Brief Definitive Report

Loss of Transporter Protein, Encoded by the TAP-1 Gene, Is Highly Correlated with Loss of HLA Expression in Cervical Carcinomas

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Summary

Malignant tumor cells can escape $CD8^+$ cytotoxic T cell killing by downregulating class I major histocompatibility complex (MHC) expression. Stable class I MHC surface expression requires loading of the heavy chain/light chain dimer with antigenic peptide, which is delivered to class I MHC molecules in the endoplasmic reticulum by the presumed peptide transporter, encoded by the transporter associated with antigen presentation (*TAP*) *1* and *2* genes. We have investigated whether loss of class I MHC expression frequently observed in different cancers could result from interference with TAP function. A polyclonal antiserum, raised against a bacterial glutathione S-transferase/human TAP-1 fusion protein, was used for the immunohistochemical analysis of TAP-1 expression in 76 cervical carcinomas. Results showed loss of TAP-1 expression in neoplastic cells in 37 out of 76 carcinomas. Immunohistochemical double staining procedures in combination with HLA-specific antibodies revealed congruent loss at the single cell level of TAP-1 and HLA-A/B expression in 28 out of 37 carcinomas. The remaining samples expressed HLA(-A) in the absence of TAP-1 ($n = 6$) or showed loss of HLA(-A/B) while TAP-1 was expressed ($n = 3$). These data strongly indicate that inhibition of peptide transport by downregulation of TAP-1 is a potential strategy of malignant cells to evade immune surveillance.

The transporter associated with antigen presentation (TAP) is encoded by the *TAP-1* and *-2* genes (1, 2), located within the class II MHC region. Their products share structural properties with members of the superfamily of ATP-binding transporters (2, 3) and their function is to translocate antigenic peptide from the cytosol into the endoplasmic reticulum (ER). There, class I H chain, L chain (β_2-microglobulin), and peptide assemble into a complex which is then transported to the cell surface. Mutant cell lines (4, 5) or mice (6) lacking *TAP-1* and/or *-2* genes do not present antigen to $CD8^+$ T-cells and show strongly reduced levels of surface class I MHC molecules, indicating that peptide should be considered the essential third subunit of the class I complex (7–9). Upon viral infection or malignant cell transformation, the ensuing alternations in gene expression result in the generation of novel sets of peptides available for binding to class I MHC products. These are potential targets for $CD8^+$ CTLs (10, 11) and can play a pivotal role in the eradication of virally or transformed cells. Loss of class I MHC expression has been observed frequently in malignant cells of different origin (12) and in virally transformed cells (13). Such loss would allow these cells to escape from CTL-mediated killing and consequently increase their oncogenic potential. Downregulation of class I MHC surface expression is often related to transcriptional inhibition of H (14, 15) or L (16) chain gene expression. In virally infected cells, additional posttranscriptional mechanisms may result in the retention of class I MHC complexes in the ER, such as binding of H chains by the adenovirus E3 glycoprotein (17, 18) or binding of H and/or L chains by cytomegalovirus H301 (19) or other proteins (20) (Bijlmakers, M. J., T. Beersma, and H. L. Ploegh, unpublished observations).

Besides deprivation of H and/or L chains, the loss of peptide could also result in reduced class I MHC surface expression. Downregulation of class I MHC expression has been found in a substantial number of cervical carcinomas, containing human papilloma-virus type 16 DNA (21, 22). Since the presence of H chain and β_2-microglobulin transcripts was observed by RNA in situ hybridization in neoplastic cervical cells

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that show loss of class I MHC surface expression, a posttranscriptional regulation has been postulated (23). Loss of TAP function and the consequent failure to produce stable class I MHC molecules could be an explanation. Therefore in this study, class I MHC and TAP-1 expression were examined in 76 carcinomas of the uterine cervix by immunohistochemistry with class I H chain–specific mAbs and a polyclonal antiserum raised against a bacterial glutathione-S-transferase (GST)/human TAP-1 fusion protein. Double staining procedures were used to correlate the TAP-1 and class I MHC expression at the single cell level.

Materials and Methods

TAP-1 Immunoblotting. A rabbit antiserum raised against the TAP-1 COOH-terminal domain, fused to GST (Airey, J., M. T. Heemels, and H. L. Ploegh, unpublished observations) was used (1:1,000) to detect the TAP-1 protein in lymphoblastoid cells (LB), the human T-lymphoblastoid T1 cell line, and the TAP-1/-2–deficient T2 cell line. 5×10^5 cells of each cell line were lysed in SDS sample buffer, and their proteins separated by 10% SDS-PAGE and transferred to nitrocellulose. Incubation with a horseradish peroxidase–conjugated anti–rabbit antibody (1:1,000) followed by chemiluminescence (ECL system; all from Amersham International, Amersham, Bucks., UK) and exposure to film (X-AR 5; Eastman Kodak Co., Rochester, NY) were used to detect the antigen–antibody complex.

