

INDUCTION OF EXPRESSION OF MHC-CLASS-II ANTIGEN ON HUMAN THYROID CARCINOMA BY WILD-TYPE *p53*

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Mutation of the tumor-suppressor gene *p53* is involved in carcinogenesis. We investigated the role of *p53* in the induction of anti-tumor immune responses by establishing a thyroid carcinoma cell line (1F3) prepared by transfection of wild-type human *p53* gene into a *p53*-deficient cell line (FRO). Our results showed for the first time the involvement of *p53* in the induction of anti-tumor immune responses, as demonstrated by: (i) expression of the major-histocompatibility-complex(MHC)-class-II antigen on 1F3, but not FRO; (ii) mRNA of class-II gene was expressed both in 1F3 and in FRO, but was stable at post-transcriptional level in FRO, which restrained protein synthesis; (iii) 1F3 induced MHC-class-II-specific CD4⁺ cytotoxic-T-cell activity through allo-antigen presentation and co-stimulation. Although our novel results are limited to the wild-type-*p53*-expressing clone from a *p53*-deficient cell line, we suggest that the absence of *p53* in carcinoma cells may reduce the induction of CD4⁺ cytotoxic-T-cell activity against carcinoma cells by diminishing the expression of class-II antigen. *Int. J. Cancer* 75:391–395, 1998.
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Mutation of *p53*, a tumor-suppressor gene, is frequently observed in a variety of human neoplasms, and is associated with loss of differentiation and uncontrolled tumor growth. It is most evident in thyroid carcinomas, since *p53* mutation is exclusively present in poorly differentiated thyroid carcinomas and confers aggressive properties in these tumors (Fagin *et al.*, 1993). On the other hand, inactivation of *p53* leads to loss of expression of thyroid-specific genes as well as determination or maintenance of differentiation of thyroid tumors (Fagin, 1995; Fagin *et al.*, 1996).

An important regulator of tumor growth is cellular immunity. Antigen recognition by T cells occurs in the context of the major-histocompatibility-complex (MHC) products, namely, clusters of differentiated (CD) 4⁺ helper or cytotoxic T cells and CD8⁺ cytotoxic T cells recognize processed antigenic peptides presented by the MHC-class-II and -class-I molecules respectively. In thyroid carcinomas, weak expression of class-II antigen may account for the limited engagement of immunocompetent cells, including cytotoxic T cells, against thyroid-tumor cells (Betterle *et al.*, 1991; Lahat *et al.*, 1992).

In the present study, we used 2 types of cell lines; a poorly differentiated human follicular-thyroid-carcinoma cell line (FRO) with marginal or no expression of *p53* (Fagin *et al.*, 1993), and a locally established cell line (1F3) transfected with cDNA of wild-type *p53* into FRO. The latter cell line expressed normal *p53* (Fagin, 1995; Fagin *et al.*, 1996). Our results showed that the loss of *p53*-tumor-suppressor gene in thyroid-carcinoma cells reduced class-II expression on these cells and resulted in the escape of cells from T-cell killing.

MATERIAL AND METHODS

Cell lines and cell culture

FRO, NPA, 1F3, FRO-c and 1N20 cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% FCS (GIBCO, Grand Island, NY) and 400 µg/ml of geneticin (Sigma, St. Louis, MO) for 1F3 and FRO-c cells or 600 µg/ml of geneticin for 1N20 cells in 100-mm tissue-culture dishes (Costar, Cambridge, MA). In

one group the cells were pre-treated with IFN-γ (300 U/ml; Shionogi, Osaka, Japan) for 24 hours.

