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# Tobacco Reduces Membrane HLA Class I That Is Restored by Transfection with Transporter Associated with Antigen Processing 1 cDNA[1]

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**HLA class I molecules are recognized by CTL that eliminate virally infected and malignantly transformed cells presenting foreign peptide—a process termed immunosurveillance. Many tumors have reduced levels of membrane HLA class I. Tumor cells with mutations that reduce HLA class I avoid immunosurveillance and continue to proliferate. As tobacco use can induce tumors, we examined the effect of tobacco extracts on membrane HLA class I. These studies show that culture of cells in media containing tobacco extracts reduces membrane HLA class I, but not other proteins, on primary keratinocytes and other cell types. Culture in tobacco extracts, but not extracts of other substances, reduces TAP1 protein, but does not reduce expression of HLA class I H chain, L chain, or the housekeeping protein $\beta$-actin. The reduction of TAP1 protein occurs within 4 h and is dose-dependent. Culture in tobacco extracts reduces TAP1 protein abundance, but not steady-state mRNA abundance. Tobacco-treated cells show defects in HLA class I biosynthesis similar to those found in TAP1-deficient cell lines. Transfection with TAP1 cDNA restores TAP1 protein abundance, HLA class I biosynthesis, and cell surface expression. Combined, these data show that culture in tobacco extracts reduces TAP1 protein abundance and membrane HLA class I levels. Reduction in membrane HLA class I could permit subsequent malignant transformation of cells to be undetected by the immune system.** ***The Journal of Immunology*****, 2002, 169: 6012–6019.**

Human leukocyte Ag class I molecules are composed of a trimolecular complex consisting of an H chain, an L chain, $\beta_2$-microglobulin ($\beta_2$-m)[4] (1), and an endogenously processed peptide selected from a large intracellular pool (1). H chain, $\beta_2$-m, and peptide are required for transport of mature HLA class I molecules to the cell surface (2, 3). Peptides are transported from the cytosol to the endoplasmic reticulum by a heterodimer, TAP1 and TAP2, (4) which is essential for membrane expression of HLA class I for most alleles (4, 5).

HLA class I molecules present peptides to roving CTL (6, 7) and cells, including tumor cells, presenting "foreign" peptides are eliminated. One mechanism to prevent CTL-mediated lysis of tumor cells is a reduction in the level of membrane HLA class I. For example, variants of a melanoma cell line with different levels of membrane HLA class I are lysed by CTL with different efficiencies; CTL effectiveness correlates directly with levels of HLA class I expression (8). Cormier et al. (9) demonstrated a dose-dependent correlation between levels of tumor-associated Ag, HLA class I, and CTL reactivity with a panel of melanoma cells. Interestingly, these two studies show that cells with intermediate levels of HLA class I molecules are killed with intermediate efficiency. This idea contradicts a widely held viewpoint that only large changes in HLA class I expression can reduce CTL-mediated killing.

Multiple cell lines are TAP-deficient (3, 10–12). HLA class I (or in murine cell lines MHC class I) assembly, maturation, and transit through the Golgi have been studied in TAP1-deficient cell lines. TAP-deficient cell lines show reduced assembly of mature HLA class I molecules compared with normal cells. The assembled molecules are less stable in TAP-deficient than in TAP normal cell lines. Fewer HLA class I molecules transit through the Golgi in TAP-deficient cell lines than in normal cells. As these cell lines are of different origins and species, combined these data show that TAP1-deficient cells have characteristic defects in all aspects of HLA class I biosynthesis (3, 10–12).

Numerous mechanisms have been associated with down-regulation of HLA class I molecules found in tumor cell lines. TAP1 and low m.w. protein (LMP) LMP2 are reduced in small cell lung carcinoma cell lines (11, 12) and transfection with TAP1, but not LMP2, restores membrane HLA class I (10). Renal carcinomas, metastatic cervical cancers, and melanomas have reduced TAP1 protein abundance with concomitant reductions in membrane HLA class I (13–15). Finally, in a murine model, Johnsen et al. (16) have shown that reductions in TAP are associated with decreased tumor surveillance and increased tumorigenesis. In all of these various models, loss or decrease in TAP1 protein abundance resulted in loss or decrease in HLA or MHC class I protein expression. In the TAP1-deficient tumor cell lines described above, TAP1 loss has been ascribed to random mutations followed by selection for the TAP1-reduced cells by CTL-mediated immunosurveillance (17).

Tobacco contains known carcinogens, but it is unclear which of the tobacco components contribute to carcinogenesis or how it

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[4] Abbreviations used in this paper: $\beta_2$-m, $\beta_2$-microglobulin; LMP, low m.w. protein; SCS, supplemented calf serum; MFI, mean fluorescence intensity.



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occurs (18). We asked whether carcinogen-containing tobacco extracts could alter the expression of membrane HLA class I in an in vitro system, where reductions in HLA class I expression would not be affected by outside selection pressure. In this study, we present evidence showing that tobacco extracts specifically reduce TAP1 protein abundance and membrane HLA class I. Importantly, tobacco-induced reductions in membrane HLA class I could prevent recognition of certain oncogenic changes by the immune system, thereby allowing transformed cells to become overt tumors.

## Materials and Methods

### *Cell culture and tobacco treatment*

HaCaT, a nontumorigenic keratinocyte cell line (19), 183, a tumorigenic cell line derived from head and neck squamous cell carcinoma (20), and HeLa were maintained in DMEM (Mediatech, Washington, DC) containing 10% supplemented bovine calf serum (SCS; HyClone Laboratories, Logan, UT). Jesthom, a B lymphocyte cell line, was maintained in RPMI 1640 (Mediatech) containing 10% SCS. Third pass primary oral keratinocytes were maintained in keratinocyte growth media (Biowhittaker/Clonetics, Rockland, ME; Ref. 21). Tobacco extracts were added at the concentrations and times indicated for each experiment. Extracts of cigarette, loose leaf, and snuff tobacco were used on multiple cell lines as noted.

