Transformation-Related Cellular Protein p53: Increased Level in Untransformed Rat Cells Following Treatment with the Tumorpromoter, Tetradecanoylphorbol-Acetate

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

p53 Induction, 12-O-Tetradecanoylphorbol-13-acetate, Phorbolester, Rat Cells

12-O-Tetradecanoylphorbol-13-acetate (TPA, 100 ng ml^{-1}), a tumor promoting phorbol ester, is able to induce enhanced levels of the transformation-associated cellular antigen p53 in normal rat2 cells which had not been previously initiated by a carcinogen. p53 was estimated in ethanol-fixed treated cells on microtiter plates with ELISA using the monoclonal antibody Pab 1620 [EMBO J. **7,** 1485, (1984)]. Induction of p53 was confirmed by immunoblotting. This effect of TPA is an additional phenotypic characteristic of tumor cells which can be induced by TPA in untransformed rodent cells.

Introduction

Tumor promotion and transformation of carcinogen-initiated animals and cells was the focus of the first era of research with phorbol esters which had been isolated from the plant *Croton tiglium,* an Euphorbiaceae species [1, 2]. Later on, numerous pleiotropic effects in many different biologic systems became evident [3]. These features comprise effects on cellular differentiation [4] and the enhancement of tumor cell phenotype of transformed cells [5]. In addition, even normal cells which are not initiated by carcinogens, may transiently express features of tumor cell phenotype, if exposed to phorbol esters. Thus, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated the loss of the glycoprotein fibronectin from the extracellular matrix of untransformed cells in culture [6]. However, this derogation of fibronectin by TPA from the cell surface is an attribute of a variety of transformed cells. Though by no means universal, an elevated excretion of plasminogen activator is another attribute of transformed cells. An increase of plasminogen activator excretion was also induced by TPA in normal untransformed cells [7].

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We have investigated TPA with respect to the expression of a cellular transformation-associated phosphoprotein, p53. This protein occurs in elevated levels in a variety of human and experimental animal tumor cells lines [8]. Nontransformed lines have very low pools of p53. The levels of the protein is correlated with the cell cycle and is regulated by a short half-life of the order of 20–30 minutes [9, 10]. This half-life is increased in transformed cells. In SV40-transformed cells, p53 is complexed with the nuclear viral large tumor (T) antigen, and the large pools of this stabilized form of p53 can be easily visualized by immunofluorescence [8]. Several monoclonal antibodies against p53 have been described. Of those, Pab 122 [11] and Pab 1620 [12] recognize epitopes that occur more or less on both mouse and human cells.

The present report describes the observation that in untransformed rat cells TPA is able to increase the pool size of p53 measured by monoclonal antibody Pab 1620.

Materials and Methods

Cell culture

Rat2 fibroblasts (rat strain F 344, obtained from Dr. Martin Lipp, Munich), mouse NIH3T3 cells (obtained from Dr. G. Schütz, Heidelberg), SV40-transformed halbC mouse kidney cells, line mKSA (ob-

tained from Dr. R. Henning, Ulm) SV40-transformed balbC mouse fibroblasts, line VLM (obtained from Dr. W. Deppert, Ulm), and African green monkey kidney cells, line TC7 (obtained from Dr. K. H. Scheidtmann) were grown in polystyrene plastic flasks in Eagle's MEM containing 5–10% fetal calf serum (FCS) and penicillin plus streptomycin.

Antisera

Monoclonal antibodies against p53 were from supernatants or ascites of hybridoma line Pab 1620 [12] and Pab 122 [13]. The titers were determined by indirect nuclear immunofluorescence with acetonmethanol (2:1)-treated (−20°, 10 min.) mKSA or other SV40-transformed mouse cells and were 1:1000 (end point dilution, ascites) and 1:50 (supernatant). Fluorescein-labelled anti-mouse immunoglobulin secondary rabbit antibodies (Nordic Immunology) were used for immunofluorescence tests and peroxidase-labelled sheep anti-mouse immunoglobulin (Sigma A 5906) for immunoblotting and ELISA.

p53 induction by TPA

TPA (Sigma, stock solution 200 μg ml^{-1} in dimethylsulfoxide) was diluted in cell culture medium. For ELISA determination, flat-bottomed microtiter plates (Dynatec, Nürtingen) were used. 5×10^3 cells were seeded per well and grown for 18 hrs before addition of TPA for different periods. One microtiter plate (96 wells) was used for each of the TPA and fetal calf serum concentrations and per induction period. At the end of the induction period, cell density was determined by directly counting the wells or after trypsination of about 5 wells per plate. For p53 determination by immunoblotting, cells were seeded and treated in 75 cm^2 flasks.

