Aberrant expression and function of p53 in T-cells immortalized by HTLV-I Tax1

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Received 14 February 1997

Abstract The expression and function of p53 tumor suppressor protein was investigated in T-cells immortalized by the Tax1 protein of HTLV-I. Conformationally wild-type p53 was expressed at elevated levels in Tax1-immortalized T-cells by post-transcriptional mechanisms when compared with normal T-cells. Luciferase assays with a reporter plasmid containing p53-binding sites revealed an impairment in the transactivating function of p53 in Tax1-immortalized T-cells. Our results suggest an important role for Tax1 in the aberrant expression and function of p53 observed in many HTLV-I transformed cells.

Key words: HTLV-I; Tax1; p53; Transcription; Transactivation

1. Introduction

Human T-cell leukemia virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL), and this virus can transform normal T-cells in vitro [1,2]. Tax1, a transcriptional *trans*-activator of this virus, is thought to be responsible for this process [1,3,4]. Primary human T-cells can be immortalized by Tax1, and these Tax1-immortalized T-cells exhibit many features similar to those of HTLV-I-transformed T-cells [5–7]. Transcriptional activation of cellular genes by Tax1 is thought to lead to T-cell transformation, but the precise mechanism of this process is still unclear.

Recently, it has been reported that the majority of HTLV-I-transformed T-cells show elevated steady-state levels of tumor suppressor p53 protein compared to normal human peripheral blood lymphocytes [8–10]. Although increased p53 protein levels are usually associated with missense mutations in the evolutionarily conserved domain of the p53 gene, most HTLV-I-transformed T-cells have wild-type p53 genes [8–10]. Wild-type p53 is somehow stabilized post-translationally in HTLV-I-transformed T-cells [8]. Increased steady-state levels of wild-type p53 protein are reported to be associated with its functional inactivation, and are thought to contribute to T-cell transformation by HTLV-I [10,11].

In this study, we examined whether the aberrant expression and function of p53 protein is also observed in T-cells immortalized by Tax1. We found that p53 with the wild-type conformation was expressed at an elevated level by a post-transcriptional mechanism, and that its *trans*-activating function was impaired in Tax1-immortalized T-cells as observed in many HTLV-I-transformed cells.

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2. Materials and methods

2.1. Cells and cell culture

PBL/DGL-Tax1A and PBL/DGL-Tax1B are immortalized T-cell lines independently obtained from human peripheral blood lymphocytes (PBL) after infection with a Tax1-expressing retroviral vector [7]. Normal human $CD4^+$ T-cells obtained from PBL using a CD4 subset enrichment column (Cellect.plus HUMAN CD-4 KIT, Biotech) were initially stimulated with phytohemagglutinin (PHA) and recombinant IL-2. PBL/DGL-Tax1A, PBL/DGL-Tax1B, and normal human $CD4^+$ T-cells were maintained in AIM-V medium (Gibco) containing 2% fetal bovine serum (FCS) and recombinant IL-2 at 200 U/ml. MT-2 is an HTLV-I-infected T-cell line [12]. Jurkat is a human acute T-lymphoblastic leukemia cell line [13]. MT-2 and Jurkat were maintained in RPMI 1640 medium containing 10% FCS. J138V5C [14], a subline of Jurkat, is a stable transformant with a human temperature-sensitive p53 mutant $p53^{Val-138}$, which behaves as wild type at low temperature (32°C) and as mutant type at high temperature (37°C). J138V5C cell line was maintained in RPMI 1640 medium containing 10% FCS and G418 at 1.6 mg/ml.

2.2. Immunoblotting

Immunoblotting was performed essentially as previously described [7]. Briefly, 5×10^5 cells were pelleted and lysed in 100 μl of 2×SDS-PAGE sample buffer. After quantitating the protein with the BCA protein assay reagent (Pierce), each lysate corresponding to 5 μg of protein was fractionated on 10% SDS-PAGE, and then proteins were transferred to Immobilon filters (Millipore). After blocking with 5% nonfat dried milk in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) overnight at room temperature, the membranes were incubated with mouse anti-p53 monoclonal antibody Ab-6 (Oncogene Science) in blocking buffer for 1 h. The membranes were then washed extensively with TBS containing 0.1% Tween 20, incubated for 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody, washed and developed with the ECL chemiluminescence reagent (Amersham) as directed by the manufacturer.

