Induction of p73β by a naphthoquinone analog is mediated by E2F-1 and triggers apoptosis in HeLa cells

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Abstract Recently, p73 was identified as a structural and functional homolog of p53. The p73 protein activates the transcription of genes downstream of p53 and induces apoptosis when overexpressed in several cell lines, similar to the tumor suppressor p53. However, the extracellular stimuli and molecular mechanisms regulating p73 activity remain to be elucidated. In this paper, we present evidence that the naphthoquinone analog, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (NA), is a novel apoptotic stimulus that induces p73β expression. Treatment with NA induced the expression of p73β mRNA and protein and its downstream genes, p21 and bax, in HeLa cells. Similar results were obtained in MCF7 cells ($p53^{+/+}$, $p73^{+/+}$). In the MCF7 cells, p53 protein level was rather decreased by NA treatment. Overexpression of p73β led to the apoptosis of HeLa cells and enhancement of NA-induced cell death. Expression of p73β was mediated by E2F-1, which was activated via release from pRB after exposure of cells to NA. We additionally observed that overexpression of pRB inhibited NA-induced apoptosis. These results imply that p53-independent p73β-dependent p21 expression is involved in NA-induced apoptosis of HeLa cells.

Key words: p73β; Naphthoquinone analog; Apoptosis; E2F-1

1. Introduction

Apoptosis is an important regulatory mechanism that eliminates unwanted cells during the development and maintenance of cell homeostasis. In many cases, the apoptotic process requires the p53 tumor suppressor protein. Several stimuli, such as DNA-damaging agents, withdrawal of growth factors and expression of myc or E1A, also cause p53-dependent apoptosis [1–4]. The cellular role of p53 is achieved primarily through its ability to transcriptionally activate specific target genes [5], including the cell cycle regulatory gene, p21 [6], and the apoptosis-regulating gene, bax [7]. Thus, the transcriptional activity of p53 is critical in p53-mediated cell growth and apoptosis. However, the integrity or activity of p53 is impaired in most malignant tumors. Although p53-deficient cancer cells are less responsive to chemotherapy, resistance is not complete, since a proportion of the malignant cells still undergo apoptosis. This p53-independent apoptosis is often accompanied by p21 induction, suggesting the involvement of another tumor suppressor protein [8–10].

Recently, p73 was identified as a p53 homolog [11]. At least four splicing variants of p73 expressed in a tissue-specific manner are recognized, specifically, p73α, p73β, p73γ and p73δ [12]. The significant sequence homology between p73 and p53 suggests that the two proteins perform similar functions [13,14]. Ectopic expression of p73 not only activates the transcription of p53 target genes, but also induces apoptosis and growth suppression in cells, irrespective of their p53 status [11,14,15]. However, a number of differences between p73 and p53 have been reported. p73 differentially transactivates only certain, not all p53-responsive genes [16,17]. The steady-state levels of p73 are not reduced by complex formation with MDM2 [18,19] that induces the ubiquitination and proteolytic degradation of p53 [20–22]. In addition, viral oncoproteins, including adenovirus E1B, SV40 large T antigen and human papilloma E6 that bind wild-type p53 and inhibit activity, do not interact with p73 [23–25]. Furthermore, the levels of p73 are not affected by exposure to DNA-damaging agents (such as actinomycin D or UV irradiation) that increase p53 levels, indicating that the two proteins have distinct cellular functions. An earlier study demonstrated that p73 is involved in cellular response to DNA damage induced by γ-irradiation and chemotherapeutic agents such as cisplatin [26]. However, the extracellular stimuli capable of inducing p73 are yet to be identified.

Menadione (known as vitamin K3) is currently a focus of research interest, since it displays a broad range of antitumor activity in human cells and imposes lower levels of toxicity than other cancer chemotherapeutic drugs with a quinone structure, such as doxorubicin, daunorubicin and mitomycin C. Menadione inhibits cell growth via a cell cycle progression delay (S/G2), alters phosphorylation and activates both p34cdc2 kinase and protein tyrosine phosphatase [27]. A thioether analog of menadione that inhibits cell growth exerts its effects via sulfhydryl arylation of cellular PTPase [28]. In addition, menadione induces G1 arrest by generating superoxide in several cell lines [28]. However, since the hydrophobic na-

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Abbreviations: NA, naphthoquinone analog; GFP, green fluorescent protein

