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TPA-INDUCED DIFFERENTIATION OF HUMAN RHABDOMYOSARCOMA CELLS INVOLVES DEPHOSPHORYLATION AND NUCLEAR ACCUMULATION OF MUTANT P53

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SUMMARY: Previous studies have shown that human rhabdomyosarcoma cells are induced to differentiate by TPA, in the absence of appreciable alterations of the muscle regulatory genes and their products (1). The question was addressed whether the tumor suppressor p53 could be a target of TPA action in these cells. Genomic analysis by a Polymerase Chain Reaction/Single-Strand Conformation Polymorphism (PCR/SSCP) and direct sequencing indicate the presence of a mutation in exon VII at codon 248 (C to T transition) and a loss of heterozygosity of p53 gene in human rhabdomyosarcoma cell line (RD). It is here shown that transcription of p53 mRNA strongly decreases in RD cells induced to growth arrest and differentiate by TPA treatment. In these cells immunoprecipitation and immunoblot analysis show that both synthesis and total cellular concentration of the protein are also reduced by TPA. Nevertheless nuclear p53 accumulation is at much higher extent, whereas ^{32} P-orthophosphate labelling, followed by immunoprecipitation, demonstrates a decrease of phosphorylation of both cytoplasmic and nuclear p53. These results indicate that TPA causes a number of alterations of mutant p53, likely mediated through a protein kinase C dependent mechanism, which might impair the transforming ability of mutant p53 in growth-arrested and differentiating RD cells.

p53 is a highly conserved phosphoprotein expressed at very low levels in normal cells (2,3). It is known that allelic deletions or point mutations of p53 are accompanied by increased incidence of tumors (4), and that wild type p53 (w.t.p53) plays a role in the suppression of cell proliferation and in the inhibition of oncogene-mediated cell transformation (5). Two hypotheses have been proposed to explain how p53 regulates cell proliferation. One is based on the ability of w.t.p53, but not of any known mutant form, to form an heterocomplex with SV40 large T antigen, as well as with other oncoproteins, or their cellular homologues, making them incompetent to up regulate DNA replication (6,7). Alternatively p53 could act at the transcriptional level, either by promoting or repressing expression of genes relevant to the cell cycle control (8). In fact, it has

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Abbreviations. TPA,12-O-Tetradecanoyl phorbol-13-acetate; SSCP, Single Strand Conformation Polymorphism; PCR, Polymerase chain reaction; PMSF,Phenyl Metyl Sulfonyl Fluoride.

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been demonstrated that transforming mutant p53, but not w.t. p53, lacks the ability to activate gene transcription (9). Furthermore, a possible regulatory factor of p53 transcriptional function has been recently identified as the product of the mdm2 oncogene. This protein forms a tight complex with p53, thus inhibiting p53-mediated transactivation and enhancing its tumorigenic potential (10). Post-translational modifications of p53 may also be important in regulating its function. p53 is a natural substrate of both casein kinase II as well as p34 cdc2 kinase (11,12) thus raising the possibility that phosphorylation may regulate p53 functions, including interactions with other proteins. A mutation of the p53 gene has been described in human rhabdomyosarcoma as well as in many other tumors (4,13, 14). The rhabdomyosarcoma-derived cell line (RD) undergoes TPA-induced arrest of growth and progressive myogenic differentiation, with no alteration of the expression patterns of the myogenic regulatory genes (1). These responses require long term TPA treatment (more than 24 hrs) suggesting a complex cascade of events including activation, likely followed by desensitization, of protein kinases and alterations of transduction of mitogenic signals. It is conceivable that these events could lead to alterations of mutant p53, which could thus acquire a non transforming role. In this report we address the problem of the existence and nature of such alterations and we show that TPA induces alterations of expression, subcellular distribution and post-translational modifications of mutant p53, likely dependent on a protein kinase C-mediated mechanism. These alterations could affect the trasforming ability of mutant p53 in growth-arrested and differentiation-induced rhabdomyosarcoma cells.