2.3. Immunoprecipitation

Cells (2×10^6) were pelleted and lysed in 200 μl of 1% NP40 lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 2.5 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF, 10 mg/ml leupeptin, 1% aprotinin at 4°C for 20 min. Insoluble material was removed by centrifugation at 4°C for 20 min at $10\,000\times g$. Cell lysates were incubated with anti-p53 monoclonal antibodies PAb1801, PAb240, and PAb1620 (Ab-2, 3 and 5, Oncogene Science) for 2 h at 4°C. PAb1801 reacts with both wild-type and mutant p53 [15], PAb240 reacts preferentially with mutant p53 [16], and PAb1620 reacts preferentially with wild-type p53 [17]. Immune complexes were precipitated by incubation with protein G-agarose for 1 h at 4°C. Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with rabbit polyclonal anti-p53 antibody RSP53(Nichirei).

2.4. Northern blot analysis

Total cellular RNA (10 μg) was resolved by electrophoresis in 1.0% agarose containing formaldehyde and MOPS, then blotted onto a nitrocellulose membrane. The membrane was hybridized with ^{32}P-labeled probe, washed with 0.2×SSC and 0.1% SDS at 65°C for 1 h, then exposed to X-ray film with intensifying screens at −70°C. The probe for human p53 was obtained by RT-PCR based on the sequence data [18] and was confirmed by DNA sequencing. It contained almost the entire coding region of p53.

PII S0014-5793(97)00280-9

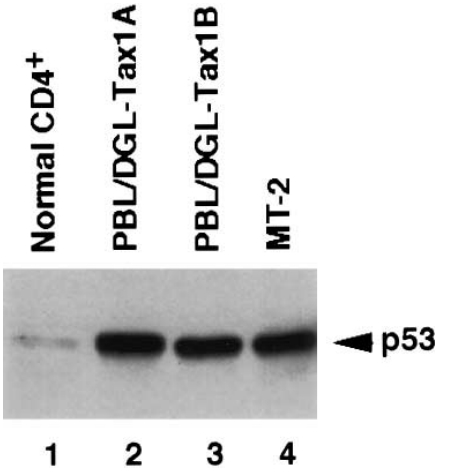

Fig. 1. Immunoblot analysis of p53 protein. Whole cell lysate corresponding to 5 μg of protein was subjected to immunoblot analysis with mouse anti-p53 monoclonal antibody Ab-6 (Oncogene Science). The p53 band is indicated by an arrow. Lanes: 1, normal human CD4+ T-cells prepared from PBL as described in Section 2; 2, PBL/DGL-Tax1 A; 3, PBL/DGL-Tax1 B; 4, MT-2. PBL/DGL-Tax1A and PBL/DGL-Tax1B are Tax1-immortalized T-cell lines independently established from PBL.

2.5. Luciferase assay

Luciferase reporter plasmids were introduced into T-cells by electroporation as described previously [5]. One day after electroporation, cells were harvested and luciferase activities were measured with the PicaGene luciferase assay system (Toyo Ink) as directed by the manufacturer. Reporter plasmids used were pCAST2Bluc, p-55Bluc, and pCMV-Luc. pCAST2Bluc has two copies of a consensus p53 binding sequence upstream of the interferon-β basal promoter, and p-55Bluc has only the interferon-β basal promoter [19]. pCMV-Luc was constructed by inserting a *Xba*I-*Hin*dIII fragment from pCMV-CAT [5] containing the human cytomegalovirus immediate early region promoter into the *Sma*I-*Hin*dIII site of PGV-B2 (Toyo Ink) after end-filling the *Xba*I site.