FRO is a poorly differentiated human follicular-thyroid-carcinoma cell line with marginal or no expression of *p53* and thyroid peroxidase mRNA (Fagin *et al.*, 1993) while 1F3 cell line was prepared locally by transfection of FRO with cDNA of wild-type *p53* using the wild-type-*p53*-expression vector pC53SN3 derived from the expression vector pCMV-Neo-Bam. 1F3 cells express normal *p53* and a weak but detectable level of thyroid peroxidase mRNA (Fagin, 1995; Fagin *et al.*, 1996). In the present study, FRO represented a *p53*-deficient cell line, based on absence of *p53* mutations in exons 5 to 8, and the presence of markedly low levels of *p53* mRNA. These characteristics suggest the presence of a structural abnormality either of *p53* itself or of some factor(s) controlling its expression as reported by Fagin *et al.* (1993). A further confirmation of the lack of *p53* in FRO cells was provided by the absence of reactivity with *p53* protein using anti-*p53* monoclonal antibody (MAB) (Fig. 1). We also prepared a mock (expression vector pCMV-Neo-Bam only)-transfected cell line (termed FRO-c) by using lipofectin (GIBCO-BRL, Gaithersburg, MD) and geneticin. This cell line was used in flow-cytometric analysis as a negative control (see below). NPA is a poorly differentiated human papillary-thyroid-carcinoma cell line with a heterozygous *p53* mutation and absence of thyroid peroxidase mRNA (Fagin *et al.*, 1993). 1N20 was a locally established cell line prepared by transfection of NPA cells with cDNA of wild-type *p53* using the wild-type-*p53*-expression vector pC53SN3. 1N20 expressed normal *p53* and thyroid peroxidase protein (Fagin, 1995; Fagin *et al.*, 1996).

FACScan flow-cytometry analysis

Staining and flow-cytometric analyses of FRO, FRO-c and 1F3 cells were performed using standard procedures described by Tanaka *et al.* (1995). Staining of cells with MABs [anti-DR MAB HU-4, anti-DP MAB B7/21, anti-DQ MAB HU-11, HU-18 or HU-46, anti-intercellular adhesion molecule-1 (ICAM-1) MAB 84H10 and control MAB thy1] was detected using FITC-labeled anti-mouse IgG antibodies and FACScan (Becton Dickinson, Mountain View, CA). Amplification of the MAB binding was provided by a 3-decade logarithmic amplifier. MABs for DR, DP and DQ antigens were prepared by one of the investigators (Y.N.), while MAB for ICAM-1 was prepared by another (Y.T.).

Western-blot analysis

Cell lysate (100 µg) isolated from each of FRO and 1F3 cells was electrophoresed in a 10% (for class II) or 7.5% (for *p53*) SDS-polyacrylamide gel and followed by blotting onto a nitrocellu-

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lose membrane filter (Toyo Roshi, Tokyo, Japan). In the next step, we added a mouse MAb against the common framework of human class II ($\times 1$), which was established by Y. Tanaka and Dr. PE. Auron, or 1 $\mu\text{g/ml}$ of mouse MAb against human p53 (PAb 1801; PharMingen, San Diego, CA), as the first antibody, followed by the addition of the second antibody [horseradish-peroxidase-conjugated F(ab')₂ fragment of sheep anti-mouse Ig antibody ($\times 1/500$ or $\times 1/2500$ respectively (Amersham Life Science, Tokyo, Japan)]. Finally, the protein was detected by enhanced chemiluminescence system (Amersham).

Immunoprecipitation

After pre-incubation of cultured FRO and 1F3 cells (80–90% confluent monolayer in 100-mm tissue-culture dishes; Costar, Cambridge, MA) in methionine-free RPMI 1640 medium (GIBCO) for 2 hr, the cells were labeled with [³⁵S]methionine (Amersham) for 18 hr. Before terminating the labelling process, we added 10 $\mu\text{g/ml}$ of cycloheximide (Sigma) for 3 or 6 hr. Immunoprecipitation was performed using 1.0 μg of mouse MAb against the common framework of human class-II (similar to Western-blot analyses) and 20 μl of agarose conjugate (protein A/G plus-agarose; Santa Cruz Biotechnology, Santa Cruz, CA). The size of each sample was adjusted by the cell number; roughly equal amounts of total protein derived from cell lysates were electrophoresed in each lane. Thereafter, the pellets were washed and electrophoresed in 10% SDS-polyacrylamide gel and followed by drying of the gel using a gel dryer (Bio-Rad, Hercules, CA) and analysis using a Bioimage Analyser (BAS-2000; Fuji Film, Tokyo, Japan).

Northern-blot analyses

Total cellular RNA was isolated from FRO and 1F3 cells by an acid-guanidinium-phenol-chloroform method using Isogen (Nippon Gene, Tokyo, Japan). In the IFN- γ -treated group, the cells were pre-treated as described above. The total RNA (10 μg) was electrophoresed in 1% agarose gel and transferred to a nylon membrane filter (Amersham). The blots were hybridized with cDNA probes of class II (HLA-DR and DP) labeled with [α -³²P]dCTP (Amersham). cDNA probes of class II were prepared by one author (A.K.). Cyclophilin was used as a housekeeping gene.