TAP-1/HLA Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues (n = 16) were obtained from patients attending the oncological gynecological outpatient department from the Free University Hospital for routine diagnostic and therapeutic procedures. Snap-frozen tissues (n = 60) were obtained from patients undergoing anesthesia before radiotherapy at the Christie Hospital. Immunohistochemical stainings were essentially performed as described (22). Briefly, 4-μm-thick paraffin sections were deparaffinized with xylene, rehydrated, and endogenous peroxidase was blocked by incubating for 30 min with methanol, containing 0.3% H_2O_2. After rinsing in PBS, pH 7.4, sections were pretreated as follows for the different primary antibodies: for HCA2 and rabbit anti–human TAP-1 sections were treated for 2 × 5 min at 95°C with Target Unmasking Fluid (Kreatech, Amsterdam, The Netherlands); for Pan Keratin (Dako Patts, Glostrup, Denmark), sections were digested for 30 min at 37°C with trypsin (0.5% wt/vol) in 0.5% $CaCl_2$ (pH 7.8); and for HC10, no pretreatment was required. Frozen sections (7 μm thick) were fixed for 10 min in acetone and immediately transferred to PBS, without further pretreatment.

After preincubation with the appropriate normal serum, sections were incubated with the following primary antibodies: for paraffin sections, mAb HC-A2 (1:500) recognizing preferentially HLA-A locus products (24); mAb HC10 (1:1,000), reactive with HLA-B and -C locus products (24); polyclonal antibody Pan Keratin (1:400), recognizing a broad spectrum of cytokeratins; and polyclonal rabbit anti–human TAP-1 serum (1:400); for frozen sections, mAbs B116-5-28 and B126-30 (1:5), specific for HLA-Bw4 and -Bw6, respectively (a generous gift from Dr. Gelsthorpe, National Blood Transfusion Service, Sheffield, UK); mAb W6/32 (1:20, Seralab Ltd., Sussex, UK), recognizing HLA-A, -B, and -C locus products, complexed to β_2-microglobulin; mAb 4E (1:10), binding to HLA-B, C and Aw19 (25); mAb HC10 (1:100); polyclonal rabbit anti–human TAP-1 serum (1:1,000); mAb LP34 (1:5) (Dako), specific for cytokeratins 6 and 18.

After incubation, sections were washed repeatedly in 4× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na-citrate), 1% (vol/vol) low-fat milk (for HC-A2 and rabbit anti–human Tap-1) or in PBS (HC10, Pan Keratin, B116, B126, W6/32, 4E, LP34). W6/32 and LP34 were detected in a two-step method with horseradish peroxidase, coupled to rabbit anti–mouse antibody (Dako). All other antibodies were detected in a three-step method, using biotinylated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA) and horseradish peroxidase, conjugated to streptavidin (Dako). Signals were visualized with diaminobenzidine (DAB) and H_2O_2, after which sections were counterstained with hematoxylin, dehydrated, and mounted in DePex (BDH, Poole, UK).

For immunohistochemical double stainings for TAP-1 and HLA-A and -B expression, sections were incubated simultaneously with rabbit anti–human TAP-1 antibody and HC-A2 or HC10, respectively. The bound TAP-1 polyclonal antibody was detected as described, with signal development with DAB/H_2O_2. The HLA-specific mAb was detected with a biotinylated rabbit anti–mouse Fab antibody fragment (Dako) and alkaline phosphatase, conjugated to streptavidin (Boehringer Mannheim, Mannheim, Germany). Signal was developed with naphthol AS-MX phosphate as substrate and Fast Blue BB as coupling agent in 0.2 M Tris-HCl (pH 8.5), according to the method of Mason and Sammons (26). Sections were not counterstained, and mounted in Kaiser's glycerin gelatin (E. Merck, Darmstadt, Germany).

Results were interpreted by three independent observers. The keratin-specific antibodies were used to confirm that carcinoma cells were of epithelial origin and to ascertain general epitope conservation. Loss of class I MHC or TAP-1 expression was confirmed in an additional experiment and was classified as positive, heterogenous, or negative, according to the criteria noted (see legend to Table 1).