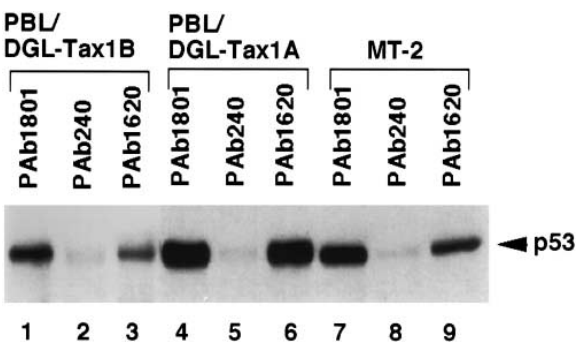

Fig. 2. Immunoprecipitaion of p53 protein. p53 was immunoprecipitated with mouse monoclonal anti-p53 antibody PAb1801 (lanes 1, 4, 7), PAb240 (lanes 2, 5, 8), and PAb1620 (lanes 3, 6, 9). Immunoprecipitates were then subjected to immunoblot analysis with rabbit polyclonal anti-p53 antibody RSP53 (Nichirei). PAb1801 and RSP53 react with both wild-type and mutant p53, PAb240 reacts preferentially with mutant p53, and PAb1620 reacts preferentially with wild-type p53. We confirmed that PAb240 could immunoprecipitate mutant p53 in CEM cells under our experimental conditions (data not shown). Lanes: 1–3, PBL/DGL-Tax1 B; 4–6, PBL/DGL-Tax1 A; 7–9, MT-2.

3. Results and discussion

3.1. Expression of p53 in Tax1-immortalized T-cell lines

Steady-state expression levels of p53 protein in two individual Tax1-immortalized T-cell lines, PBL/DGL-Tax1A and PBL/DGL-Tax1B, were examined by immunoblot analysis. Both of these T-cell lines showed markedly increased p53 protein expression compared to normal human CD4+ T cells (Fig. 1), and this level of expression was almost the same as that of the HTLV-I transformed T-cell line MT-2. To determine the conformational phenotype of the p53 protein expressed in these cells, we performed immunoprecipitation assays using three monoclonal antibodies, PAb1801, PAb240, and PAb1620 (Fig. 2). PAb1801 immunoprecipitates p53 protein in both wild-type and mutant conformation, PAb240 immunoprecipitates mutant forms of the protein, and PAb1620 immunoprecipitates wild-type forms of the protein [15–17]. In two Tax1-immortalized T-cell lines as well as in MT-2 cells, PAb1801 and PAb1620 precipitated equivalent amounts of p53, and only a very small amount of p53 was precipitated by PAb240. These results indicated that most of the p53 protein expressed in Tax1-immortalized T-cell lines as well as in MT-2 cells had the wild-type conformation. Northern blot analysis revealed that the levels of p53 mRNA did not differ significantly between normal CD4+ T-cells, PBL/DGL-Tax1A, PBL/DGL-Tax1B, and MT-2 cells (Fig. 3). Thus, the level of wild-type p53 protein was increased post-transcriptionally in Tax1-immortalized T-cell lines, probably due to prolongation of protein half-life as is the case with HTLV-I-transformed T-cells.

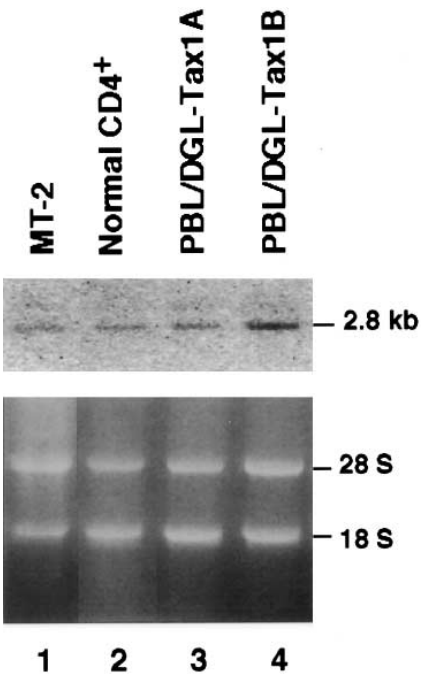

Fig. 3. Northern blot analysis of p53 mRNA. Total cellular RNA (10 μg) was subjected to Northern blot analysis with p53-specific probe. Ethidium bromide staining of rRNAs is shown in the lower panel. The sizes of the bands are indicated on the right. Lanes: 1, MT-2; 2, normal human CD4+ T-cells; 3, PBL/DGL-Tax1 A; 4, PBL/DGL-Tax1 B.

