has only been reported for the in vitro cell lines Daudi and RIE. Our results, however, give evidence for a surprisingly high frequency of β2m-deficient human colon carcinomas. The detection of such β2m-deficient tumors in vivo excludes the objection that such defects leading to an HLA- phenotype of tumor cells arise artificially during long-term cell culture. Other human tumors that were reported for loss of the two-chain complex remain to be investigated for the presence of β2m and free H chain.

MHC class I deficiency may provide a selective advantage to tumor cells, since it may facilitate escape from antitumor immune attack mediated by cytotoxic T cells. According to present knowledge, β2m is required for post-translational processing and transport to the cell surface of all HLA-A, -B, and -C gene products (12, 23). Therefore, the exclusive abrogation of its expression appears to be a very efficient mechanism for eliminating the restriction elements required by cytotoxic T cells for the recognition and destruction of tumor cells.

Summary

Expression of MHC class I antigens requires the intracellular assembly of HLA-A, B, C H chains and β2-microglobulin (β2m). We have investigated the expression of free H chain, β2m, and their mRNAs in tissue sections of colon carcinomas that were defective for the native two-chain molecule. In all of these tumors, β2m protein and mRNAs were found to be completely absent from the neoplastic cells, whereas free H chains and their respective mRNAs were present in abundance in the tumor cell cytoplasm. The selective abrogation of β2m expression represents a unique mechanism leading to a complete loss of class I antigen surface expression in vivo.

We are grateful to Dr. P. Möller for his gift of the tumor specimens. We thank Mr. N. Haag and Ms. M. Kaiser for excellent technical assistance and Drs. G. J. Hämmerling, N. Koch, B. Kyewski, G. Moldenhauer, and J. Moreno for helpful discussions.

Received for publication 20 September 1988.

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