### *Metabolic radiolabeling and immunoprecipitation*

Jesthom were treated with 0.2% final concentration of extract from snuff tobacco overnight. At 24 h, the cells were washed and resuspended in fresh RPMI 10% SCS. Metabolic radiolabeling was performed as described (22). Briefly, cells were starved for 1 h in $Met^-/Cys^-$ RPMI, followed by addition of 300 $\mu$Ci $^{35}S$ $Met^+/Cys^+$ radiolabeling mix (NEN, Boston, MA) per $5 \times 10^6$ cells and incubated for 2 h in a 37°C 7.5% $CO_2$ incubator (pulse). Following radiolabeling, cells were resuspended in nonradioactive RPMI-10% SCS and cultured for time periods indicated (chase).

Immunoprecipitation and gel electrophoresis was performed as described (22). For each sample, 2 $\mu$l of total lysate was spotted on Whatman filter paper (Whatman, Clifton, NY) before immunoprecipitation as a control for cell labeling. Images were exposed to a Phosphorscreen (Molecular Dynamics, San Jose, CA) quantified using ImageQuant software (Molecular Dynamics), and the amount of immunoprecipitated HLA class I was normalized to total labeling in each sample.

### *Transfection of tobacco-treated cells*

Jesthom were treated with tobacco extracts for 24 h. After treatment, an aliquot of treated and untreated cells were collected for flow cytometry and Western blot, the 0 time point. The remainder of the tobacco-treated cells were divided into the following groups: 1) treated, untransfected; 2) treated TAP1 transfected; and 3) treated vector transfected. The transfections were performed as described (23). Twenty-four hours after transfection, an aliquot of untreated and tobacco-treated cells from the three groups was collected for flow cytometry and Western blot. The untreated and tobacco-treated cells from the three groups were cultured in RPMI 10% SCS for an additional 24 h (a total of 48 h after transfection, 72 h after the start of the experiment). Then an aliquot from untreated and each group of tobacco-treated cells was collected for flow cytometry and Western blot while the remainder of the cells were metabolically radiolabeled, cultured for "chase" times, and immunoprecipitated.

### *Extract preparation*

Loose leaf tobacco (Pinkerton Tobacco, Owensboro, KY), snuff tobacco (Swisher International, Wheeling, WV), and dried leaf (Earl Gray Tea; McNulty's, New York, NY) extracts were prepared according to the method of Murrah et al. (24) by making a 10% (w/v) solution in water. Briefly, the leaves were stirred until thoroughly wetted, then allowed to soak for 2 h, stirring continuously at room temperature, followed by centrifugation to obtain the supernatant, and adjusting the pH to 7.4, with 10 N NaOH. The extracts were filtered through a 0.22-$\mu$ filter (Millipore, Bedford, MA). Cigarette tobacco extracts (Marlboro Phillip Morris, Richmond, VA) were made by removing the filter, slitting open the paper, removing the tobacco, and preparing a 10% (w/v) solution in water as described above. Lozenge extracts were prepared by gently crushing LifeSavers (Nabisco, East Hanover, NJ), weighing, and dissolving the powder in an appropriate amount of water to make a 10% solution. The pH was adjusted to 7.4 with 10 N NaOH and the solution filtered as described. Extracts were aliquoted in small batches and stored at −20°C until needed. Once the extracts were thawed, they were used immediately.

### *Flow cytometry*

Flow cytometry was performed as described (23). Briefly, cells were stained with CVC7, an anti-clatherin Ab as an isotype control (25), W6/32, against assembled HLA class I (CVC7 and W6/32 were prepared as supernatants from hybridoma cell lines obtained from American Type Culture Collection, Manassas, VA), followed by goat anti-mouse IgG-FITC (Fisher, Houston, TX). A total of 10,000 cells per group were analyzed on a FACsort using CellQuest (BD Biosciences, San Jose, CA), and dead cells were eliminated by propidium iodide uptake. For most experiments, the reduction in membrane HLA class I in tobacco-treated cells was normalized to the amount of HLA class I on control (untreated) cells.

### *Intracellular flow cytometry*

Cells were collected as appropriate and stained using the BD PharMingen intracellular flow staining kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were collected as appropriate and washed in 1 ml of wash buffer. Cells were stained with 4 $\mu$g/$\mu$l 7-aminoactinomycin D (Molecular Probes, Eugene, OR) for 1 min followed by a wash in 1 ml wash buffer. Cells were fixed using 100 $\mu$l cytofix/cytoperm at room temperature for 20 min, and washed in 500 $\mu$l perm/wash buffer. A total of 50 $\mu$l of supernatants of either 148.3, an anti-TAP1 mAb (13), or CVC7 as an isotype control and an equal amount of wash buffer were added and cells were incubated for 15 min on ice and washed as described. Cells were resuspended in 20 $\mu$l goat anti-mouse FITC and incubated on ice for 15 min. Flow cytometry was performed as described above using 7-aminoactinomycin D staining as an exclusion gate for live cells.

### *Western blot analysis*

Cells were treated with tobacco extracts for the times indicated, and protein collected by the methods described in Ref. 26. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 20 $\mu$g of each sample was analyzed by 10% SDS-PAGE (the linear range for both anti-TAP1 antisera and anti-$\beta$-actin covered 10–40 $\mu$g total protein loaded). Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Genie transfer apparatus (Idea Scientific, Minneapolis, MN). Western blot staining was performed using HC-10, an mAb against some HLA class I H chain alleles (27), BBM1, an mAb against $\beta_2$-m (23), anti-TAP1 antisera or an anti-actin mAb (Sigma-Aldrich, St. Louis, MO) followed by a goat anti-mouse or goat anti-rabbit HRP conjugate (Fisher). Blots were developed using Pierce SuperSignal West (Pierce, Rockford, IL) and exposed to film for appropriate periods of time. Western blots were scanned and analyzed using Un-Scan-IT software (Silk Scientific, Orem, UT).

