Induction of Neuronal Differentiation by p73 in a Neuroblastoma Cell Line*

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The p53-related p73 and p63 genes encode proteins that share considerable structural and functional homology with p53. Despite similarities, their deletion in mice has different outcomes, implying that the three genes may play distinct roles in vivo. Here we show that endogenous p73 levels increase in neuroblastoma cells induced to differentiate by retinoic acid and that exogenously expressed p73, but not p53, is sufficient to induce both morphological (neurite outgrowth) and biochemical (expression of neurofilaments and neural cell adhesion molecule (N-CAM); down-regulation of N-MYC and up-regulation of pRB) markers of neuronal differentiation. This activity is shared, to different extents, by all p73 isoforms, whereas the transcriptionally inactive mutants of p73 isoforms are ineffective. Conversely, blockage of endogenous p73 isoforms with a dominant negative p73 results in the abrogation of retinoid-induced differentiation (11). The p73 gene has been mapped to a region (1p36.33) that is frequently deleted in neuroblastomas (undifferentiated neuronal tumors (12, 13)), suggesting that loss or altered expression of p73 in vivo may be a determinant of the undifferentiated phenotype of neuroblastoma. Moreover, neurons from p53 null mice and neurons from wild type mice cultured with p53 antisense oligonucleotides in vitro show accelerated spontaneous differentiation (14). This suggests the hypothesis that loss of the p53 heterodimeric partner of p73 may liberate more uncomplexed or homodimerized p73 and allow expression of its differentiation-inducing function.

Accordingly, we have investigated the role of p73 in the differentiation of neuroblastoma cell lines, a well studied model of neuronal differentiation and death (15–17).

MATERIALS AND METHODS

Cell Culture—N1E-115 cells were grown in Dulbecco’s modified essential medium supplemented with 7.5% (v/v) fetal bovine serum, 1.2 g of bicarbonate per liter, 1% nonessential amino acids, and 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid at 37 °C in a humidified atmosphere of 5% (v/v) CO2 in air. Cells were differentiated by reduction of serum to 2.5% and addition of 1 μM all-trans-retinoic acid (RA) to the culture medium.

Cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol, with 1.5 μg of p53 or p73 isoform expression vectors, together with 0.5 μg of a LacZ (cytomega-
lovirus-β-galactosidase marker vector CLONTECH, Palo Alto, CA) or GFP-spectrin fusion protein (18) expression vector. Cells were then cultured for 96 h in normal growth medium and then fixed on the dishes, stained for β-galactosidase activity, and photographed. Cell differentiation was evaluated by counting 150 β-galactosidase-positive cells. Cells were scored as differentiated if the length of the neurite extensions was at least three times the diameter of the cell body.

Plasmids—Human p73 isoforms and p53 cDNAs in pcDNA3 have been described (2). p73 transcription inactive mutants were obtained by mutating alanine 156 into valine by site directed mutagenesis per-
turer’s protocol. The mutation of the corresponding alanine into valine site-directed mutagenesis kit (CLONTECH) according to the manufacturer’s protocol. The mutation of the corresponding alanine into valine in p53 has been reported to have a dominant negative function (19). All four p73 A156V mutants were inactive for p21 promoter transcription (data not shown). The p73p.A84 mutant of p73, lacking the aminoterminal transcription activation domain, was generated by amplifying the β isofrom using a 5’ primer from base pair 363 to 385 containing an in-frame NheI restriction site and an Sp6 primer as 3’ primer and then cloned into pCDNA-3 in-frame with a hemagglutinin tag into pcDNA-hemagglutinin (2) using the NheI and NolI unique restriction sites. A 1267-base pair fragment of the human N-CAM promoter (20) (nucleotides −1274 to 107) was amplified by polymerase chain reaction from genomic DNA. The amplified product was cloned into the pGL2 basic vector (Promega, Madison, WI) using standard methods and checked with dyeoxy sequencing. The GFP-spectrin expression vector

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§ The abbreviations used are: RA, all-trans-retinoic acid; GFP, green fluorescence protein; N-CAM, neural cell adhesion molecule.
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**Determination of Cell Cycle and Apoptosis**—To estimate DNA fragmentation, cells transfected with either the control vector or p73 isoforms and p53, together with a GFP-spectrin expression vector at a 1:2 ratio, were collected at 3, 6, and 24 h after transfection, and an equal number of cells were used for DNA content analysis (4, 2d, 4d, and 6d, respectively) using 1 μm RA. C designate control mock-treated cells. α and β designate cells transfected with α and β, respectively. The α and β forms of p73, both recognized by the antibody, were up-regulated by RA treatment, whereas no modulation of p53 was observed. β-Actin immunoblot (-actin) was used as a control.