Changes in HLA-DP/cyclophilin mRNA level after treatment with 5,6-dichlorobenzimidazole riboside (DRB)

After pre-incubation in serum-free RPMI 1640 medium for 20 hr, we treated FRO and 1F3 cells with 25 $\mu\text{g/ml}$ DRB (Sigma) for 3 or 6 hr. In the next step, Northern blotting was performed using the protocol described above, and the density of class-II and cyclophilin mRNA was accurately measured by a Bioimage Analyser (BAS-2000). Finally, we compared the density of each band in FRO and 1F3 cells.

Cytotoxic activity of CD4⁺ T cells

First, FRO and 1F3 cells were treated with mitomycin C (100 $\mu\text{g/ml}$; Sigma) for 1 hr. After washing, the cells ($1 \times 10^6/\text{ml}$) were incubated with allogenic CD4⁺ T cells ($2 \times 10^6/\text{ml}$) in the presence of RPMI 1640 medium (Nissui) containing 10% FCS (GIBCO) and interleukin (IL)-2 (10 ng/ml; Shionogi) for 7 days. Allogenic CD4⁺ T cells were prepared by exhaustive negative selection from peripheral-blood mononuclear cells of normal donors using immunomagnetic beads and multi-antibody cocktail as described (Tanaka *et al.*, 1993). The obtained CD4⁺ cytotoxic T cells were adjusted to a concentration of 4×10^6 cells/ml. In the next step, 100 μl of these cells were incubated with 100 μl of ⁵¹Cr sodium chromate (Amersham International, Aylesbury, UK) labeled FRO or 1F3 cells ($1 \times 10^5/\text{ml}$) with or without anti-class-II, anti-class-I, anti-lymphocyte-function-associated-antigen-1 (LFA-1) MAbs (10 $\mu\text{g/ml}$) for 4 hr in 96-well round-bottomed tissue-culture plates (Nunc, Roskilde, Denmark). After centrifugation of the tissue-culture plates at 100 g for 5 min, 100 μl of the culture supernatant was counted in a gamma counter (auto-gamma scintillation spectrometer; Packard). The percentage of specific lysis was calculated using the following formula: % specific lysis = [(experimental release – control release)/(maximum release – control release)] \times 100,

where maximum release was determined by incubating ⁵¹Cr-labeled target cells in the presence of 1% Nonidet P-40 (Sigma), while control release was determined by incubating target cells without effector cells. The values were expressed as means \pm standard.

Statistical analysis

In the evaluation of changes in HLA-DP/cyclophilin mRNA level after treatment with DRB and cytotoxic activity of CD4⁺ T cells, values were expressed as mean \pm SD of triplicate experiments. Differences in the mean values were examined for statistical significance by Student's *t*-test in the former experiments. A *p* value less than 0.05 denoted a statistically significant difference.

RESULTS

Expression of p53 protein in FRO and 1F3 cells

To document the effectiveness of transfection of cDNA of wild-type p53, we evaluated p53 expression in FRO and 1F3 cells by Western-blot analysis. As shown in Figure 1, while we could not detect reactivity with p53 protein in FRO using anti-p53 MAb; however, such reactivity was detected in 1F3 cells.

Synthesis and expression of class-II (HLA-DR and DP) proteins in FRO, FRO-c and 1F3 cells

In the first series of experiments, we used flow-cytometric analysis to examine the effect of p53 on the expression of functional cell-surface antigens, by using various MAbs against cell-surface molecules, including MHC-class-I and class-II products and ICAM-1. Among these, MHC-class-II product was the only molecule that was differentially expressed on 1F3, FRO, and FRO-c cells; 1F3, which possesses p53, highly expressed HLA-DR and DP antigens, while FRO, which lacks p53, and FRO-c expressed both proteins only marginally.