### *RNA collection and Northern blots*

Total RNA was collected using RNAzol (Teltest, TX) as previously described (22), and samples were quantified from their OD. A total of 40 $\mu$g of each RNA was analyzed on a 1% formaldehyde-agarose gel (22). TAP1 mRNA was assessed using a cDNA probe for TAP1 (28) and a cDNA for 18S ribosomal RNA as a loading control (25). All probes were labeled using a random priming kit (Roche, Indianapolis, IN) as previously described (22). Membranes were hybridized in 6× SSC (29) at 55°C and washed in 0.1× SSC:1% SDS at 55°C for 1 h. Membranes were exposed to a Phosphorscreen and samples were quantitated using ImageQuant as previously described (22). For each experiment, the amount of TAP1 mRNA was normalized to the amount of 18S mRNA for each sample.

## Results

### *Physiologic use of tobacco extracts reduced membrane HLA class I*

Tumors associated with tobacco use have reduced membrane HLA class I. To identify the mechanism causing reduced HLA class I expression, we tested the ability of tobacco extracts to reduce HLA class I on cultured cells using indirect flow cytometry. Continuous culture in media containing different preparations of tobacco extracts reduced membrane HLA class I on primary keratinocytes and a variety of cell lines (Jesthom, HaCaT, HeLa, and 183) by up to 70% (Fig. 1 and Table I). The result is more dramatic on homogenous cell lines than on the heterogeneous primary keratinocyte cell population, but a portion of primary keratinocytes responded to tobacco by reducing membrane HLA class I (Fig. 1).

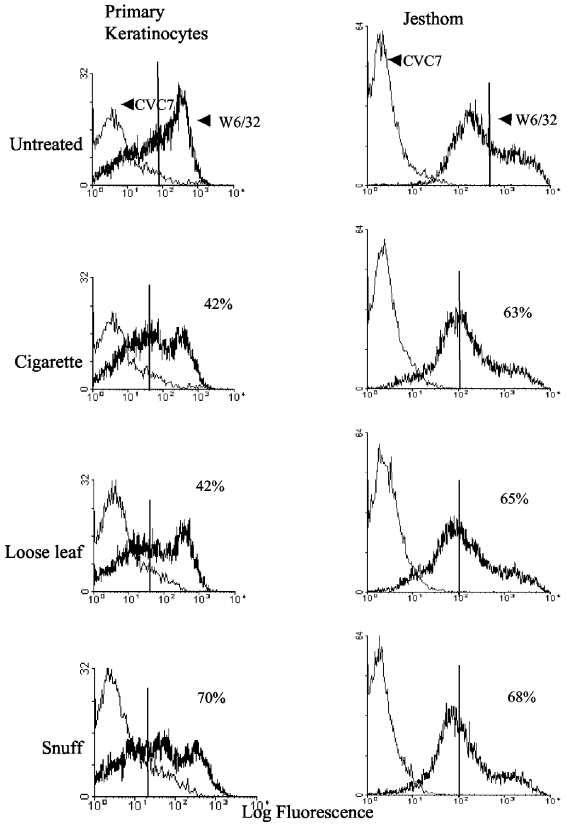


**FIGURE 1.** Tobacco extracts reduce membrane HLA class I levels. Cells were cultured overnight in media containing each tobacco extract. The fluorescence distribution of the isotype control mAb, CVC7 (thin line), and the HLA class binding mAb, W6/32 (heavy line), is shown on primary keratinocytes (*left panels*) and Jesthom (*right panels*). *First row*, Binding to untreated (control) cells; *second row*, cells treated with cigarette tobacco extracts; *third row*, cells treated with loose leaf tobacco extracts; and *bottom row*, cells treated with snuff tobacco extract. The vertical line indicates the geometric mean for each population. For primary keratinocytes, the means are 71, 41, 40, and 21 for control, cigarette, loose leaf, and snuff, respectively. For Jesthom, the means are 402, 150, 143, and 134 for control, cigarette, loose leaf, and snuff, respectively. The percentage of reduction from the control is indicated by the number for each tobacco extract. Primary keratinocytes have been examined three times, Jesthom have been examined four times.

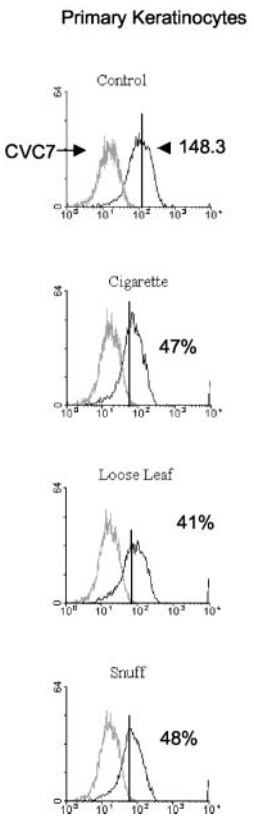


**FIGURE 2.** Tobacco extracts reduce TAP1 protein abundance. Primary keratinocytes were cultured with tobacco extracts for 20 h and stained for intracellular flow cytometry using the TAP1-specific mAb 148.3 (heavy line) or the isotype control Ab CVC7 (thin line). *Top panel*, The fluorescence distribution on untreated cells; *second panel*, cigarette tobacco extract-treated cells; *third panel*, loose leaf tobacco-treated cells; and *bottom panel*, snuff tobacco-treated cells. The percentage of reduction in TAP1 is indicated for each extract. The means are 121, 64, 71, and 63 for control, cigarette, loose leaf, and snuff, respectively. This experiment has been repeated twice.