MATERIALS AND METHODS

Cell cultures, labelling and antibodies. Rhabdomyosarcoma cells were obtained from American Type Culture Collection (ATCC), grown and treated with the tumor promoter TPA as previously described (15). Labelling with 50uCi/ml [^{35}]S-methionine (1000 Ci/mMole, NEN) and with 250 μCi/ml of [^{32}]P-orthophosphoric acid (37 MBq, NEN) were performed respectively in methionine-free and phosphate-free medium 3hr before the end of treatment. Monoclonal antibodies used in these studies were the anti p53 antibody PAb 1801 (Ab2 Oncogene Science, Manhasset, NY) and anti human-PCNA monoclonal antibody (Boehringher). PAb 1801 reacts in RD cells better of any other monoclonal antibody directed to mutant forms of p53. The antibodies were used at 1:50 dilution for immunoblotting and 1:100 for immunoprecipitation procedure. Rabbit anti-mouse IgG (Zymed) was used as secondary antibody in both immunoblotting and immunoprecipitation.

PCR/SSCP analysis and direct sequencing. For Single Strand Conformation Polymorphism analysis (SSCP), 100ng of total genomic DNA were used according to protocols already published (16,17) with few modifications. For PCR analysis 100ng DNA were amplified with 25pmol of specific primers for exon VII (upstream P7-5: 5'-GTGTTGTCTCCTAGGTTGGC-3', downstream P7-3: 5'-GTCAGAGGCAAGCAGAGGCT -3') in 10mM Tris-HCl pH 9, 50mM KCl, 1mM $MgCl_2$ with 200μM dNTP, 1 μCi -[^{32}]P-dATP (3000Ci/mMole, NEN) and 0.5 unit of Taq polymerase (Promega), using 35 cycles (2' 94°C, 2' 60°C, 2' 72°C) in a thermal cycler (Perkin-Elmer). Aliquots of denatured and non-denatured amplified DNA were run onto a 6% non-denaturing acrylamide gel containing 10% glycerol for 14 hrs at room temperature. Mutations were screened for qualitative difference in migration of single stranded DNA in comparison with placental DNA (SIGMA) as normal control. For direct sequencing the 188 bp fragment from p53 exon VII was obtained by PCR amplification according to standard procedures. Briefly, 100ng of RD genomic DNA were amplified (30 times 1' 94°C , 1' 56°C , 2' 72°C , in thermal cycler) in 25μl of 10mM Tris-HCl pH9, 50mM KCl, 0.01% gelatine, 1.5mM $MgCl_2$, 0.1% Triton X-100 using 25pmol of each primer that we have used for PCR/SSCP,

200μM dNTP and 0.5 units of recombinant Taq polymerase (Promega).The amplified PCR product was run onto 1.5% agarose gel and the band further purified onto 0.6% low melting agarose gel and eluted according to usual procedure. Sequence analysis was performed following sequenase protocol (Stratagene).

Northern blot and hybridization analysis. RNA was prepared with the procedure of guanidine isothiocyanate and caesium chloride gradient following already published methods (18). Total RNA (20μg) was separated electrophoretically, transferred to nitrocellulose and probed with random-prime-labeled cDNA specific for human p53 (ATCC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm equal loading of RNA. Filters were exposed for autoradiography on RP-X-OMAT films (KodaK).

Extracts, subcellular fractionation, immunoblotting and immunoprecipitation. Total extracts were prepared by homogenizing cells in 10mM Tris-HCl pH 7.5 additioned with 2mM PMSF, 200 U/ml Aprotinin, 10 μg/ml each Leupeptin, Trypsin inhibitor and Antipain as protease inhibitors, 20 mM NaF and 0.1 mM sodium orthovanadate as phosphatase inhibitors. Nuclear fractions were prepared by sedimenting the total extracts at 800xg and the supernatant cytoplasmic fractions were clarified after 100,000xg sedimentation. The immunoblotting procedure was performed according to standard protocols (19). The amount of proteins was measured according to the Lowry procedures (20). For immunoprecipitation total extracts, nuclear and cytosolic fractions were extracted in 50mM Tris-HCl pH 8, 0.5% NP40, 150mM NaCl, 10mM EDTA (NET Buffer) additioned with the above mentioned inhibitors, and clarified at 20,000xg. Immunoprecipitation was performed as previously described (21). The immunoprecipitates were analysed by acrylamide gels according to Laemmli (22) and autoradiographed on RP-X-OMAT films (Kodak). The quantitative evaluation of p53 has been performed by densitometric scanning of immunoblotting and immunoprecipitation autoradiographies.