IFN- γ is known to enhance the expression of class-II antigen. The addition of IFN- γ further enhanced the expression of class-II antigen in 1F3 cells but not FRO or FRO-c cells (Fig. 2a). These results suggested that changes in 1F3 were not due to the effect of transfection of pCMV-Neo-Bam vector and that the control FRO-c cell line was similar to FRO. Therefore, we used FRO as a negative control instead of FRO-c in the remaining experiments described in this study. We did phenotypic analysis first, because these examined cell-surface molecules are expressed and function on the cell-surface membrane. We could not detect any difference in the expression of functional cell-surface antigens between NPA and

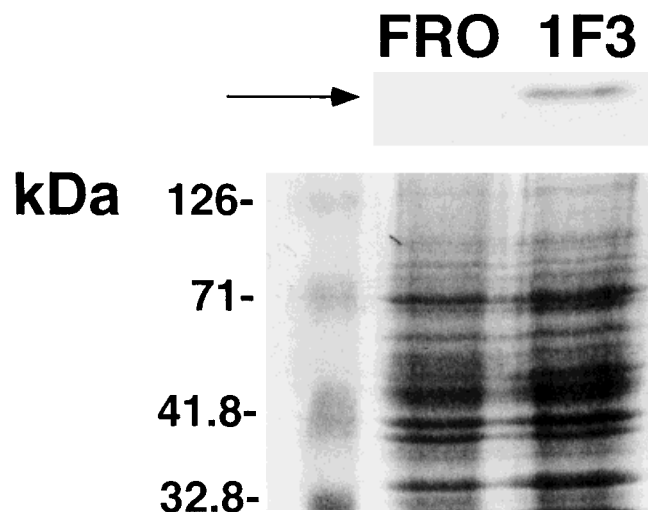


FIGURE 1 – Western-blot analysis of expression of p53 in lysates of FRO and 1F3 cells. Arrow indicates the specific band of p53 protein. Lower panel: loaded protein level of the nitrocellulose membrane filter stained by Coomassie blue. The size marker is indicated by kDa.

1N20 (data not shown), and the level of expression of class-II antigen on both cell lines was high (percentage of positive cells, NPA, 96.1%; 1N20, 97.8%). Therefore, only FRO and 1F3 cells were used in the subsequent experiments.

We also used Western-blot analysis to measure quantitatively the level of these molecules in the cytoplasm. Synthesis of class-II protein was strongly observed in cell lysates of 1F3 but was markedly reduced in FRO cells (Fig. 2b). The same results were also confirmed by immunoprecipitation of class-II proteins. The precipitated class-II proteins in lysates of FRO cells were weaker than those in lysates of 1F3 cells (Fig. 3a). Furthermore, the reduction of class-II protein following treatment with cycloheximide, a protein-synthesis inhibitor, occurred more slowly in FRO than 1F3 cell lysates (Fig. 3b). These results demonstrated that the synthesis and expression of class-II proteins were enhanced both on the cell-surface membrane and in the cytoplasmic proteins in 1F3 cells compared with FRO cells, whereas class-II protein in the cytoplasm of FRO cells was more stable than in 1F3 cells.

mRNA expression of class-II (HLA-DR and DP) in FRO and 1F3 cells

In the next series of experiments, we assessed mRNA transcription of class-II genes in FRO and 1F3 cells by Northern-blot analysis. In contrast to the expression of proteins in these cells, the expression of class-II mRNA was higher in FRO than in 1F3 cells (Fig. 4a). In particular, the level of DR mRNA was enhanced by the addition of IFN- γ to both cells. To explore the mechanism of the discrepancy between mRNA transcription and protein production of class-II products, we examined the differences in post-transcriptional regulation between 1F3 and FRO cells. Treatment of these cells with DRB, an inhibitor of RNA synthesis, reduced both HLA-DP/cyclophilin (Fig. 4b) and HLA-DR/cyclophilin (data not shown) mRNA levels in 1F3 cells more markedly and more quickly than in FRO cells. These results indicated that the reduced translation of class-II mRNA to protein in the post-transcriptional level resulted in reduced production and expression of class-II proteins in FRO cells lacking p53.