p53 determination by ELISA

An ELISA protocol [11] was modified as follows: Following the TPA induction period, microtiter plate cultures were washed with PBS, fixed with ethanol for 15 minutes at −30 °C, dried at room temperature and conserved at −30 °C until evaluation, if necessary. Immediately before the assay, the cultures were treated with 200 μl H_2O per well for 30 minutes at 20 °C and then decanted. Ascites Pab 1620 was diluted 1:50, 1:100 etc. down to 1:3200, and hybridoma supernatant 1:2, 1:5, 1:10 down to 1:160 in PBS containing 1% FCS. Negative controls were mouse normal serum and phosphate-buffered saline (PBS). Fifty μl of each dilution were transferred to 8 wells in parallel. After 1 hour at 37 °, the antibody was decanted and the cells were washed three times for 3 minutes with PBS containing 0.05% Tween 20, 50% glycerol and 0.1% gelatin, pH = 7.40. Then peroxidase labelled anti-mouse antibody diluted 1:400 in PBS containing 1% FCS was added. After 1 hour at 37 °, the cultures were washed as above. Bound peroxidase was then assayed by addition of 100 μl per well of the following reaction mixture: 55 mg of 1,2-diaminobenzene (Sigma) were freshly dissolved in 100 ml 40 mM Tris HCl plus 150 mM NaCl, pH = 7.4. After 15 minutes at 20 ° the color development was stopped and stabilized with 20 μl 2N H_2SO_4. OD at 492 nm was determined with a Titritek photometer (Flow Laboratories). For each group of 8 wells comprising one antibody dilution, the mean and standard deviation of the OD were calculated and plotted against the dilution. The resulting regression curve cuts the OD-axis at an intersection point OD_{Pab}. This value was back-ground-corrected by substracting the OD of the antibody-free control (C on abscissa, Figs. 1 and 2). The resulting net OD (p, indicated in Fig. 1a) was used to estimate the relative pool size of p53.

p53 determination by immunoblotting

Cells were cultivated and induced by TPA in 75 cm^2 flasks and finally washed with cold PBS. For p53 extraction, 0.5 ml per 2.5×10^7 cells of a solution containing 6.3% SDS, 4.5% 2-mercaptoethanol in 0.4 M Tris HCl, pH = 6.8 were added, sonicated for 10 sec. (Branson sonifier B12, setting 5, microtip) and heated for 10 min at 100 °. Then 10% of glycerol was added. For protein estimation, 20 μl aliquots were added to 100 μl N NaOH, diluted with 1 ml H_2O and OD was measured at 280 nm. For SDS polyacrylamide gel electrophoresis (PAGE; 10% acrylamide), 6 μl per 1 OD of the cell extract were used. Following electrophoresis, the gels were washed three times in a solution containing 4 volumes of 25 mM Tris, 192 mM glycine, pH = 8.5, plus 1 volume methanol. The separated proteins were then electrophoretically blotted from the gel onto cellulose nitrate sheets (Schleicher & Schüll, BA-85) using the same solution; the electric field was 10 volts per 5 cm for the first hour, and 30 volts for 18 hrs.

The blots were then treated with 500 mM NaCl, 20 mM Tris, pH = 7.5 (TBS) for 10 minutes and then for 30 minutes in the same solution containing 3% gelatin in order to saturate free protein binding valences of the blots. For p53 detection the blots were first treated for 1 hour at 20 ° with monoclonal antibody Pab 122 or 1620 supernatant diluted 1:20 in TBS containing 1% gelatin. The blots were washed with H_2O and then twice (10 minutes) in TBS plus 0.05% Tween 20 and then floated in a 1:400 dilution of sheep anti-mouse globulin-peroxidase conjugate (Sigma) in TBS plus 0.05% Tween 20 for 40 min. After washing in H_2O and PBS-0.05% Tween 20 as above, the bound peroxidase complex was visualized by floating the blots in a freshly prepared mixture of 30 mg 4-chloronaphthol in 10 ml methanol plus 30 µl H_2O_2 (30% solution) in 50 ml TBS. After 20 to 40 min, lilac-blue bands indicating p53 became visible and were conserved by washing with H_2O and photography.

