Brief Definitive Report

SELECTIVE LOSS OF β_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, β_2-microglobulin (β_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/β_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 β_2m, or whether the expression of one of the two subunits is selectively abrogated. We investigated the expression of uncomplexed H chain and β_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 β_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/β_2m complex; BBM.1 (11) and B.1.G.6 (10) against free or complexed β_2m; and HC-10 (12) against a determinant present on free HLA-B and HLA-C H chains. Additionally, a polyclonal rabbit antiserum (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.

Immunohistological Staining. Immunohistochemical staining was performed as previously described (6). Briefly, acetone-fixed serial 5-μm frozen sections of selected colon carcinomas (a gift of Dr. P. Möller, Heidelberg) were incubated for 1 h with purified mouse mAbs at 10 μg/ml, diluted ascitic fluid in case of HC-10, or rabbit anti-H chain antiserum, 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 antiserum, 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-

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ized by incubating with aminoethylcarbazole/H_2O_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 β_2m 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 10^8 cpm/μg input plasmid DNA (HLA-B8) and 1.2×10^8 cpm/μg (β_2m).

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 (2×10^5 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× 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× SSC, 0.05% NaPPi, 1% sodium thiosulfate, 14 mM 2-ME followed by a 24-h wash (five changes) using 0.5× 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 β_2m; (*b*) free or complexed β_2m; 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 β_2m, 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 β_2m paralleled the stainings with antibodies directed to the two-chain complex (Fig. 1 *B*), in-

TABLE I

Immunohistological Stainings and In Situ Hybridizations

		Immunohistology		
Colon carcinoma	Cell type	mAb W6/32 and mAb B.9.12.1 (H chain/β_2m complex)	mAb BBM.1 and mAb B.1.G.6 (β_2m)	mAb HC-10 and RaHC (free class I H chain)
No. 1-15	Tumor cells	–	–	+
	Stromal cells; normal epithelium	+	+	+
			In situ hybridization	
			Antisense RNA probe	
			β_2m	HLA class I H chain
No. 10, 13, and 15	Tumor cells		–	+
	Stromal cells; normal epithelium		+	+