Cytotoxic activity of CD4⁺ T cells induced by FRO and 1F3 cells

Finally, we assessed the relevance of differential expression of class-II antigens and the influence of p53 on the induction of allogenic cytotoxic activity of CD4⁺ T cells against FRO or 1F3 cells. CD4⁺ cytotoxic activity against 1F3 cells (bearing both wild-type p53 and class-II antigens) was stronger than that against FRO cells (lacking both p53 and class-II expression). Of note was that 1F3-induced CD4⁺ cytotoxic activity was inhibited completely by the anti-class-II MAb as well as by MAbs against LFA-1, α L β 2 integrin on T cells (Table I). These results suggested that the induced expression of class-II protein on 1F3 cells bearing wild-type p53, but not on FRO cells lacking p53, could induce antigen-specific CD4⁺ cytotoxic T-cell activity against 1F3 cells through T-cell-receptor(TCR)-mediated signaling as well as co-stimulatory signals induced by adhesions between T-cell LFA-1 and ICAM-1 on 1F3 cells.

DISCUSSION

There is ample evidence for an important role of cytokines in the growth and function of cancer cells. In particular, IFN- γ plays a key role in the induction of class-II antigen expression which is important for antigen presentation. The major finding of the present study was that transfection of wild-type p53 into thyroid-carcinoma cells induced the expression of class-II antigen on the cells, which in turn resulted in the induction of alloantigen-presentation activity for CD4⁺ cytotoxic T cells through TCR-mediated signaling and co-stimulatory signal via interaction of LFA-1 to ICAM-1.

Whereas the role of CD8⁺ T cells in the immune-mediated destruction of tumor cells is well defined, the role of CD4⁺ T cells in anti-tumor immunity is less clear, with evidence supporting both effector and suppressor functions. Kosugi *et al.* (1987) showed that depletion of CD4⁺ T cells diminishes the anti-tumor immune response induced by CD8⁺ T cells, suggesting the involvement of