RESULTS

Structural alteration of the genomic locus of p53. The presence of mutant p53 in RD cells, has been demonstrated in a number of sarcomas in vivo (13, 14). In RD cell line we performed single-strand conformation polymorphism analysis (PCR/SSCP), amplifying segments of exons V, VI, VII, VIII and IX, where most of the mutations have been observed on tumor DNAs (23). We found altered migration of amplified DNA only in exon VII. Fig.1 shows the autoradiography of electrophoretic profile of the amplified DNA of exon VII from RD and human placental DNA as a control (SSCP). An altered pattern of migration is evident both in the denatured single strand and in non denatured double strand DNA fragments from RD cells (lane RD, D and ND) compared to human placental samples (lane P, D and ND). These results are indicative of the presence of mutations in exon VII of the p53 gene suggesting the absence of the normal allele. Fig.1 also shows the results of PCR and direct sequencing demonstrating a C to T transition at p53 codon 248 (CGG to TGG, arginine to tryptophan).

p53 mRNA expression. The expression of p53 mRNA was studied in RD cells treated with TPA for different times. As shown in Fig.2, the level of p53 mRNA drastically decreases in RD cells growth-arrested after 2 days of TPA treatment and in differentiating cells after 5 days of treatment, whereas the level of p53 mRNA shows only slight changes in growing untreated RD cells during the culture time examined.

Subcellular distribution and accumulation of p53 and PCNA. It has been shown that subcellular distribution of p53 is modulated during cell cycle (24). In addition w.t. p53 is known to down modulate the expression of Proliferating Cell Nuclear Antigen (PCNA) (25), whose nuclear accumulation specifically increases in replicating cells. Thus p53 or PCNA subcellular

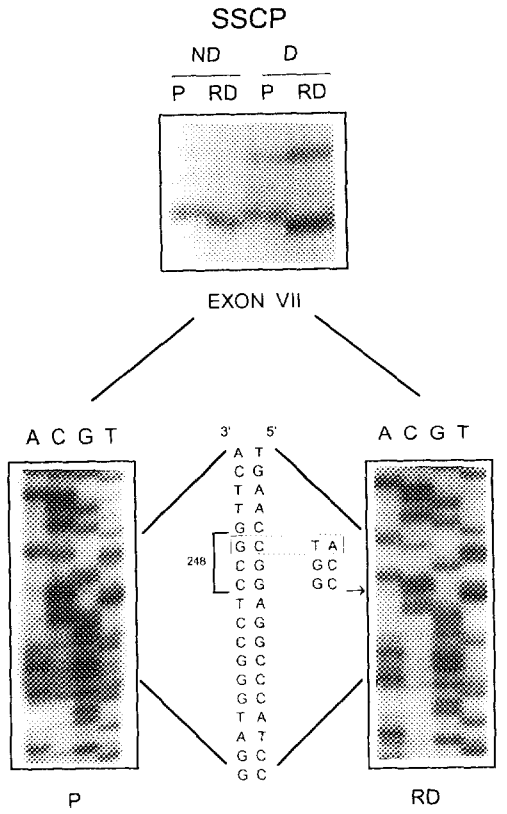

Fig.1. PCR/SSCP analysis and direct sequencing of exon VII from p53 gene. In the upper portion of the Fig., ND and D stand for non denaturing and denaturing conditions, respectively. P is human placental, RD is rhabdomyosarcoma DNA. For the sequence, reading on the gel is on the non coding strand. The codon at which the mutation occurs is indicated and the point of C to T transition is boxed.

distribution may correlate with the resting or growing state of the cell. As shown in Fig.3 the nuclear level of PCNA drastically decreases by day 2 of TPA treatment (Panel A), while it increases in untreated growing RD cells. This result indicates that, as previously shown (15), in RD cells 2 days of TPA treatment is the time interval sufficient to switch off the mitogenic pathways. On the contrary, a remarkable nuclear accumulation of p53 (approximately two fold by densitometric scanning) is evident in TPA-treated respect to untreated cells by day 2, persisting up to day 5 of TPA treatment (Panel B), when the RD cells have already acquired a myogenic phenotype. Concomitantly, a decreased amount of p53 is found in the cytoplasmic fractions of TPA-treated cells (Panel C). It is important to notice that nuclear accumulation of p53 occurs in the presence of a decrease of the total amount of p53 in TPA-treated cells (Panel D).