Results

Quantitative estimation of p53 in SV40-transformed and untransformed cells by ELISA

The technique for p53 estimation was established by means of p53 high (SV80, mKSA, VLM) and low (TC7, rat2, NiH3T3) expression cell lines as described in Materials and Methods. Regression curves and *p*-values of 4 of the cell lines are shown in Fig. 1a–d (in Fig. 1a a prozonal depression was not taken into account). The *p*-values of the cell lines VLM and TC7 were 0.7 and 0.2, respectively (curves not shown). Obviously, the results demonstrate significant pools of p53 in the SV40-transformed cells and the aptitude of Pab 1620 for ELISA. The un-

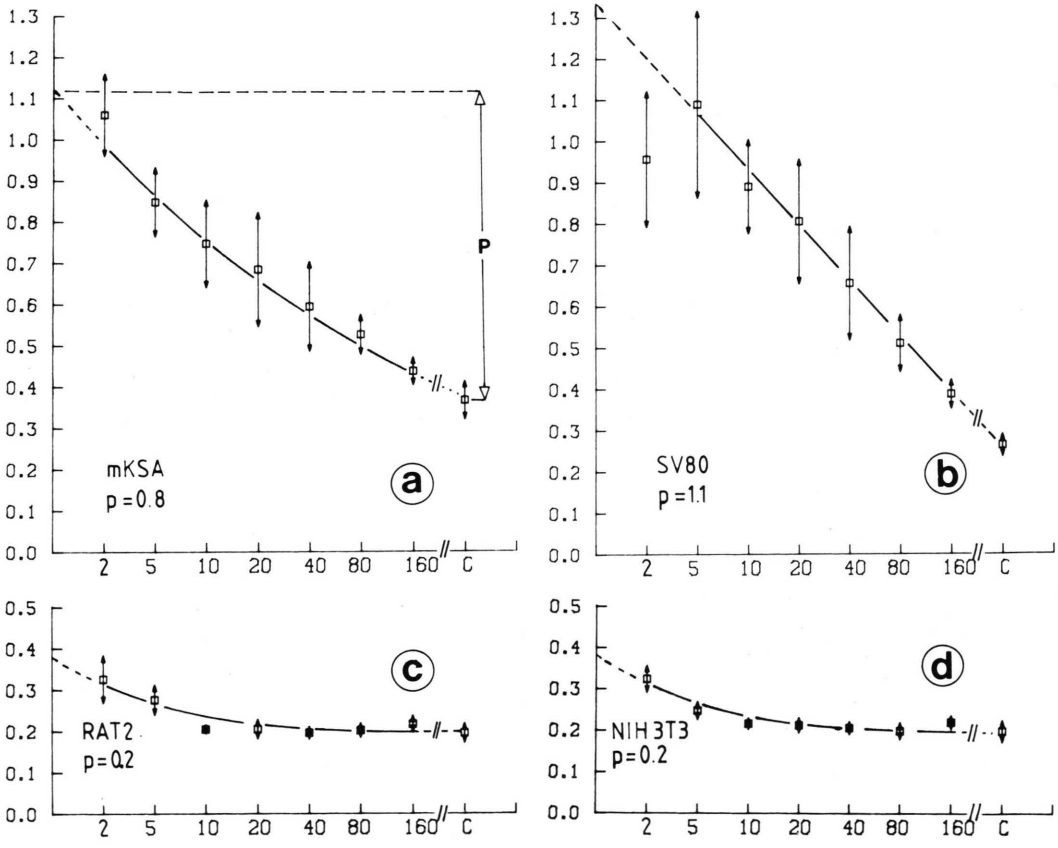

Fig. 1. Estimation of p53 of 4 cell lines by ELISA. Ordinate, $OD_{492\ nm}$ (mean and standard deviation of 8 determinations per antibody dilution in parallel). Abscissa, dilution of Pab 1620 supernatant; C, antibody-free control; *p* is explained in Materials and Methods.