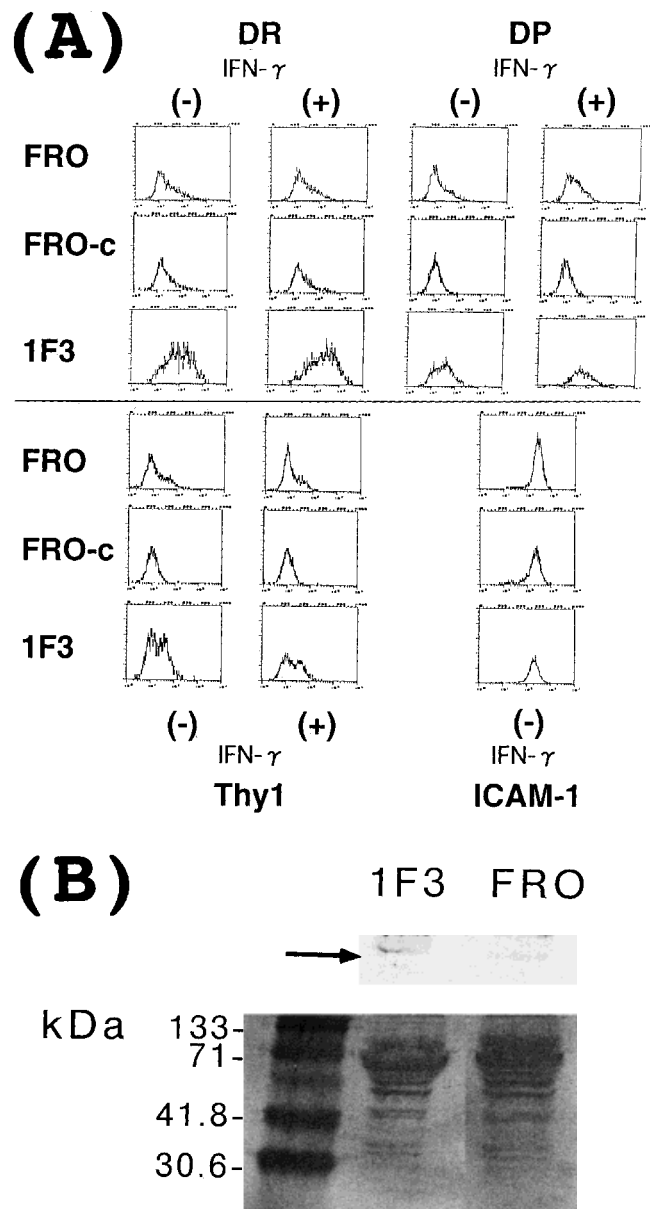


FIGURE 2 – (a) FACS analysis of the expression of class-II (HLA-DR and DP) proteins on FRO, FRO-c and 1F3 cells. The ordinate represents a histogram of the cell number stained with MAbs at each logarithmic fluorescence intensity on the abscissa. Thy1 MAb, which specifically binds to mouse T cells, was used as a negative control. Mean fluorescence intensity (MFI) was 341, 281 for FRO, FRO-c and 384 for 1F3 cells. Compared with the negative control, FRO cells were stained with MAbs to class II, DR (MFI, 418) and DP (342), while FRO-c cells were stained with MAbs to class II, DR (385) and DP (291). 1F3 cells were stained with MAbs to class II, DR (571) and DP (432). IFN- γ further enhanced MFI to 660 (DR) and 529 (DP) in 1F3, but did not enhance MFI in FRO (DR, 422; DP, 373) and FRO-c (DR, 411; DP, 274). (+), IFN- γ treatment; (-), no IFN- γ treatment. Note that the level of expression of ICAM-1 on FRO, FRO-c and 1F3 cells is high in all cell lines (MFI; 629, 603 and 604, respectively; percentage of positive cells, 96.3, 99.3 and 97.1% respectively). Although not shown in this figure, FRO, FRO-c and 1F3 cells were positively stained with MAb to DQ (471, 450 and 610 respectively). The MFI in DQ was enhanced to 705 in 1F3 cells by the addition of IFN- γ , but not in FRO and FRO-c cells (453 and 455 respectively). (b) Western-blot analysis of expression of class II in FRO and 1F3 cell lysates. Arrow indicates the specific band of class-II protein. Lower panel, loaded protein level of the nitrocellulose membrane filter stained by Coomassie blue. The size marker is indicated by kDa.

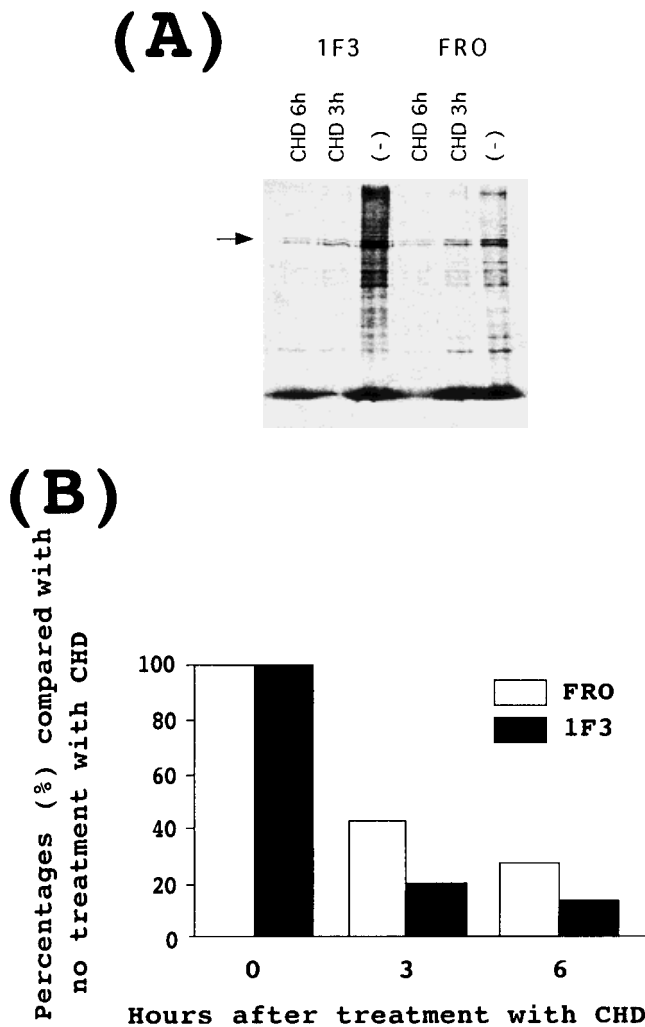


FIGURE 3 – (a) Immunoprecipitation analysis of class-II expression in lysates of FRO and 1F3 cells. Arrow indicates the specific band of class-II protein. (–); without cycloheximide treatment; CHD 3h and CHD 6h, cycloheximide treatment for 3 hr and 6 hr respectively. (b) Disappearance of class-II protein following treatment with cycloheximide in lysates of FRO and 1F3 cells. Changes in the level of class-II protein are expressed relative to the control (no cycloheximide).