Synthesis and post-translational modification of p53. Extracts from ^{35}S-methionine and ^{32}P-ortophosphoric acid-labelled RD cells at 2 days of TPA treatment, were immunoprecipitated with anti p53 PAb 1801. Analysis of the immunoprecipitates shows that ^{35}S-methionine-labelled p53 increases in the nuclear fraction of TPA-treated respect to untreated cells (approximately two fold by densitometric scanning), while it decreases in the cytoplasmic fraction (Fig.4 panel

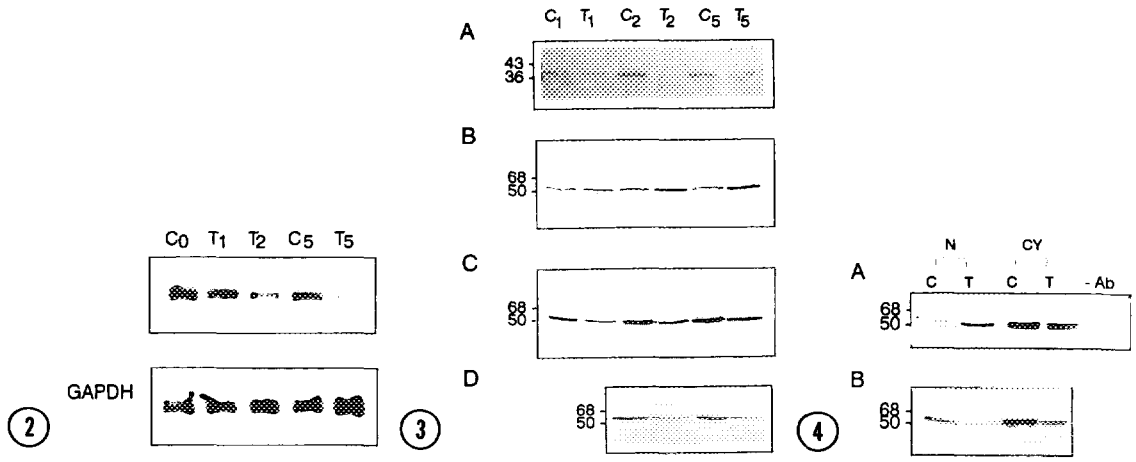

Fig.2. Autoradiography of Nothern blot of 20 µg mRNA from untreated (Co, C5) and treated RD cells (T1,T2,T5) with TPA (10^{-7}M final concentration), for 1,2,5 days hybridized with p53 probe, and with a GAPDH probe as control. Co is the onset of TPA treatment.

Fig.3. Autoradiography of Western blot of equal amount of protein from TPA-treated RD for 1,2,5 days (respectively T1,T2,T5), and from untreated RD (C1,C2,C5): **(A)** nuclear extracts probed with monoclonal anti-PCNA antibody; **(B)** nuclear extracts probed with PAb 1801 anti p53; **(C)** cytoplasmatic extract probed as in B; **(D)** total extracts, from 2, 5 days untreated and TPA-treated RD, probed as in B and C. Molecular weight markers are: bovine albumin (68K), L-glutamic dehydrogenase (50K), ß-actin (43K), glyceraldeide 3-P dehydrogenase (36K).

Fig.4. Autoradiography of SDS-PAGE of nuclear (N) and cytoplasmatic (CY) extracts from 2 days untreated (C) and TPA-treated (T) RD, immunoprecipitated with PAb 1801 anti-p53. RD labelled with ^{35}S-methionine **(A)** and with ^{32}P-orthophosphoric acid **(B)**. Immunoprecipitation with nonspecific IgG (-Ab).