**RESULTS AND DISCUSSION**

**Induction of p73 during Retinoid-induced Differentiation**—N1E-115 murine neuroblastoma cells induced to differentiate with 1 μM RA (17, 21) showed a time-dependent accumulation of p73 proteins that parallels their morphological differentiation (Fig. 1), suggesting that some p73 functions are recruited during neuronal differentiation. In contrast, p53 levels were unchanged along the entire time course of neuronal differentiation (Fig. 1).

**p73 Induces Neurite Outgrowth via Gene Transactivation**—To directly assess the activity of p73 isoforms in neuroblastoma cells, N1E-115 cells were transiently transfected to express each of the four p73 isoforms or p53. N1E-115 cells were transfected with 1.5 μg of p53 or the different p73 isoform expression vectors, together with 0.5 μg of a LacZ reporter (the cytomegalovirus-β-galactosidase vector from CLONTECH) or a GFP-spectrin fusion protein expression vector (18) in order to follow the fate of transfected cells (Fig. 2). After 3–4 days, cells transfected with all the four p73 isoforms showed morphological changes characteristic of neuronal differentiation, i.e. increased number of cells with neurites, increased neurite length, and increased number of neurites per cell (Fig. 2). The p73 isoforms showed different abilities to induce differentiation (Fig. 2, a–g), with the β isoform producing the most pronounced changes (Fig. 2, a and e). Cells transfected with empty vector (Fig. 2b) or p53 (Fig. 2c) displayed no signs of differentiation. As expected, a proportion of p53 transfectants showed apoptotic features (Figs. 2c and 3b). Thus, neuronal differentiation in vitro can be triggered by p73 overexpression alone, in the absence of any differentiation inducer, and this activity is not shared by p53. Under the same conditions, transcriptionally inactive p73 mutants (Δ156V) have proven incapable of inducing differentiation (Fig. 2, h and i), showing that the transactivating ability of p73 is required and also suggesting that transcription of downstream targets specific for p73, and not p53, is necessary to induce differentiation. A quantitative analysis of p73, p73 mutants, and p53 (differentiative potential) activity is shown in Fig. 2i. Up to 60% of the cells expressing the p73 isoforms displayed differentiated features, whereas less than 15% of the cells differentiate when either the control plasmid (C), p53, or the transcriptionally inactive mutants (p73m) are transfected. Only a small percentage of the cells transfected with the p73-isoforms undergo apoptosis (Fig. 3B), but as expected, similarly to p53, p73 is capable of blocking cells in G0/G1 phase of the cell cycle (Fig. 3C). The effect on the cell cycle is likely mediated by the induction of p21 (Fig. 3, D and E). Cells differentiated with RA show a similar induction of p21 (Fig. 3D).

**Induction of Differentiation-related Genes**—We next evaluated the ability of the p73 isoforms to regulate the expression of differentiation-related genes. N1E115 cells were co-transfected with p73, p53, or pcDNA3 expression vectors together with the GFP-spectrin vector. Four days after transfection, cells were fixed, and indirect immunofluorescence staining was carried out using monoclonal antibodies against the 160-kDa neurofilament or against N-CAM and a R-phycoerythrin-conjugated secondary antibody to mouse IgG. As shown in Fig. 4, N1E-115 cells transiently transfected with a GFP expression plasmid with the p73 isoforms were positively stained with antibodies against both neurofilaments (Fig. 4A) and N-CAM (Fig. 4B), whereas cells transfected with the control vector or p53 were either completely negative or slightly positive. Thus, in addition to inducing morphological changes, exogenously expressed p73 isoforms were also sufficient to drive the expression of biochemical
Fig. 2. Induction of neuronal differentiation by exogenously expressed p73 isoforms in neuroblastoma cells. a, N1E-115 murine neuroblastoma cells transfected with 1.5 μg of the p73β vector and 0.5 μg of the vector expressing the GFP-spectrin fusion protein. Cells were then
In order to demonstrate that p73 is not only sufficient but is indeed required for neuronal differentiation of neuroblastoma cells in vitro, we evaluated the effect of blocking p73 functions with the p73β-Δ84 dominant negative mutant, lacking the amino-terminal transcription activation domain, on RA-induced neuronal differentiation of N1E-115 cells. p73β-Δ84 acts as a dominant negative mutant for p73 transcriptional activity on the p21 promoter (Fig. 3E). As shown in Fig. 6A, differentiation of N1E-115 cells with 1 μM RA is accompanied by a 3-fold increase in N-CAM promoter-driven luciferase expression at 24 h. Transfection with p73β results in a 12.5-fold activation, and the co-treatment of p73β-transfected cells with RA results in a greater than 28-fold increase of luciferase activity, showing a cooperative positive effect of p73 expression and RA on the N-CAM promoter (Fig. 6A). When cells were co-transfected with p73β-Δ84, induction of the promoter by RA was completely blocked at all concentrations (Fig. 6B). Because the N-CAM promoter is not induced by p53 in these cells (Fig. 5C) and there is no potentiation of the RA effect by p53 (data not shown), the observed changes in N-CAM activation after RA treatment may be attributed to a selective interference on endogenous p73 functions by the mutant. Thus, p73 transcriptional functions appear to be required for RA to induce N-CAM activation and neuronal differentiation.