CD4⁺ T cells in immunity. Furthermore, immunophenotypic comparisons between regressing and non-regressing human skin tumors demonstrated a major role for CD4⁺ T cells in the regression of these tumors (Hunt *et al.*, 1994). In addition, several studies indicated that the Fas-mediated apoptosis of target cells is involved in CD4⁺-T cell-mediated cytotoxicity (Stalder *et al.*, 1994). Baker (1995) has proposed that the loss of class-II-antigen expression prevents immune recognition and targeting of papillary thyroid carcinomas by T cells, and Matsubayashi *et al.* (1995) reported that the degree of lymphocytic infiltration around the tumor or inside the tumor in papillary thyroid carcinoma correlates well with a favorable prognosis *in vivo*. Furthermore, lymphocytic infiltration is more marked in follicular thyroid carcinomas than in tumors with papillary histology, however, such infiltration is ineffective, since it does not correlate with parameters of cell-mediated immunity (Juhász *et al.*, 1989). Based on the present findings and the above earlier results, we propose that the loss of class-II-antigen expression prevents immune recognition and targeting not only of papillary but also of follicular thyroid carcinomas as

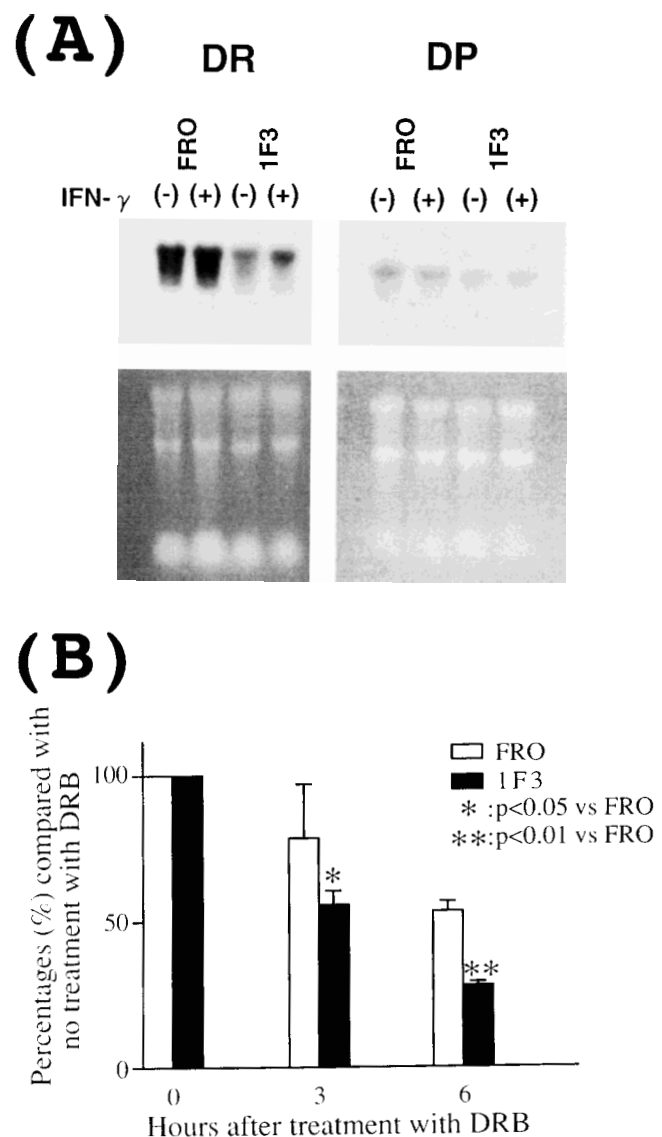


FIGURE 4 – (a) Northern-blot analyses of mRNA expression of class II (HLA-DR and DP) protein in FRO and 1F3 cells. (+); treatment with IFN- γ ; (–); no IFN- γ . Lower panel indicates loaded RNA level of the nylon membrane filter stained by ethidium bromide. Roughly equal amounts (10 μ g) of RNA were electrophoresed in each lane. (b) Changes in the level of HLA-DR/cyclophilin mRNA after treatment with DRB in FRO and 1F3 cells. Values are mean \pm SD of triplicate experiments.

evident in the FRO cell line lacking p53 used in this study. It should be remembered, however, that most differentiated thyroid carcinomas do not harbor p53 mutations, or have mutations in one copy of the gene, yet many are not palatable targets for cytotoxic cells. This fact emphasises the importance of other factors apart from p53 in class-II expression and those factors that interfere with the engagement of CD4⁺ cytotoxic T cells to their targets.