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ture of menadione may generate physicochemical changes in the cell membrane leading to cytotoxicity, several vitamin K3 analogs were recently synthesized. Of these, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (NA) was identified as the most potent growth inhibitor and apoptotic inducer. NA induces G1 arrest through cdc25A phosphatase inhibition in hepatocarcinoma cells [29]. Recently, it was reported that NA not only triggers apoptosis but also restores the tolerance to apoptosis mediated by tumor necrosis factor-α in HeLa cells. We also showed that NA induced the cleavage of p65 (a component of nuclear factor-κB) at the NH_2-terminus via the activation of caspase-3 [30]. We examined the mechanism of NA-mediated cell death under the assumption that p73β is involved in apoptosis of HeLa cells. Our data reveal that p73β is generated at the transcriptional level during NA-induced apoptosis. Expression of p21 and Bax is additionally induced in a p73β-dependent manner. p73β overexpression accelerates NA-induced apoptosis. In addition, induction of the p73β protein is mediated by E2F-1, which is activated through release from pRB (a negative regulator of E2F-1) during NA-induced apoptosis.

2. Materials and methods

2.1. Cell culture, reagents and vector constructs

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C. All reagents used for cell culture were purchased from Gibco BRL (USA). The pcDNA3-HA-p73β construct was generously provided by Dr. Daniel Cauput (Sanofi Recherche, France). From pcDNA3-HA-p73β, GFP-73β was constructed by subcloning into the *Bam*HI/*Xba*I restriction sites of pEGFP-C1.

2.2. Cell viability, apoptotic cells and FACS analysis

Cell viability was determined by the trypan blue exclusion method at different time points. For analysis of apoptosis, HeLa cells were transfected with pEGFP-C1-p73β or pEGFP-C1. After 48 h of transfection, cells were observed under a Nikon fluorescence microscope. Apoptotic cells were identified by their rounded morphology, in contrast to the spread-out morphology of non-apoptotic cells. Apoptotic cell numbers were counted and are presented as a percentage of the total population of cells expressing green fluorescent protein (GFP). The apoptosis of GFP-expressing cells was additionally measured by fluorescence activated cell sorting (FACS) analysis. Cells were trypsinized, washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were re-washed with PBS, incubated with 1 μg/ml RNase for 30 min and stained with 100 μg/ml propidium iodide (Sigma). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson) for calculation of the relative DNA content. The double fluorescence-expressing cells were analyzed for apoptosis.

2.3. DNA fragmentation

Cells were lysed in 400 μl lysis buffer (10 mM Tris–HCl pH 8.0, 100 mM NaCl, 25 mM EDTA pH 8.0, 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/ml proteinase K), and incubated at 50°C for 15 h. Genomic DNA was prepared by phenol/chloroform extraction and precipitated with absolute ethanol. After treatment with RNase A (0.1 mg/ml) for 2 h, the DNA sample was separated by electrophoresis on a 1.5% agarose gel and visualized under UV with ethidium bromide.

2.4. Northern blot analysis

Total RNA was extracted from cells as described previously. RNA (30 μg) was separated on 1% formaldehyde agarose gels, transferred onto nitrocellulose membranes (Hoefer, USA) and hybridized to human p73β cDNA probes labeled with [^{32}P]dCTP by the random primer method, using the manufacturer's instructions (Boehringer Mannheim, Germany). The membrane was hybridized for 24 h at 42°C in a solution of 50% deionized formamide, 5×saline sodium citrate (SSC), 5×Denhardt's solution, 0.5% SDS and 200 μg/ml salmon sperm DNA, and washed twice with 2×SSC and 0.1% SDS at 42°C.

2.5. Immunoprecipitation and Western blot analysis

Cells were lysed in TNN buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride). Total cell lysates (100 μg) were subjected to SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline comprising 0.5% Tween-20 (TBST) with 5% skimmed milk, and incubated with primary antibodies, polyclonal antibody against p73β generated from GST-p73β, monoclonal antibody against p53 (Santa Cruz, sc-126), polyclonal antibody against p21 (Santa Cruz, sc-397), polyclonal antibody against Bax (Santa Cruz, sc-439), and monoclonal antibody against GFP (Roche, 1814 460). Next, membranes were washed with TBST and treated with secondary antibody conjugated to peroxidase. Protein was detected with an enhanced chemiluminescence (ECL) assay using Amersham ECL reagents, followed by exposure to film. For immunoprecipitation, 400 μg total protein was pre-cleaned with 30 μl protein A agarose beads, and incubated on ice for 3 h with fresh protein A beads in the presence of 1 μg polyclonal antibody against pRB (Santa Cruz, sc-050). Immunoprecipitates were washed three times with TNN buffer and subjected to SDS–polyacrylamide gel electrophoresis. The amount of E2F-1 bound to pRB was determined by Western blot analysis, using a polyclonal antibody against E2F-1 (Santa Cruz, sc-192).