Results and Discussion

Specificity of TAP-1 Serum. The rabbit anti–human TAP-1 serum recognizes a polypeptide of ~80 kD in immunoblots on cell extracts from a panel of EBV-transformed human lymphoblastoid cell lines (Fig. 1). Specificity of the serum for TAP-1 protein was demonstrated by the lack of reactivity in Western blots from T2 cells, which are devoid of TAP-1 protein (4, 5). Transfection with the TAP-1 cDNA restored reactivity with the anti-TAP-1 serum (27) (Heemels, M. T., and H. L. Ploegh, unpublished observations). Specificity of the immunocytochemical staining with the TAP-1 antiserum was further confirmed by the lack of staining of cytospins of TAP-1/-2–negative T2 cells (data not shown).

TAP-1 Immunohistochemistry. The serum was used for immunohistochemical detection of TAP-1 protein in formalin-fixed, paraffin-embedded (n = 16), and acetone-fixed, snap-frozen (n = 60) tissue sections of cervical carcinomas. Normal epithelial cells, present in the same section, and infiltrating cells of the immune system, always stained positively. In neoplastic cells, three different TAP-1 expression patterns could be observed. Complete loss of TAP-1 expression in virtually all neoplastic cells was observed in 4 out of 16 paraffin-embedded and 11 out of 60 frozen carcinomas. In 9 paraffin-embedded and 13 frozen carcinomas, heterogeneous loss of TAP-1 expression in neoplastic cells was found. Positively staining tumor areas could be observed adjacent to areas that clearly show loss of staining (Fig. 2 *A*). Staining for TAP-1

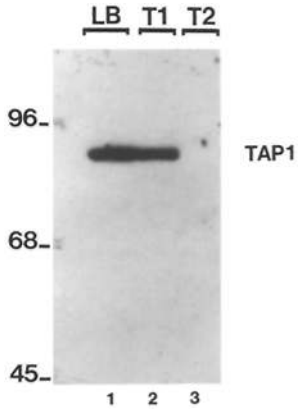

Figure 1. Immunoblotting of the TAP-1 subunit on cell extracts from the EBV-transformed cell line LB, the cell line T1, and its TAP-1- and -2-deficient variant T2 (29, 30). The complete absence in the T2 extract of the ~80 kD polypeptide corresponding to TAP-1 indicates the specificity of the antiserum.

protein was localized in the cytoplasm (Fig. 2 *B*), which is in accordance with the proposed ER localization of the TAP-1 subunit (28). The remaining three paraffin-embedded and 36 frozen carcinomas were uniformly positive for TAP-1 protein. Preliminary RNA in situ hybridization data employing a TAP-1–specific probe indicate that loss of staining for TAP-1 protein is related to reduced signals for TAP-1 mRNA (Cromme, F. V., and J. M. M. Walboomers, unpublished observations), further confirming that negative immunohistochemical staining with the TAP-1 serum represents reduced steady state levels of TAP-1 protein.

Correlation between TAP-1 and Class I MHC Expression. The different staining patterns for the TAP-1 protein in relation