It has been suggested that p53 peptides binding to HLA-A2.1 may act as useful immunogens for the generation of HLA-A2.1-restricted tumor-specific cytotoxic T lymphocytes *in vitro* and *in vivo* (Ropke *et al.*, 1995; Stuber *et al.*, 1994). However, the relevance of p53 to class-II antigen is still not well understood. In the present study, we showed that, contrary to the protein level, the mRNA transcription of class-II is somewhat higher on p53-lacking FRO cells than on p53-bearing 1F3 cells. p53 was originally

TABLE I – CYTOTOXIC ACTIVITY OF CD4⁺ T CELLS INDUCED BY FRO AND 1F3 CELLS

Cytotoxic T cells induced by	Target cells	Specific lysis (%)
1F3	1F3 treated with none	13.9 ± 0.4
1F3	1F3 treated with anti-class-I Ab	14.9 ± 0.6
1F3	1F3 treated with anti-class-II Ab	0 ± 0.2
1F3	1F3 treated with anti-LFA-1 Ab	0 ± 0.3
FRO	FRO	4.9 ± 0.1

Values are mean ± SD. Effector-to-target-cell ratio was 40:1 in this experiment.

reported as a transcriptional factor suppressing the expression of several genes (*c-myc*, *IL-6*, etc.) at the transcriptional level (Margulies and Sehgal, 1993; Ragimov *et al.*, 1993). However, other studies have also reported enhancement by wild-type *p53* of not only *p21* (*CIP1/Waf1*) transcription but also accumulation of *p21* (*CIP1/Waf1*) protein at a post-transcriptional level in human breast epithelial cells (Gudas *et al.*, 1995). Our results demonstrate that the mRNA of both HLA-DP and HLA-DR in 1F3 cells was more quickly reduced by treatment with an inhibitor of RNA-synthesis (DRB), as compared with FRO cells. These results suggest that the reduced expression of class-II proteins in *p53*-lacking FRO cells was due to the restrained post-transcription and synthesis of class-II protein and that *p53* could be involved in the post-transcriptional regulation of several proteins, including class-II antigens as shown in 1F3 cells. Our results also showed that IFN- γ increased class-II-mRNA transcription in FRO cells, but not its protein synthesis in the same cells. It has been reported that *p53* is related to interferon regulatory factor-1 (IFNRF-1), a major transcription factor induced by IFN- γ and one of the transcription

factors for IFN- γ (Sato *et al.*, 1995; Tanaka *et al.*, 1996). Thus, an alternative interpretation of our results is that *p53* might induce class-II-protein synthesis through the involvement of IFNRF-1.

Taking these results together, we propose that, in carcinoma cells, *p53* functional protein is involved in the induction of anti-tumor CD4⁺ cytotoxic-T-cell activity against carcinoma cells through the synthesis of MHC-class-II products by post-transcriptional regulation. We are currently investigating the mechanism through which wild-type *p53* specifically modifies the stability of class-II antigen.

Although the data presented in this study are limited to one carcinoma cell line, it should not be concluded that the observed changes are simply due to clonal variation, since the low frequency of generation of authentic exogenous wild-type-*p53*-expressing transfectants limits the extrapolation of these findings to other independently generated clones, as discussed by Fagin *et al.* (1996). Thus, our findings should not be considered as a general phenomenon but as applicable solely to the cell line and clone used in the present study (FRO and 1F3 respectively).

In conclusion, our data suggest that in the absence of wild-type *p53* in carcinoma cells, there is a disturbance of expression of class-II antigen and low induction of CD4⁺ cytotoxic-T-cell activity through TCR and co-stimulatory molecules. These changes would ultimately lead to less differentiation or to uncontrolled growth of tumor cells.

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