The expression of membrane Ig (mean fluorescence intensity (MFI) for untreated, cigarette, loose leaf, and snuff treated are 22, 21, 27, 25, respectively) or $\beta_1$ integrin (MFIs of 69, 65, 66, 71, same order as above) were not reduced by treatment with any tobacco extract. We also tested discontinuous culture (1 h per day) of cells in tobacco extracts and saw similar reductions in HLA class I after 3 h of treatment (data not shown). Combined, these data show that extracts of tobacco reduced HLA class I, but not other membrane proteins, in primary keratinocytes and several cell lines in a physiologically relevant manner.

### *Tobacco extracts reduce the abundance of TAP1 protein*

As multiple proteins are required for proper assembly of HLA class I molecules, treatment with tobacco extract could reduce

Table I. *Percentage of reduction on membrane and intracellular protein abundance by extracts of cigarette, loose leaf, and snuff tobacco*[a]

| Cell Line | mClass I[b] | | | TAP1 | | |
|---|---|---|---|---|---|---|
| | Cigarette | Loose leaf | Snuff | Cigarette | Loose leaf | Snuff |
| HaCaT | 50 | 44 | 65 | 30 | 29 | 33 |
| 183 | 56 | 55 | 57 | 40 | 40 | 33 |
| HeLa | 57 | 50 | 39 | N.T.[b] | N.T. | N.T. |
| Jesthom | 63 | 64 | 68 | N.T. | 39 | 40 |
| Primary keratinocytes | 42 | 42 | 70 | 45 | 39 | 46 |
| Average ± SD[c] | 55 ± 5 | 53 ± 7 | 61 ± 14 | 38 ± 8 | 33 ± 8 | 38 ± 6 |

[a] For each cell population data are expressed as the percentage of reduction of membrane expression of HLA class I or intracellular abundance of TAP1 in tobacco-treated compared to control cells. Data are compiled from multiple experiments. All data were generated by flow cytometry, TAP1 data were generated by intracellular flow cytometry.
[b] mClass I, membrane class I; N.T., Not tested.
[c] For mClass I and TAP1 the overall reduction of each extract on different cell populations was averaged and SDs were generated.

membrane HLA class I by directly reducing the abundance of H chain and/or $\beta_2$-m or by reducing abundance of one of the other proteins involved in HLA class I assembly. To examine the mechanism underlying the tobacco-induced reduction in HLA class I, protein levels of HLA class I H chain, $\beta_2$-m, TAP1, TAP2, LMP2, LMP7, and the housekeeping protein $\beta$-actin were analyzed by intracellular flow cytometry and Western blot. Culture in media containing extracts of cigarette, loose leaf, and snuff tobacco reduced the protein abundance of TAP1 by 30–55% in primary keratinocytes and several cell lines analyzed by intracellular flow cytometry (Fig. 2 and Table I). These results were confirmed by Western blot analysis and show that TAP1 protein levels declined by 4 h (Fig. 3, *a* and *b*), but longer incubation times did not cause further reductions in TAP1 (Fig. 3, *c* and *d*). As dried leaf mixtures contain a myriad of compounds, one possibility was that other dried leaf preparations would have similar affects. We examined the ability of a preparation of tea to reduce TAP1 (Fig. 4). In multiple experiments, other dried leaf extracts had no affect on TAP1 protein abundance, or as predicted on membrane HLA class I expression (data not shown). Thus, these data show that only dried tobacco leaf preparations reduce TAP1 protein abundance. Although culture in media containing tobacco extract reduces TAP1, it did not reduce HLA class I H chain, $\beta_2$-m, or $\beta$-actin proteins even after 24 h of incubation (Fig. 3, *a–d*). Furthermore, tobacco extracts did not reduce protein levels for TAP2, LMP2, or LMP7 (data not shown). These data show that extracts from three different preparations of cigarette, loose leaf, and snuff, tobacco but not other dried leaf preparations, reduced TAP1 protein abundance and membrane HLA class I rapidly and specifically in primary keratinocytes and several different cell lines.

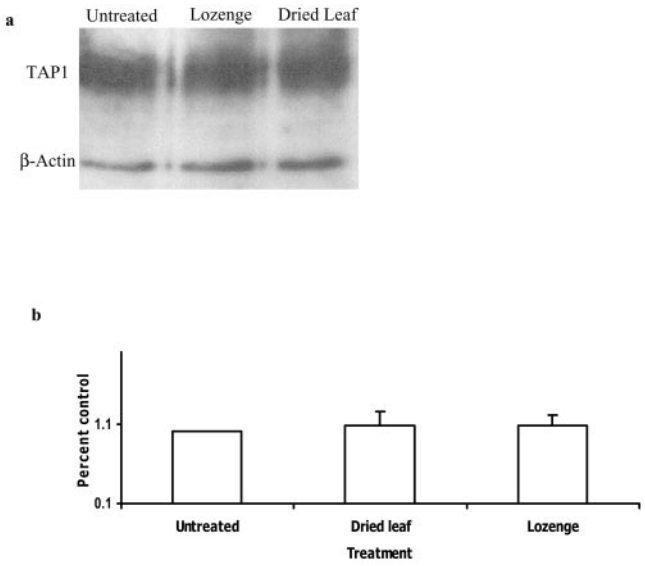


**FIGURE 4.** Tobacco extract, but not extracts of other substances, specifically reduces TAP1 protein abundance. *a*, Jesthom were cultured for 24 h with extracts of dried leaf or lozenge. Western blots show the levels of TAP1 and $\beta$-actin. *b*, Densitometry showing the mean and SD of TAP1 protein normalized to $\beta$-actin and expressed as percentage of control. This experiment was performed three times.