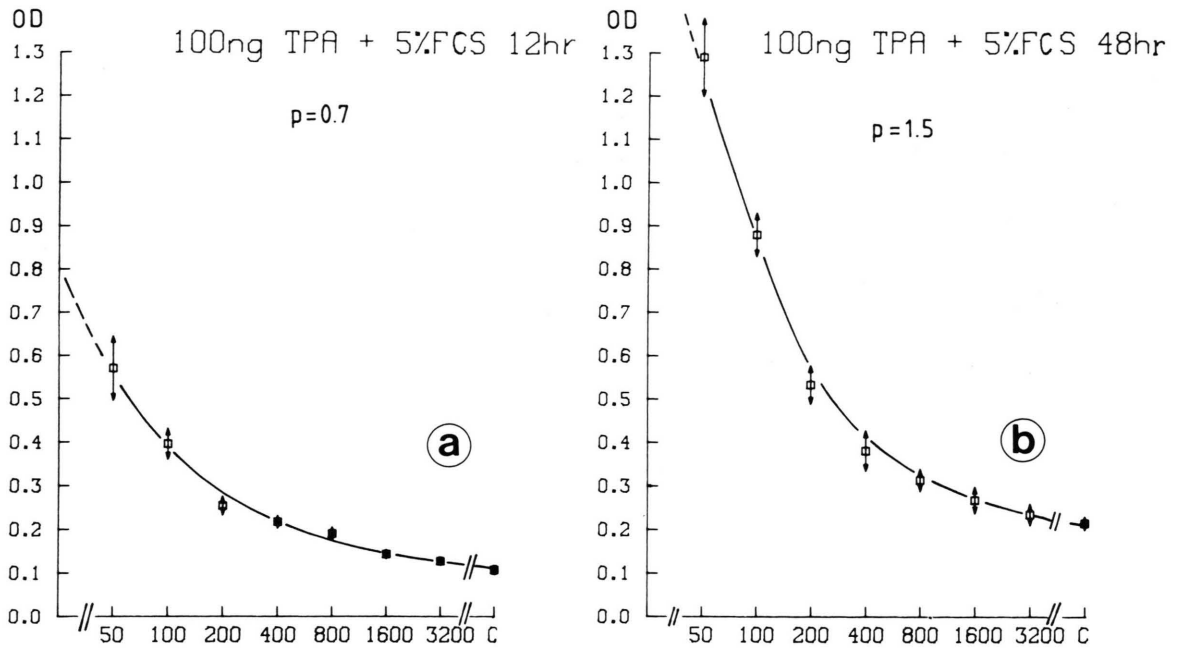

Fig. 2. Estimation by ELISA of p53 in TPA-treated rat2 cells. Incubation periods of 12 hrs (a), and 48 hrs (b). Ordinate, $OD_{492\ nm}$; abscissa, dilution of Pab 1620 ascites; C, antibody-free control.

transformed cell lines showed a significantly lower slope of the regression curves. Using this technique, induction of p53 by TPA was estimated in untransformed rat2 cells as shown in the next paragraph.

Induction of p53 in untransformed rat2 cells by TPA

p53 estimation by ELISA

Microtiter cultures of rat2 cells were grown in Eagle's MEM containing 10% FCS until confluence and were treated for 12, 24 and 48 hrs with 100 ng ml^{-1} of TPA in the presence of 0.5 and 5.0% FCS. Controls were cultivated without TPA. At the end of the incubation periods, cell numbers per well were determined. p53 pools were estimated as above using ascites from Pab 1620. The OD of the peroxidase reactions was plotted against ascites dilution (1:50 to 1:3200 as above). The regression curves and *p*-values (defined in Materials and Methods) of two of the experiments (induction with 100 ng TPA ml^{-1} for 12 and 48 hrs) are demonstrated in Fig. 2a and b. The results of a series of 5 induction experiments are listed in Table I. The *p*-values of p53 in TPA-treated and untreated cells are normalized for 10^4 cells per well (p_n) and the ratio $f = p_{n,\ tPA}+/p_{n,\ TPA}\varnothing$ is depicted in a graph (Fig. 3).

Table I. Estimation of p53 from TPA-treated rat2 cells by ELISA; synopsis of 5 pairs of treated and untreated cells. Columns: TPA, + (100) or ∅ (0 ng ml^{-1}); *t*, induction period; FCS, serum concentration during induction period; *n*, mean cell number per well (8 parallel assays per dilution step); *p*, relative amount of p53 per well (for details see text); p_n, *p* normalized for 10^4 cells per well.

TPA	*t* [hrs]	FCS [%]	*n* [$\times 10^{-4}$]	*p*	p_n
+	24	0.5	0.8	0.9	1.1
∅	24	3*	0.8	0.4	0.5*
+	48	0.5	1.1	1.4	1.3
∅	48	3*	1.1	0.4	0.4*
+	12	5	0.7	0.7	1.0
∅	12	3*	0.6	0.4	0.7*
+	24	5	0.7	0.9	1.2
∅	24	3*	0.8	0.4	0.5*
+	48	5	1.1	1.5	1.4
∅	48	3*	1.1	0.4	0.4*

* p_n for 0.3% FCS in $TPA^{\varnothing}$ controls were not significantly different (data not shown).