Concluding Remarks—Our results indicate that endogenous p73 is induced during neuroblastoma cell differentiation, that overexpression of the p73 isoforms in neuroblastoma cells is per se sufficient to cause neuronal differentiation, and that p73 transcriptional functions are required for RA-induced differentiation of neuroblastoma cells in vitro. Recent evidence indicates that another p53 homologue, p63, is also involved in the differentiation process. However, the specific role of p63 in neuronal differentiation has yet to be elucidated.

Induction of neuronal differentiation of neuroblastoma cells in vitro is accompanied by N-MYC down-regulation (15) and an increase of pRb levels (17). Therefore, we compared the effects of p73 on N-MYC and pRb levels with that of treatment with 1 μM RA (Fig. 5). After transfection with p73β, N-MYC protein levels decreased to levels similar to those of RA-treated cells (Fig. 5). No changes were observed in cells transfected with vector alone or the p53 plasmid. Under the same conditions, an increase of pRb was seen. Immunoblot analysis also confirmed the increase of N-CAM in cells transfected with p73β but not in those transfected with p53 to levels comparable to those obtained with RA-induced differentiation (Fig. 5).

Blockage of p73—In order to demonstrate that p73 is not only sufficient but is indeed required for neuronal differentiation of neuroblastoma cells in vitro, we evaluated the effect of blocking p73 functions with the p73β-Δ84 dominant negative mutant, lacking the amino-terminal transcription activation domain, on RA-induced neuronal differentiation of N1E-115 cells. p73β-Δ84 acts as a dominant negative mutant for p73 transcriptional activity on the p21 promoter (Fig. 3E). As shown in Fig. 6A, differentiation of N1E-115 cells with 1 μM RA is accompanied by a 3-fold increase in N-CAM promoter-driven luciferase expression at 24 h. Transfection with p73β results in a 12.5-fold activation, and the co-treatment of p73β-transfected cells with RA results in a greater than 28-fold increase of luciferase activity, showing a cooperative positive effect of p73 expression and RA on the N-CAM promoter (Fig. 6A). When cells were co-transfected with p73β-Δ84, induction of the promoter by RA was completely blocked at all concentrations (Fig. 6B). Because the N-CAM promoter is not induced by p53 in these cells (Fig. 5C) and there is no potentiation of the RA effect by p53 (data not shown), the observed changes in N-CAM activation after RA treatment may be attributed to a selective interference on endogenous p73 functions by the mutant. Thus, p73 transcriptional functions appear to be required for RA to induce N-CAM activation and neuronal differentiation.

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control of mammalian cell differentiation in a tissue-specific manner (24, 25). p63 null mice fail to maintain the apical ectodermal ridge essential for limb development and have truncated limbs. They also have defective epidermal differentiation, with no hair follicles, teeth, or mammary, lachrymal, or salivary glands. Our data show that, at least in neuroblastoma cells, p73 determines neuronal differentiation. The absence of p73 due to deletion and monoallelic expression in human neuroblastomas, albeit not the primary cause of transformation, could determine the dedifferentiated phenotype of neuroblastomas. p73 expression from its normally silent allele in stage IV-S cases without N-MYC amplification may be involved in the induction of spontaneous regression often observed in this subset of neuroblastoma patients. Although structurally related to p53, p73 also has distinct cellular functional activities (26–29). Both cause growth arrest by acting on similar targets, 

![Fig. 4. Induction of N-CAM and neurofilaments](image-url)
p73 responds to specific differentiation inducers and contributes to neuronal differentiation.

While this article was in preparation, Yang et al. (31) reported in nature the knockout mouse for p73. In agreement with our data, the mice showed neurological defects, among other features.

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