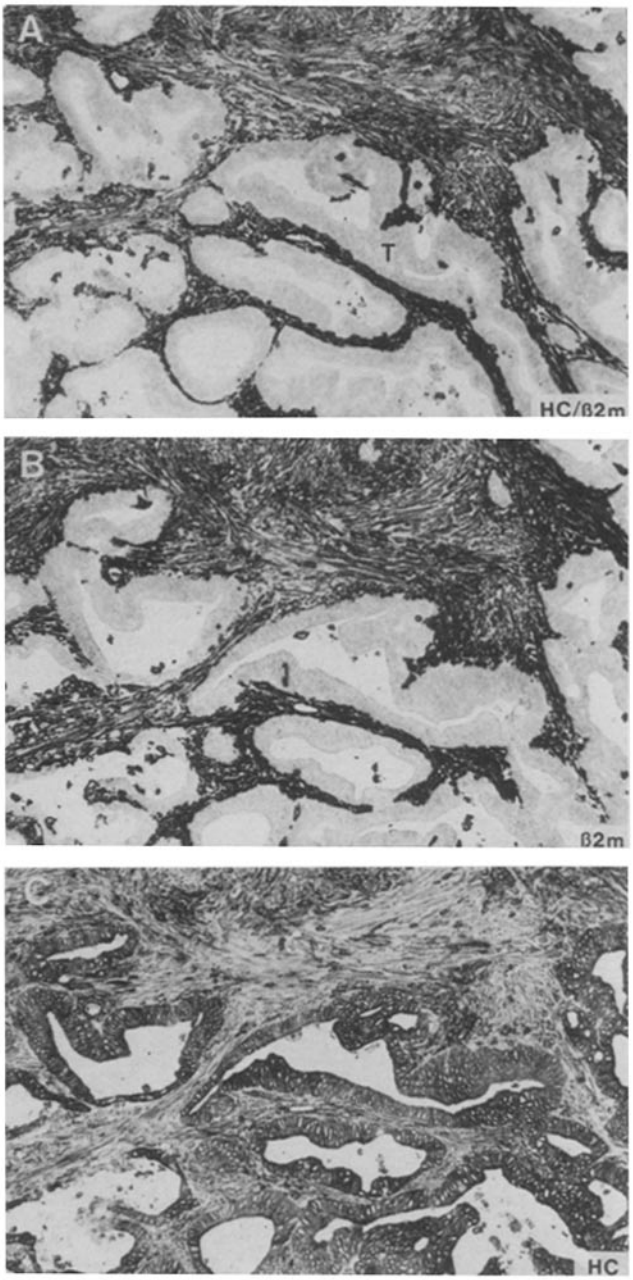

FIGURE 1. Immunoperoxidase stainings of serial frozen tissue sections of a colon adenocarcinoma (case No. 15); counterstain of nuclei. (*A*) mAb W6/32 recognizing the class I H chain/β_2m complex strongly stains the cells in the stroma intersecting the tumor while the pseudoglandular structures formed by the tumor cells (*T*) are entirely negative. (*B*) The staining for β_2m with mAb BBM.1 is superimposable to the staining with W6/32, indicating the absence of free or bound β_2m 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 β_2m 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 β_2m-deficient tumor cells.

In Situ Hybridizations. In situ hybridizations were performed with three of these tumors in order to address the question of whether β_2m mRNA was expressed. ^{35}S-labeled antisense RNA probes specific for β_2m 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 β_2m probe labeled the tumor cells only at background levels (Fig. 2). As expected, normal colon epithelium was labeled with both β_2m and H chain probes (not shown). By in situ hybridization, however, it cannot be decided whether this lack of β_2m 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/β_2m complex on the cell surface due to the complete absence of β_2m protein, expresses β_2m mRNA (14). This mRNA is, however, not functional due to a mutated initiation codon (15). The mutant mouse thymoma cell line R1E lacks β_2m due to genetic defects in both β_2m genes. Low levels of H-2K/D have been detected at the cell surface of R1E cells after transfection of the β_2m gene (16, 17). In human small cell lung cancer (18) and melanoma cell lines (19), a coordinate low level of H chain and β_2m transcription and translation has been found, which could be considerably increased by IFN treatment. The mouse teratocarcinoma cell line F9 has been found

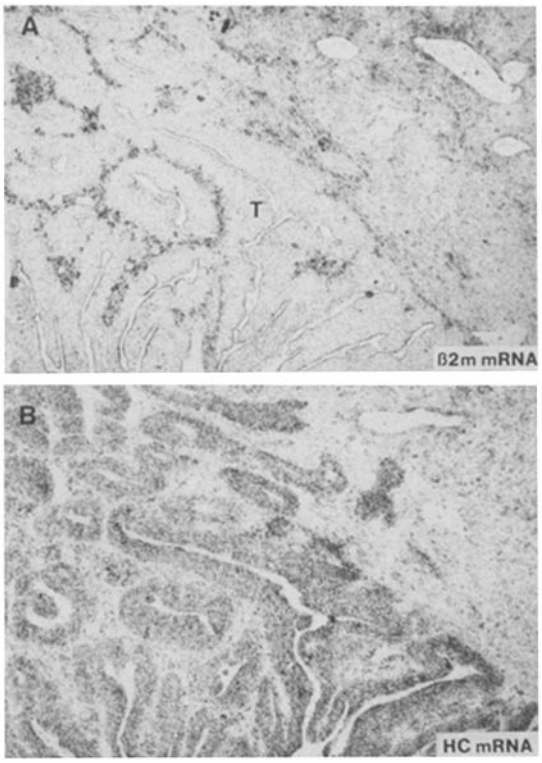

FIGURE 2. In situ hybridizations of serial frozen sections of colon carcinoma No. 15; no counterstain. Grains appear at the sites of tissue-bound ^{35}S-labeled antisense RNA probe. (*A*) The β_2m-specific probe hybridizes markedly to cells in the tumor stroma (compare with Fig. 1 *B*) while it binds to the tumor structures (*T*) only at background density as it was estimated by comparison with control sections pretreated with RNAse before hybridization (not shown). (*B*) In contrast, the HLA H chain-specific RNA probe strongly hybridizes to the carcinoma cells (compare with Fig. 1 *C*). Stromal cells are less densely labeled. × 60.

to have a transcriptional block of both H chain and β_2m 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 β_2m in the absence of detectable HLA H chains.

Hence, a selective loss of β_2m expression has only been reported for the in vitro cell lines Daudi and R1E. Our results, however, give evidence for a surprisingly high frequency of β_2m-deficient human colon carcinomas. The detection of such β_2m-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 β_2m 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, β_2m 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 β_2-microglobulin (β_2m). We have investigated the expression of free H chain, β_2m, and their mRNAs in tissue sections of colon carcinomas that were defective for the native two-chain molecule. In all of these tumors, β_2m protein and mRNA 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 β_2m expression represents a unique mechanism leading to a complete loss of class I antigen surface expression in vivo.

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