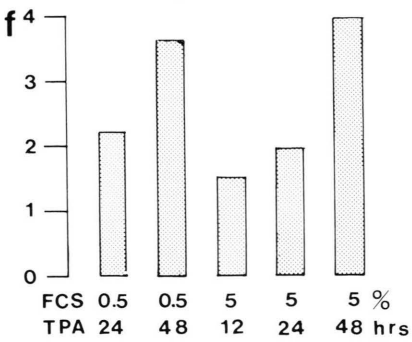

Fig. 3. Induction of p53 in TPA-treated rat2 cells (synopsis of 5 experiments listed in Table I). f, ratio of normalized amounts of p53, (p_n, see Table I) in treated vs. untreated cells; FCS, serum concentration during TPA incubation; TPA, duration of induction period.

The results demonstrate a several-fold increase of the p53 pool beginning between 12 and 24 hrs after TPA addition and an augmentation of this increase by higher FCS concentration.

The identity of p53 which had been induced in the above experiments was then proven by the additional independent detection by immunoblotting described in the next paragraph.

p53 detection by immunoblotting

Rat2 cells were grown in 75 cm^2 flasks to confluence and treated with 100 ng ml^{-1} TPA for 48 hrs. As detailed in Materials and Methods, treated and untreated cultures were extracted with detergent at 100 °, the extracted proteins were separated according to size by PAGE, blotted by electrophoresis onto cellulose nitrate paper and reacted with the monoclonal anti p53 antibodies Pab 122 and 1620. Bound monoclonal immunoglobulin was visualized with peroxidase-labelled second antibody. An extract from the SV40-transformed mKSA cell line served as a positive control. We found a pronounced p53 signal when blotting the extract from the mKSA as well as from rat2 cells following TPA treatment, however, no signal from the untreated rat2 cells (Fig. 3). Obviously the mobility in SDS PAGE of p53 from rat was higher than that from mouse cells. This result was obtained only with Pab 122; Pab 1620 was unable to react with p53 which is denatured by heat and high detergent concentration according to the extraction protocol (blot not shown).

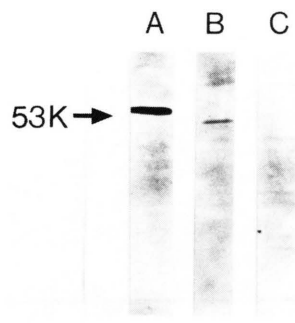

Fig. 4. Immunoblot of p53 extract from each 10^6 transformed control cells (mKSA, lane A) and TPA-treated (48 hrs, 100 ng ml^{-1} TPA) (lane B) as well as untreated rat2 cells (lane C).

Discussion

Elevated cellular pools of p53, an attribute of tumor cells [8], were observed here also in untransformed rat2 cells which have been treated with the tumor promoter TPA, in the absence of any initiating carcinogen. This increase has been augmented by elevated concentrations of FCS in the medium and occurred during an incubation period of 2 days (longer incubation periods caused a loss of cells from the culture vessel and were therefore not used for p53 evaluation).

This novel effect of TPA on untransformed cells was not unexpected since other tumor cell characteristics such as enhanced loss of fibronectin, and enhanced escretion of plasminogen activator following TPA treatment are known [6, 7]. However, the mechanism of p53 induction as well as the question as to whether TPA influences either the formation, or the processing of p53 or of a particular p53 molecule species or of its messenger, are unknown and deserve further elucidation. Also the distribution of the enhanced p53 pool in the cell is unknown.

In addition, it may be of interest whether the p53 induction can be exerted only by the "full" promoter, TPA, or whether a promotion stage II-competent mitogen like 12-O-retinoylphorbol-13-acetate (RPA, [13]) may be sufficient for p53 induction.

As to the antigenic epitopes of the TPA-induced p53, these are obviously homologous to those of p53 in transformed cell since they react with the same monoclonal antibodies (Pab 122 and 1620) and also the heat or detergent lability was the same.

Some of the questions above may be answered by pulse-chase labelling of p53, by determination of p53-specific RNA and by induction experiments with RPA.

The biological significance of the observed effect for transformation is not defined. Elevated levels of p53 occur also during a certain stage of embryonic development of mice [14]. It is, however, unknown, whether the observed effect may be interpreted in terms of a transient retrodifferentiation.

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