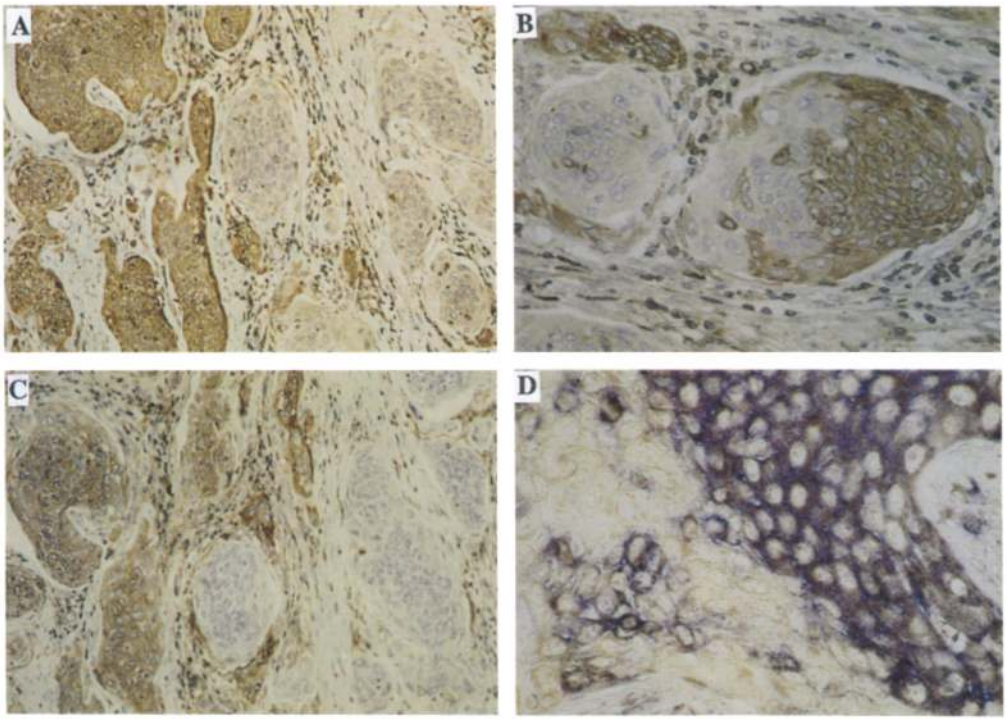

Figure 2. Immunohistochemical staining using rabbit anti-human TAP-1 serum (*A* and *B*), HC10 (HLA-B/C H chains; *C*), and double staining with rabbit anti-TAP-1 and HC10 (*D*) on paraffin-embedded tissue sections from a cervical carcinoma. (*A* and *B*) Low and high magnification, respectively, of staining for TAP-1 protein. Positive staining for TAP-1 is localized in the cytoplasm of some neoplastic areas (*brown*), whereas other adjacent areas clearly show loss of staining. (*C*) Staining for HLA-B locus products on the consecutive tissue sections shows that areas with loss of TAP-1 also exhibit loss of HLA-B expression. (*D*). Double staining with HC10 (*blue*) and rabbit anti-TAP-1 (*brown*). Membranous staining for HLA-B is observed only in neoplastic cells that also exhibit cytoplasmic staining for TAP-1. Remaining cells are negative for both antigens.

Table 1. *Patterns of Expression of TAP-1 and HLA in Cervical Carcinomas*

TAP phenotype		HC-A2			HC10			TAP phenotype		W6/32			HC10*		
Paraffin	16	+	+/−	−	+	+/−	−	Frozen	60	+	+/−	−	+	+/−	−
+	3	**3**	0	0	**3**	0	0	+	36	**36**	0	0	**33**	0	3
+/−	9	0	**7**	2	0	**9**	0	+/−	13	1	**11**	1	0	**11**	2
−	4	0	0	**4**	0	0	**4**	−	11	3	2	**6**	0	0	**11**

Summary of the immunohistochemical staining results for TAP-1 and HLA expression of 76 cervical carcinomas (73 squamous cell and 3 adenocarcinomas), as determined with antibodies HC-A2 (HLA-A), W6/32 (HLA-A/B/C), and HC10 (HLA-B/C). Three TAP-1/HLA phenotypes were recognized: positive (+), when virtually all cells are labeled; negative (−), when virtually all neoplastic cells show loss of staining as compared to normal epithelial and infiltrating immune cells in the same section; heterogeneous (+/−), when positively staining tumor areas were observed adjacent to negatively staining areas, usually between 25 and 75% of the neoplastic cells in a specimen. When the TAP-1 and HLA phenotypes are identical (*bold numbers*), this indicates congruency at the single cell level.

* Pattern confirmed with antibodies 4E, Bw4, and Bw6.