### *The reduction in TAP1 is dose-dependent*

Jesthom were cultured in different doses of snuff tobacco extract and TAP1 protein abundance was analyzed by Western blot. The

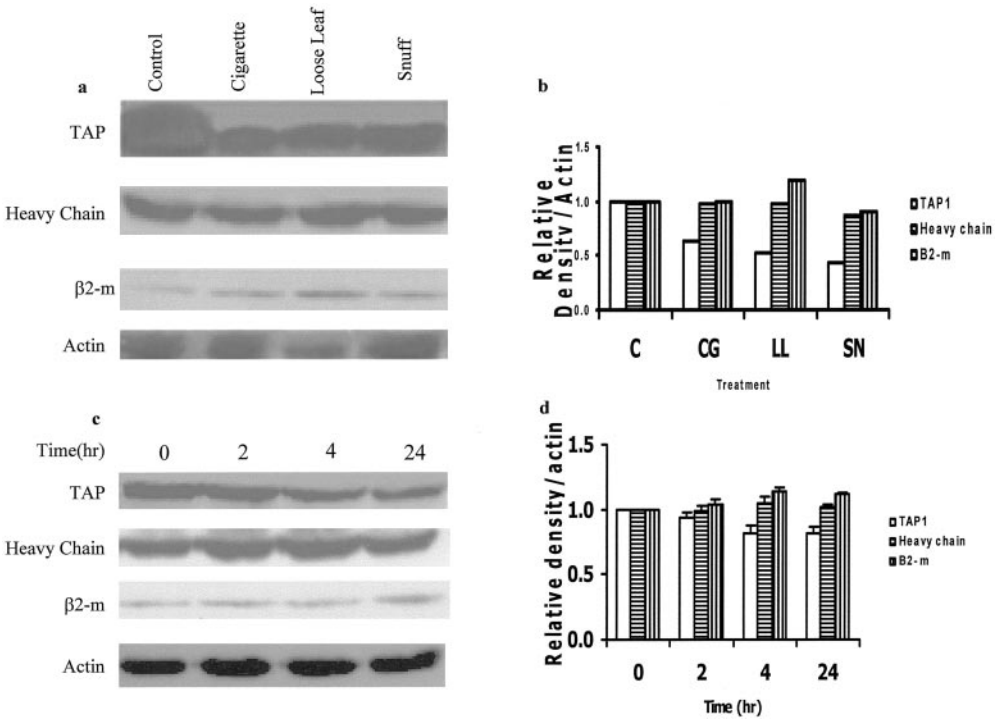


**FIGURE 3.** Tobacco extracts specifically reduce TAP1 protein abundance within 4 h. *a*, Western blot of HaCaT treated for 4 h with cigarette, loose leaf, or snuff extract. *Top panel*, TAP1, *second panel*, HLA class I H chain; *third panel*, $\beta_2$-m; and *bottom panel*, the housekeeping protein $\beta$-actin. *b*, Densitometry showing the relative levels of TAP1, HLA class I H chain and $\beta$-$_2$m normalized to $\beta$-actin and expressed as percentage of untreated cells. This experiment was performed twice. *c*, Western blot of Jesthom treated with snuff tobacco extract for 2, 4, and 24 h. Proteins are shown in the same order as in *a*. *d*, Densitometry showing mean and SD of the relative levels of TAP1, HLA class I H chain, and $\beta_2$-m normalized to $\beta$-actin and expressed as percentage of untreated cells. This experiment was performed four times.

~30% reduction in TAP1 is saturated by 0.01%; higher doses did not cause additional reductions (Fig. 5). Similar results were seen with culture in different doses of cigarette and loose leaf (data not shown). The half maximal dose for reduction by snuff tobacco extract is ~0.05%. Multiple experiments show that culture in 0.4% tobacco extract markedly reduces cell viability especially during longer incubations (data not shown). Based on these data, we have performed our experiments using a saturating 0.2% tobacco extract.

*Tobacco does not alter TAP1 mRNA abundance*

Decreased protein abundance can result from changes in turnover of the protein itself or by a decrease in steady-state mRNA abundance (30). To determine which of these mechanisms is responsible for the decrease in TAP1 protein abundance, cells were cultured in tobacco extract for the times indicated, total RNA was collected and analyzed by Northern blot. Treatment with tobacco extract did not reduce TAP1 mRNA steady-state abundance, even after 24 h in culture (Fig. 6). Thus, these data show that culture in tobacco extract reduced TAP1 protein abundance by a mechanism that does not involve altering mRNA abundance.

*Culture in tobacco reduced assembly, stability, and maturation of HLA class I molecules*

In other systems, loss of TAP1 causes characteristic reductions in assembly, stability, and maturation of HLA class I (10–12). To determine whether the tobacco-induced reduction in TAP1 causes similar changes in biosynthesis of HLA class I molecules, Jesthom were treated with tobacco extract to reduce TAP1 protein levels. Two days later, cells were metabolically radiolabeled followed by 6 h of nonradioactive chase. Aliquots of cells were removed at the time periods indicated and immunoprecipitated using the mAb W6/32, which only recognizes fully assembled HLA class I molecules, followed by gel electrophoresis. In untreated cells, the amount of the W6/32 recognizable form of HLA class I increased during the chase time. These data are consistent with previous findings showing that HLA class I molecules are stable for ~24 h (25). In tobacco-treated cells, the amount of the W6/32 recognizable HLA class I was similar to that of control cells at the beginning of the experiment, but instead of continued assembly, HLA class I decreased over time (compare the Endo $H^-$ lanes within each gel in Fig. 7, *a*, *b*, and *d*). When normalized to total cell labeling, there was a 2-fold increase in HLA class I in untreated Jesthom compared with a 30% reduction in HLA class I in tobacco-treated cells (Fig. 7*d*). These data show that HLA class I was less stable in tobacco-treated cells.