3. Results

3.1. p73β is transcriptionally activated during NA-induced apoptosis in HeLa cells

To establish whether NA induces apoptosis, HeLa cells were treated with 15 μM NA for the indicated time periods, and cell viability was examined with trypan blue dye exclusion. Six hours after treatment with NA, cell viability was diminished by 50%, and most of the cells underwent apoptosis within 24 h (Fig. 1A). Apoptosis by NA was confirmed by DNA fragmentation, which was detected faintly at 12 h and strongly at 24 h. DNA fragmentation was also observed in preparations treated with 10 μM NA for 24 h, which increased in a concentration-dependent manner (Fig. 1B). Proteolytic cleavage of poly(ADP ribose) polymerase (PARP) by caspase is a predominant biochemical hallmark of apoptosis. PARP cleavage by NA during the course of apoptosis was examined. Within 24 h of NA treatment, cleavage of PARP into a 89 kDa fragment was observed (Fig. 1C). PARP protein was not detected at 48 h, indicating that most of the protein was proteolytically degraded at this stage. Our results confirm that NA induces the apoptosis of HeLa cells.

To investigate whether p73β is involved in NA-induced apoptosis, protein levels in HeLa cells were measured after exposure to NA. As shown in Fig. 2A, p73β expression began to increase at 12 h after treatment with 15 μM NA in a time-dependent manner. p73β was induced by 10 μM NA and reached maximum levels with 15 μM. An increase in mRNA levels of p73β was additionally noted, indicating that p73β is transcriptionally induced by NA in HeLa cells (Fig. 2B).

The transcription factor p73β stimulates the expression of several genes, including the cyclin-dependent kinase inhibitor, p21, and the proapoptotic factor, bax. To determine whether p73β induced by NA possesses transcriptional activity, the expression of the two downstream genes, p21 and bax, was examined. Protein levels of p21 and Bax increased in concurrence with that of p73β. Northern blot analyses revealed an increase in the mRNA levels of p21 and bax (Fig. 2B). Because p53 protein is inactivated by human papilloma virus 18