Table 1
Transactivating function of p53

Cell line	p53-mediated transactivation (fold induction)
PBL/DGL-Tax1 A	13.9 ± 8.8
PBL/DGL-Tax1 B	11.1 ± 3.1
MT-2	7.0 ± 1.3
J138V5C (32°C)	1024.0 ± 124.5
J138V5C (37°C)	6.7 ± 3.0

5 μg of pCAST2Bluc, p-55Bluc, and pCMV-Luc were transfected into each cell line by electroporation. J138V5C cells were prepared as two sets. Following 4 h incubation at 37°C, one set of the J138V5C cells was transferred to 32°C and the other was kept at 37°C. About 24 h after electroporation, cells were harvested and luciferase activities were measured. pCAST2Bluc has two copies of the consensus p53 binding sequence upstream of the interferon-β basal promoter, and p-55Bluc has only the interferon-β basal promoter. p53-mediated transactivation is presented as fold induction, which was determined by dividing the activity seen in cells transfected with pCAST2Bluc by that seen in cells transfected with p-55Bluc. Prior to it, each activity was normalized against the activity seen in cells transfected with pCMV-Luc to correct the differences in the transfection efficiency between each cell line. The results are the average ± standard deviations of three independent experiments.

3.2. Function of p53 in Tax1-immortalized T-cell lines

The transactivating function of p53 in Tax1-immortalized T-cell lines was examined. A luciferase reporter plasmid containing two copies of a consensus p53 binding sequence (pCAST2Bluc) and the reporter plasmid with no p53 binding sequence (p-55Bluc) were transfected into cells, and luciferase activities were measured. To evaluate the level of p53-mediated transactivation, we used the J138V5C cell line as a control. J138V5C is a subline of Jurkat stably transformed with a human temperature-sensitive p53 mutant $p53^{Val-138}$ [14]. At non-permissive temperature (37°C), $p53^{Val-138}$ behaves as mutant type and J138V5C cells grow normally. However, at permissive temperature (32°C), $p53^{Val-138}$ behaves as wild type and J138V5C cells undergo apoptosis. It has been demonstrated that the transactivating activity of $p53^{Val-138}$ is equivalent to that of wild-type p53 at permissive temperature but is almost undetectable at non-permissive temperature [20]. The value of p53-mediated transactivation, which was determined by dividing the activity seen in cells transfected with pCAST2-Bluc with that seen in cells transfected with p-55Bluc, was more than 1000 in J138V5C cells at 32°C (Table 1). In contrast, the values of p53-mediated transactivation in the two Tax1-immortalized T-cell lines and in the MT-2 cell line were much lower and were only 7–14, which were equivalent to that in J138V5C cells at 37°C. Therefore, the level of p53-mediated transactivation in J138V5C cells at 32°C was 70–140 times higher than that in Tax1-immortalized T-cells and MT-2 cells. When protein expression levels were compared, J138V5C cells expressed 10-fold greater amounts of p53 protein than the Tax1-immortalized T-cell lines and the MT-2 cell line (Fig. 4). To determine whether the difference in expression levels could account for the differences in the levels of transactivation, the dose-response relation of p53-mediated transactivation was analyzed. pCAST2Bluc was co-transfected with increasing amounts of wild-type p53 expressing vector $pCMVp53^{W}$ [20] into Jurkat cells expressing no endogenous p53 protein, and the luciferase activity was measured. As shown in Fig. 5, it was clear that a 10-fold increase in the level of expression did not result in an increase in transactivation equivalent to that observed in J138V5C cells at 32°C. Our data from this dose-response analysis were consistent with a previous report [21]. Thus, these results strongly suggest that p53 in Tax1-immortalized T-cells as well as in MT-2 cells was functionally impaired even though it had wild-type conformation.