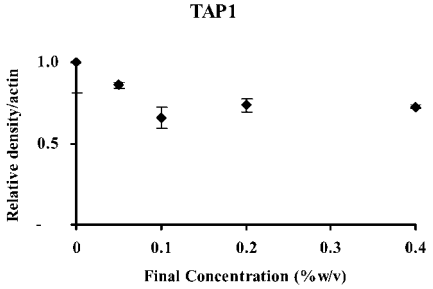


**FIGURE 5.** The effect of tobacco extract is dose dependence. Jesthom were treated with snuff extract and analyzed by Western blot. Densitometry showing the mean and SD of TAP1 protein normalized to $\beta$-actin and expressed as percentage of control is shown. The *x*-axis shows the final concentration of snuff extract. This experiment was repeated four times.

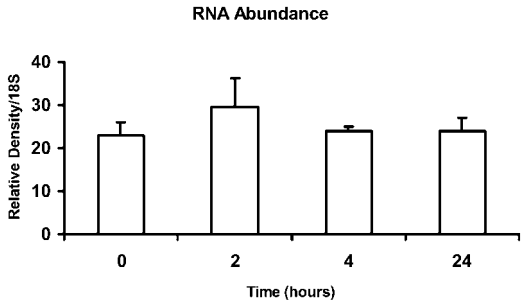


**FIGURE 6.** Tobacco extracts do not modify the steady-state abundance of TAP1 mRNA. HeLa cells were cultured in media containing tobacco extract for the times indicated. The zero time point indicates control (untreated) cells. Densitometry analysis of four separate Northern blots is shown as the mean and SE of TAP1 mRNA normalized to 18S mRNA.

The carbohydrate moieties on HLA class I become resistant to cleavage by Endo H after modification in the Golgi; molecules residing in the endoplasmic reticulum remain Endo H sensitive (31). Treatment with Endo H shows that the amount of HLA class I that moves into the Golgi increased 2-fold during the chase period in untreated cells (compare the Endo $H^+$ lanes within each gel of Fig. 7, *a*, *b*, and *e*). In contrast, the Endo H-resistant form of HLA class I in tobacco-treated cells increased by 60% at 2 h, but decreased to baseline at longer times (Fig. 7, *a*, *b*, and *e*). Thus, HLA class I in tobacco-treated cells did not remain in an assembled form that can mature over longer times. Combined, these data show that tobacco treatment altered the biosynthesis of HLA class I by reducing assembly, stability, and maturation through the Golgi. In summary, these data show that cells with tobacco-induced reductions in TAP1 have defects in HLA class I biosynthesis comparable to TAP1-deficient cell lines (10–12).

*Transfection with TAP1 cDNA restores HLA class I assembly, stability, maturation, and cell surface expression*

If the alterations in HLA class I biosynthesis resulted from the decrease in TAP1, then restoring TAP1 protein abundance should restore HLA class I biosynthesis. We treated cells with tobacco extract to reduce TAP1 protein levels (day 0). Tobacco-treated cells were then transfected with TAP1 cDNA or the vector pRS-Vneo DNA (day 1). Two days later (day 3), metabolic radiolabeling and immunoprecipitation were done as described above. Transfection with TAP1 cDNA restored HLA class I assembly (compare the Endo $H^-$ lanes in Fig. 7, *a–c*, and *d*. Because the HLA class I remained in a form recognized by W6/32, these data show that the stability of HLA class I was also restored (Fig. 7, *c* and *d*). There was a 3-fold increase in the Endo H-resistant form of HLA class I in the TAP1-transfected cells over the 6-h chase period (Fig. 7, *c* and *e*). Thus, these data show that HLA class I maturation through the Golgi is restored by transfection with TAP1 cDNA (Fig. 7, *c–e*). Transfection with TAP1 cDNA also restored TAP1 protein abundance and membrane HLA class I as measured by Western blot and flow cytometry, respectively (Fig. 8). These data show that transfection with TAP1 restored HLA class I assembly, stability, and maturation through the Golgi.

## Discussion

Tobacco use is associated with many different tumor types including non-small cell lung carcinomas, head and neck squamous cell carcinomas, renal carcinomas, and cervical carcinomas (18). Many of the same tumor types exhibit reduced membrane HLA class I (7). The current dogma is that random mutations occur reducing