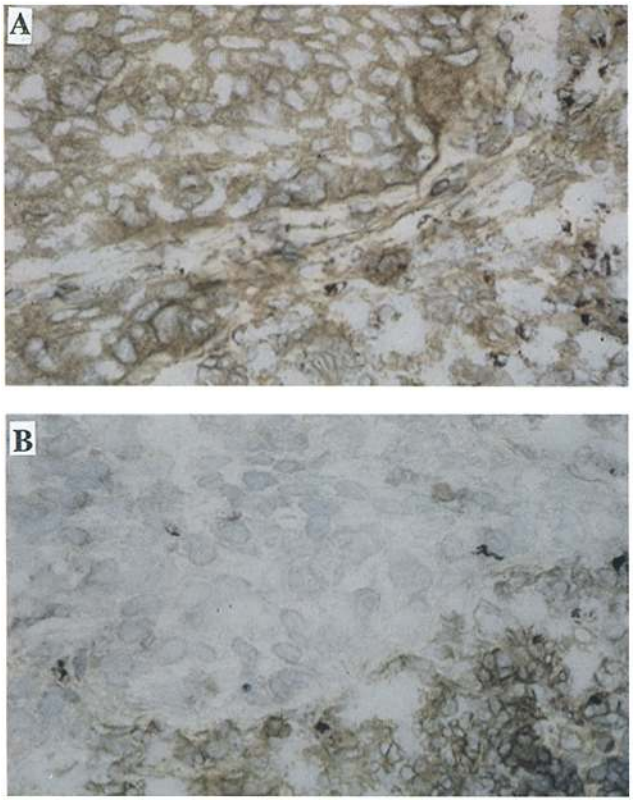

Figure 3. Immunohistochemical staining using W6/32 (HLA-A/B/C; *A*) and rabbit anti-TAP-1 (*B*) on consecutive tissue sections of a snap-frozen cervical carcinoma. (*A*) Staining with W6/32 clearly shows positive membranous staining of neoplastic cells and infiltrating immune cells. (*B*) Corresponding neoplastic area is negative for TAP-1 protein, whereas immune cells exhibit cytoplasmic staining.

to class I MHC expression are summarized in Table 1. Class I MHC expression was studied on consecutive tissue sections using HLA-A and -B H chain locus–specific mAbs (HC-A2 and HC10, respectively) in the paraffin-embedded specimens. Frozen tissues were analyzed using an antibody recognizing complexed HLA-A/B/C locus products (W6/32) and antibodies specific mainly for HLA-B/C H chains (HC10, 4E). In most cases, Bw4- and Bw6-specific antibodies confirmed the staining pattern obtained with HC10 and 4E, indicating that the latter two antibodies mainly react with B locus products in immunohistochemistry.

In general, the pattern of HLA expression was similar to that of TAP-1, as shown with HC10 in Fig. 2 *C*. Areas that show loss of staining for TAP-1 (Fig. 2 *A*) are also negative for HLA-B (Fig. 2 *C*). Immunohistochemical double staining procedures revealed a simultaneous loss of TAP-1 and HLA-B expression at the single cell level (Fig. 2 *D*). Staining for class I has predominantly a membranous localization (blue), whereas TAP-1 protein is detected in the cytoplasm of the same neoplastic cells (brown). From the 37 carcinomas that show heterogeneous or total loss of TAP-1 expression, HLA-B locus expression was congruently lost in 35 cases (Table 1, HC10 column). No examples of expression of HLA-B locus products with loss of TAP-1 protein were seen, indicating that cell surface expression of HLA-B alleles in vivo strongly depends on TAP functioning.

However, six carcinomas showed positive staining with W6/32 whereas TAP expression was lost (Table 1, i.e., four W6/32 positive and two W6/32 heterogenous carcinomas). A typical example is shown in Fig. 3. Neoplastic cells clearly exhibit positive staining with W6/32 (Fig. 3 *A*), whereas TAP-1 protein cannot be detected (Fig. 3 *B*). Since the identical area is also negative for HLA-B/C expression (data not shown), at least some HLA-A alleles can probably be stably expressed at the cell surface in the absence of TAP-1 protein in cervical carcinomas. HLA serotyping of these six patients revealed HLA-A2 positivity in three cases (Keating, P. J., F. V. Cromme, P. L. Stern, and P. A. Dyer, unpublished observations). This is in accordance with the residual HLA-A2 expression observed in the human T2 cell line, which is devoid of TAP-1 protein (29, 30). The expression of other HLA-A alleles in the remaining three lesions indicates that additional A locus products besides HLA-A2 can be stabilized in the absence of TAP-1.

Furthermore, eight carcinomas lack single cell congruency between TAP-1 and HLA expression, since they show reduced levels of HLA-A or -B expression while TAP-1 protein can be detected (Table 1, i.e., two HC-A2, one W6/32, and five HC10 negative cases). Because HLA-A and -B locus-specific transcripts could be detected in all carcinomas analyzed with RNA in situ hybridization (23), additional post-transcriptional mechanisms besides loss of TAP-1 might be involved that interfere with peptide loading of class I MHC molecules in the ER. These may include loss of peptide processing, loss of TAP-2, or lack of additional cytosolic factors, necessary for peptide translocation.

In conclusion, results from this study indicate that down-regulation of peptide transport may constitute a mechanism by which malignant cells escape killing by CTL. Absence of TAP-1/-2 expression accompanied by loss of class I MHC expression has recently been reported in small cell lung carcinoma cell lines (31). This report shows that this phenomenon occurs in human malignancies in situ. In addition, the observed high correlation between TAP-1 and class I MHC downregulation may to a large extent explain the post-transcriptional control of class I MHC expression frequently observed in cervical carcinomas. Preliminary results on lung (n = 10), colon (n = 5), and mammary (n = 5) carcinomas indicate that loss of immunoreactivity for TAP-1 is not only restricted to cervical carcinomas, but seems to be a more general phenomenon.

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