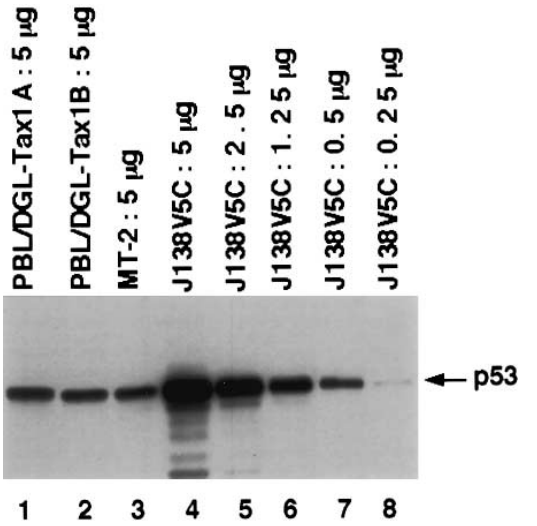

Fig. 4. Comparison of p53 protein levels. Whole cell lysate corresponding to the indicated amount of protein was subjected to immunoblot analysis with mouse anti-p53 monoclonal antibody Ab-6 (Oncogene Science). The band for p53 is indicated by an arrow. Lanes: 1, PBL/DGL-Tax1 A cultured at 37°C; 2, PBL/DGL-Tax1 B cultured at 37°C; 3, MT-2 cultured at 37°C; 4–8, J138V5C cultured at 32°C for 24 h.

3.3. Conclusion

In this study we found that the steady-state p53 protein level significantly increased in Tax1-immortalized T-cell lines

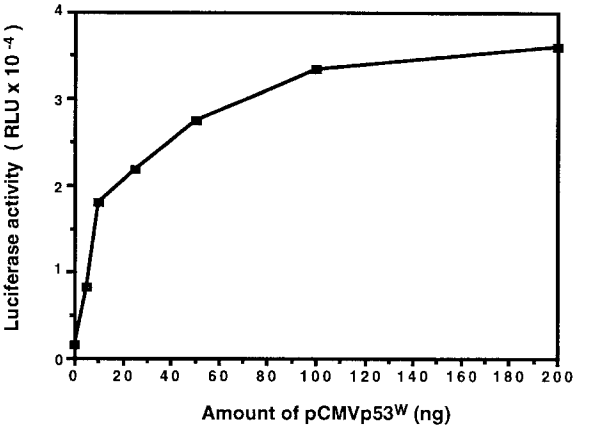

Fig. 5. Dose-response analysis of p53-mediated transactivation. Increasing amounts of $pCMVp53^{W}$ (wild-type p53 expression vector) were co-transfected with 5 μg of pCAST2Bluc (luciferase reporter plasmid containing p53 binding sites) into Jurkat cells, and luciferase activities were assayed. To equalize the total amount of DNA transfected in each experiment, appropriate amounts of pKS/CMV (empty backbone vector) were added. Values are averages of two independent experiments. Within this range of DNA amounts, we confirmed by using pCMV-Luc that the product increased linearly with an increase in the transfected expression vector plasmid (data not shown). RLU, relative light units.

compared to normal T-cells, although the p53 protein expressed was conformationally wild-type. There were no significant changes in the p53 mRNA levels, suggesting that p53 was elevated by a post-transcriptional mechanism. Luciferase assays with a reporter plasmid containing synthetic p53 binding sites revealed that the transactivating function of p53 was severely impaired in Tax1-immortalized T-cells. All these results strongly suggest an important role for Tax1 in the aberrant expression and function of p53 observed in many HTLV-I transformed cells. However, it seems that Tax1 does not directly induce such changes in p53, since the early passage of Tax1-transduced primary T-cells does not show any changes in the expression and function of p53, and this aberration of p53 expression and function is observed only in fully immortalized cells such as PBL/DGL-Tax1A and PBL/DGL-Tax1B described in this study (Akagi et al., unpublished data). Dysfunction of p53 may be a rather late event in the Tax1-induced immortalization process. Functional inactivation of p53 has been reported to be an important step in immortalization in many systems [22–28], and investigation of the mechanism by which the p53 function is impaired in Tax1-immortalized T-cells may be necessary to understand the process of T-cell transformation by HTLV-I.

Acknowledgements: We thank Dr. T. Kiyono (Aichi Cancer Center) for the luciferase reporter plasmids, p-55Bluc and pCAST2Bluc. We also thank Dr. T. Sekine (National Cancer Center) for supplying recombinant IL-2. This work was supported in part by Grants-in-Aid for Cancer Research, Grants-in-Aid for a 2nd-term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, and Grants-in-Aid from the Ministry of Education, Science and Culture.

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