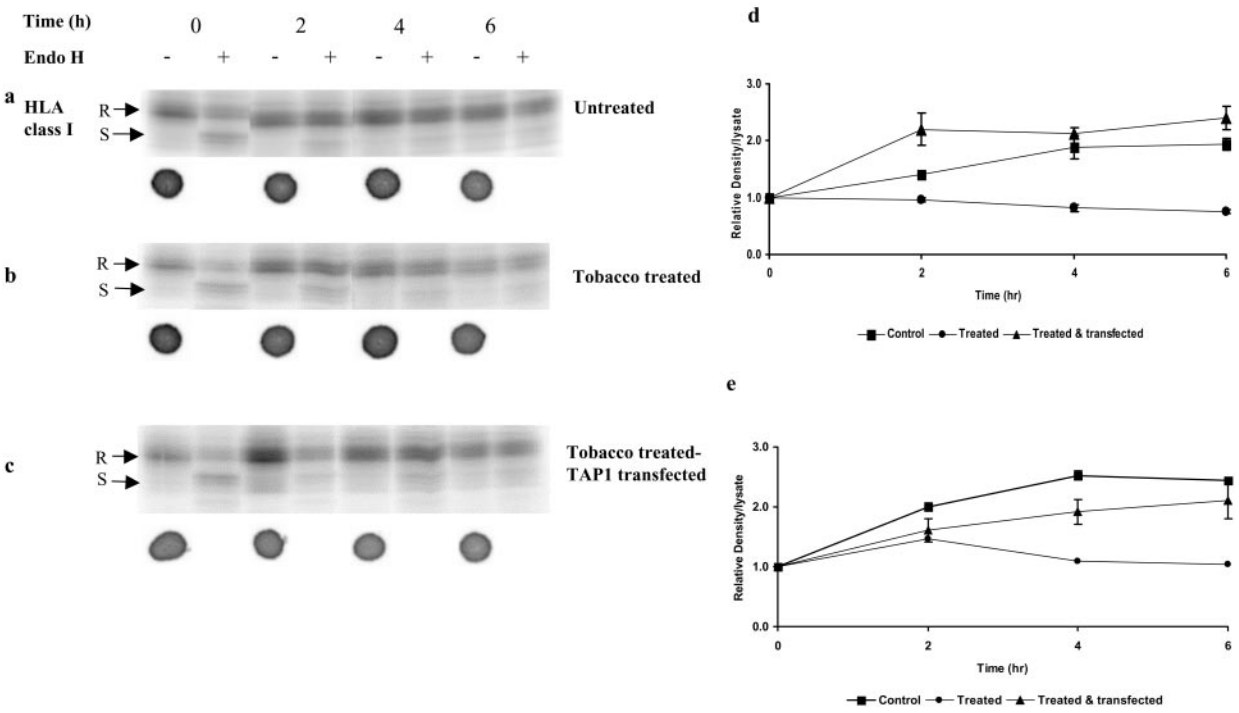


**FIGURE 7.** Transfection with TAP1 cDNA restores HLA class I biosynthesis. Jesthom were cultured in media with or without snuff tobacco extract and treated cells were divided into treated, untransfected, or transfected with TAP1 cDNA. SDS-PAGE gel showing W6/32 immunoprecipitation of assembled HLA class I over time (indicated at the *top*) in *a*, untreated; *b*, tobacco-treated; and *c*, tobacco-treated TAP1-transfected cells. Two microliters of total cell lysate was spotted on the membrane for each sample as a control for labeling efficiency (shown below each gel). After immunoprecipitation, samples were split and half were treated with Endo H as indicated. The Endo H-resistant (R) and -sensitive (S) forms of HLA class I are indicated. *d*, Densitometry of these gels showing the mean and SD of the amount of total HLA class I (normalized to total cell labeling) at each time point. *e*, Densitometry of these gels showing the mean and SD of the amount of the Endo H-resistant form of HLA class I (normalized to total cell labeling) at each time point. Graph is labeled the same as in *d*. This experiment was repeated three times with similar results.

membrane HLA class I and confer a survival advantage to these mutant cells (32). In this study, using flow cytometry, we present evidence showing that extracts of cigarette, loose leaf, and snuff tobacco specifically reduce membrane HLA class I, but not other membrane proteins including membrane Ig and $\beta_1$ integrin. Multiple cell populations of markedly different origins are susceptible to tobacco-induced reductions in membrane HLA class I (Fig. 1 and Table I). As HLA class I is reduced by three different preparations of tobacco extracts in multiple cell populations, including primary keratinocytes, it is unlikely that the reduction in HLA class I caused by the tobacco extracts results from a unique characteristic of a specific cell type. Because water soluble extracts from cigarettes, loose leaf, and snuff reduced membrane HLA class I, these data show that the effect is not preparation-dependent. Tobacco does not need to be pyrolized to be effective. Finally, our data also show that discontinuous incubation (treatment for 1 h per day) in tobacco extract reduced membrane HLA class I. Because Jesthom have a doubling time of ~20 h, these data show that the affect of tobacco extract persists beyond mitosis. This result is supported by the transfection experiments (Fig. 7), where transfected cells were cultured for an additional 72 h post tobacco treatment and did not regain membrane HLA class I. Thus, short-term exposure to tobacco could promote relatively long-term alterations in HLA class I expression. Combined, these data show that physiologic use of tobacco extracts reduce HLA class I.

Assembly of HLA class I molecules requires multiple proteins including the TAP heterodimer (33) that transports peptides from the cytosol to the endoplasmic reticulum. In the endoplasmic reticulum, peptides combine with nascent HLA class I H chain:$\beta_2$-m polypeptides. Peptide is required for stable assembly and transport to the cell surface (3). Using flow cytometry, intracellular flow cytometry, and Western blot analysis, tobacco extracts were examined for the ability to reduce membrane Ig, $\beta_1$ integrin, membrane HLA class I, HLA class I H chain, $\beta_2$-m, TAP1, TAP2, LMP2, and LMP7 protein abundance. Tobacco extracts reduce TAP1 protein abundance within 4 h (Fig. 2 and Table I), but have no affect on HLA class I H chain, $\beta_2$-m, TAP2, LMP2, or LMP7 protein abundance even after 24 h in culture (Fig. 3 and data not shown). Tobacco treatment does not alter membrane Ig, $\beta_1$ integrin, or $\beta$-actin protein abundance even after 24 h of treatment (Fig. 3), showing that tobacco extracts are not generally repressing cellular metabolism. The two different techniques, Western blot and intracellular flow cytometry, were performed using a polyclonal rabbit antisera and a murine mAb, respectively. Although these Ab preparations were generated against different epitopes, the results are similar, strongly suggesting that the reductions in TAP1 protein abundance are not the result of a hidden or altered epitope.