A; respectively N, CY). On the other hand, ^{32}P-orthophosphate-labelled p53 decreases in the nuclear, as well as in the cytoplasmic fraction of TPA-treated cells (Fig.4, panel B). Since in these cells the amount of p53 increases in the nuclear fraction, as shown above, the specific activity of nuclear phosphorylated p53 is approximately 3 times lower respect to untreated RD cells. Conversely, since the amount of the cytoplasmic p53 decreases (Fig 3 Panel C) its specific activity remains unchanged in the cytoplasmic fraction respect to the untreated cells.

DISCUSSION

Human rhabdomyosarcoma, as well as other tumors, bears mutations of the p53 gene, which contribute to maintain the transformed phenotype (4). In this report we provide evidences that RD cells bear a mutation in the exon VII at codon 248 (C to T transition) and loss of heterozygosity of the p53 gene, as found in other rhabdomyosarcoma cell lines (13, 14). Since growth arrest and differentiation can be induced in RD cells by TPA treatment (15), and these changes do not involve an altered expression pattern of the myogenic regulatory genes (1), we addressed the question as to whether reversal of the transformed phenotype involves alterations of mutant p53. The results obtained show that TPA affects p53 expression at the transcriptional, translational and post-translational levels. Both morphological (not shown) and

immunobiochemical assays indicate that p53 is constantly present in the nucleus of RD cells. Nevertheless immunoblotting analysis shows a conspicuous increment of p53 nuclear accumulation in growth-arrested TPA-treated RD cells even in the presence of decreased p53 expression at mRNA and protein levels. These results demonstrate that TPA induces: i) a decrease of p53 mRNA level; ii) a decrease of p53 synthesis rather than an increase of degradation; iii) an increase of nuclear accumulation of dephosphorylated p53 in the presence of a reduction of the total amount of p53. It is conceivable that the lower level of mutant p53 in these cells may be required for TPA-dependent post-translational modification to be effective in inducing nuclear translocation and in restoring a non transformed phenotype. In addition, it is interesting to note that p53 nuclear accumulation parallels the reduction of PCNA, a proliferative marker which is known to be down modulated by p53 expression (25). It has been shown that temperature-dependent conformational changes of mutant p53 induce growth arrest and nuclear translocation in rat cell lines (26, 27, 28). In line with this finding it can be suggested that in RD cells TPA induces conformational changes of mutant p53 permissive for its growth suppressor function. Alternatively the acquired growth suppressor function of mutated p53 might be due to TPA- mediated altered interactions with other proteins which are known to regulate p53 function and subcellular distribution (Reviewed in ref. 29). It has been shown that p53 protein, with mutation at codon 248, lacks DNA-binding properties (30) and has potent transcriptional activity when fused with GAL4 DNA binding domain (31, 32). The interactions with other proteins, conformational changes as well as nuclear association and DNA binding may be regulated by phosphorylation or dephosphorylation. A number of reports have demonstrated that phosphorylation events might regulate growth suppressor function of p53 and retinoblastoma protein (Rb). Okadaic acid, a tumor promoter and potent inhibitor of phosphatases 1A, 2A, has been reported to induce hyperphosphorylation of Rb and p53 thereby improving their growth suppressor function (33). According to other authors, on the other hand, growth inhibition by anchorage deficiency is associated with reduced tyrosine phosphorylation of mutant p53 (34). We find that p53 is significantly less phosphorylated in 2 days TPA-treated RD cells. Considering that growth-arrested RD cells conspicuously accumulate p53 in the nuclei, the decreased level of p53 phosphorylation could be relevant for a more tight nuclear association. A number of reports have shown that p53 is a substrate of p34 cdc2 kinase as well as casein kinase II which in turn can be stimulated by a number of mitogens, including serum and TPA (35). On the other hand we cannot exclude that protein kinase C down modulation, following long TPA treatment, is implicated in the phosphorylation state of p53. This hypothesis is sustained by our preliminary experiment, not shown here, demonstrating that nuclear accumulation occurs after treatment with inhibitors of protein kinase C such as staurosporine. Though the mechanisms responsible for TPA-induced changes of mutant p53 are still unclear, our data indicate that in growth-arrested and differentiated human rabdomyosarcoma cells, mutant p53 undergoes changes of its synthesis, subcellular distribution and dephosphorylation, wich may restore its tumor suppressor function.

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