Tobacco is a highly complex mixture (18) containing heavy metals, nitrates, alkaloids, polyphenols, carbonyl compounds, and carcinogens as a partial list (34). Tobacco is a plant, and dried preparations of tobacco will contain cellulose, proteins, nucleic acids, carbohydrates, and lipids. Extracts of cigarette, loose leaf, and snuff tobacco, but not extracts of other dried leaf or lozenge, reduce TAP1 but not $\beta$-actin protein abundance, showing that the effect is specific to tobacco (among the additives tested; Fig. 4). Tobacco extracts exert a dose-dependent effect that saturates at 0.1% (Fig. 5), while higher doses can cause considerable cell death. A saturating 0.2% final concentration of all extracts was used for our experiments. We tested several common compounds

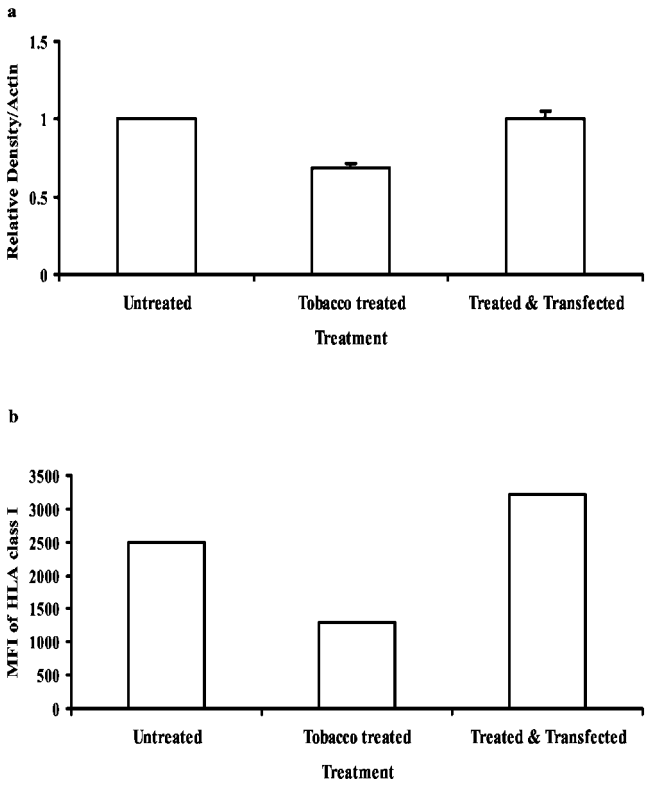


**FIGURE 8.** Transfection with TAP 1 cDNA restores TAP1 protein abundance and membrane expression of HLA class I. *a*, Densitometry of the mean and SE of TAP1 normalized to $\beta$-actin and expressed as percentage of untreated cells from four experiments. *b*, MFI of flow cytometry of membrane HLA class I using the mAbW6/32. The isotype control Ab, CVC7, had an MFI of 7 in this experiment. This experiment was repeated three times with similar results.

including nicotine, cancer-associated nitrosamines, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butone/*N*′-nitrosonornicotine, and an active metabolite methyldiazonium ion over a wide range of concentrations ($1 \times 10^{-3}$–$1 \times 10^{-9}$ M) by culturing Jesthom for 24 h followed by flow cytometry. None of the tobacco components reduced membrane HLA class I levels (data not shown). Thus, future studies on the identification of the substance in tobacco will likely require testing fractions and subfractions for the ability to reduce membrane HLA class I.

Tobacco-induced changes in transcription levels of TAP1 could underlie the loss of HLA class I which would allow a cell to escape immunosurveillance. We examined mRNA steady-state abundance (30) after treatment with tobacco extract using Northern blot analysis. These data show that tobacco extracts do not reduce TAP1 mRNA steady-state abundance (Fig. 6). Thus, tobacco extracts reduce TAP1 protein abundance without altering RNA levels, suggesting that tobacco acts directly on TAP1 proteins.

HLA class I biosynthesis is disrupted in multiple TAP1-deficient cell lines (10, 35, 36). To determine whether the tobacco-mediated reduction in TAP1 caused similar changes in HLA class I biosynthesis, we compared assembly, maturation, and stability in tobacco-treated and untreated cells (Fig. 7). These data show a characteristic reduction in HLA class I assembly and stability in tobacco-treated vs nontreated cells. In tobacco-treated cells, fewer HLA class I molecules reach the Golgi compared with nontreated cells. To demonstrate that the reduction in TAP1 protein abundance was directly responsible for the reduction in HLA class I expression, either TAP1 cDNA or vector DNA were transfected into tobacco-treated cells. Transfection with TAP1 cDNA restores HLA class I biosynthesis. This result also supports our conclusions that tobacco extracts do not reduce HLA class I H chain, $\beta_2$-m, TAP2, LMP2, or LMP7. Thus, the mechanism by which tobacco putatively causes a loss of immunosurveillance includes suppression of membrane HLA class I through a reduction in TAP1 and not some intermediate molecule.

There is a correlation between loss of membrane HLA class I and loss of TAP proteins in several different systems. Cell lines derived from human melanomas have reduced TAP1 protein abundance with a concomitant reduction in membrane HLA class I (15). A cell line derived from a human small cell lung carcinoma also has reduced TAP1 protein abundance and membrane HLA class I, and transfecting TAP1 restores membrane HLA class I (10). In a murine system, Johnsen et al. (16) have shown that tumor cells deficient in TAP1 with reduced H-2 cell surface expression can avoid CTL-mediated immunosurveillance and are highly tumorigenic. In all of these systems, reduction in TAP1 is associated with a reduction in membrane HLA class I (or H-2), and when tested, a loss of immunosurveillance.

The mechanisms underlying the initial reduction in TAP1 protein abundance are unknown. Previously, it was assumed that decreases in membrane HLA class I are due to random mutations; cells with reduced HLA class I are selected because they can evade CTL-mediated immunosurveillance (16). Our novel findings show that tobacco extracts specifically reduce membrane HLA class I by reducing TAP1 protein abundance. As tobacco extracts reduce membrane HLA class I in vitro, these data show that the loss of HLA class I cell surface expression does not result from selection pressure. To the best of our knowledge, this is the first demonstration that a nonbiological reagent can cause reductions in membrane HLA class I. We speculate that the reduction in TAP1 protein abundance with the concomitant reduction in membrane HLA class I could permit cells to avoid immunosurveillance. The HLA class I-reduced population of cells would then be able to undergo tobacco-driven oncogenic changes and form tumors without being detected by the immune system.

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