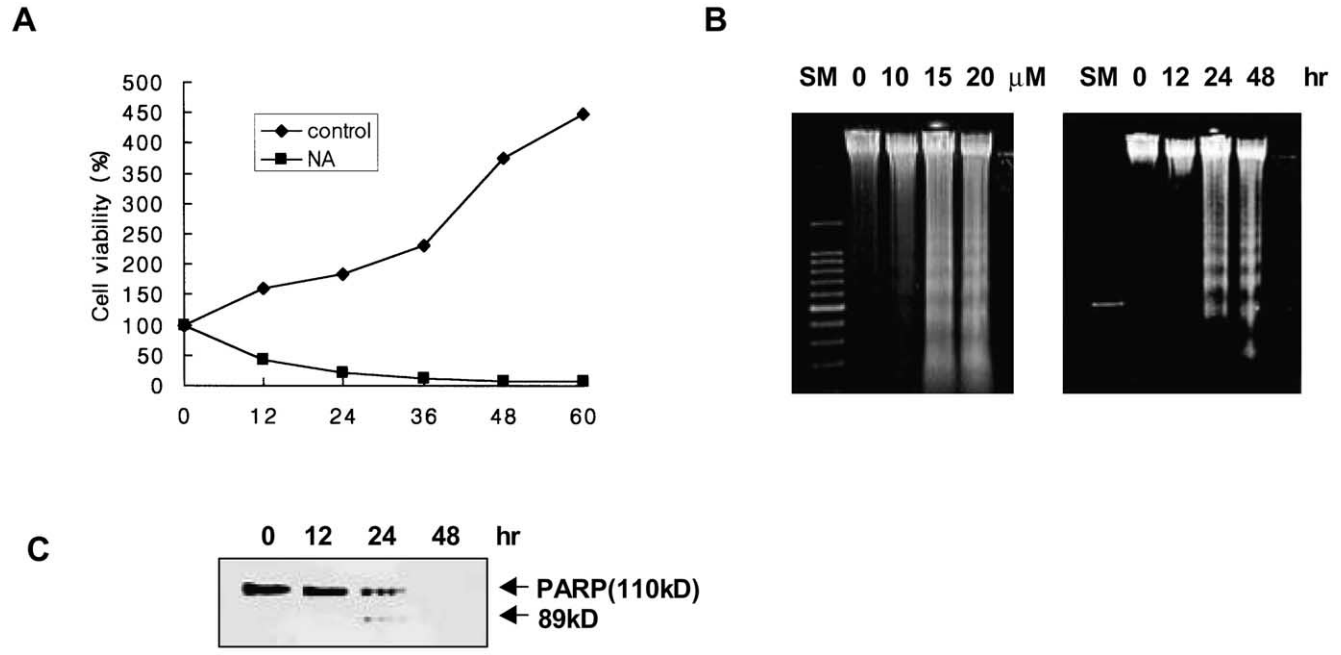

Fig. 1. NA induces apoptosis in HeLa cells. A: HeLa cells were treated with 15 μM NA, and cell viability was determined by trypan blue exclusion at the indicated time points. B: Internucleosomal DNA fragmentation was investigated in a time- and dose-dependent manner. Cells were treated with 0–20 μM NA for 24 h (left panel) or 15 μM NA for the indicated time periods (right panel). Total genomic DNA was prepared, separated on a 1.5% agarose gel and visualized by ethidium bromide staining. C: Cleavage of PARP after treatment with NA was monitored by Western blot analysis using a monoclonal antibody against the C-terminus of PARP.

in HeLa cell, we can rule out the possibility that the induction of p21 and bax may be due to the p53. In order to confirm that NA induced p21 and bax through a p73-dependent, p53-independent pathway, we performed the same set of experiments in human breast carcinoma MCF7 cells, in which both p53 and p73 are wild type. As shown in Fig. 3A, the amount of p73 protein increased significantly after treatment with NA. Levels of both bax and p21 increased, although the increments were not as great as for p73. In contrast to p73, p53 protein levels did not increase, but rather decreased in a time-dependent manner, and disappeared at 48 h after NA treatment (Fig. 3A). mRNA level was also examined and the results were similar to that of HeLa cells (Fig. 3B). These results indicate that the expression of p21 and bax induced by NA is not mediated by p53, but p73β. Therefore, we suggest that p73β participates in NA-induced apoptosis through its transcriptional function.

3.2. Overexpression of p73β accelerates NA-induced apoptosis

To determine whether p73β acts as a positive regulator of

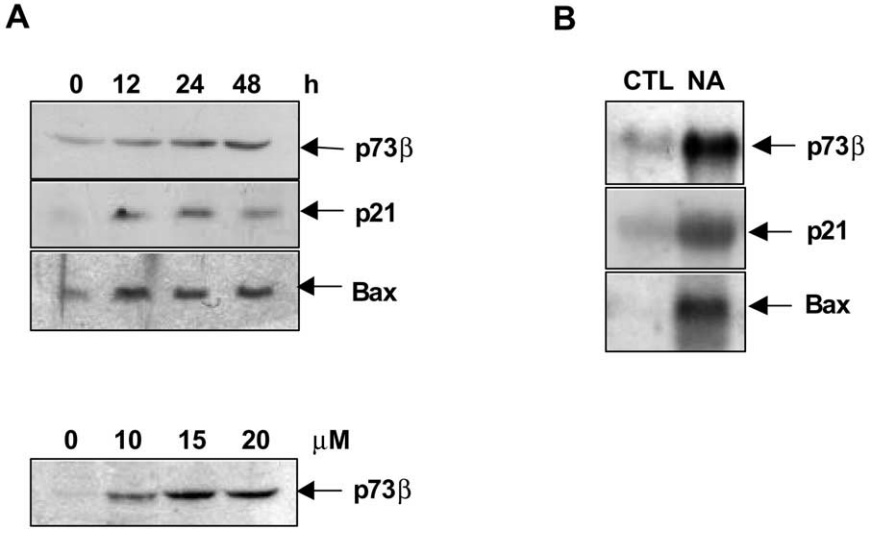

Fig. 2. Expression of p73β and its downstream genes is increased during NA-induced apoptosis in HeLa cells. A: Protein levels of p73β, p21 and Bax were examined by Western blot analysis at the indicated time points after treatment with 15 μM NA or at 24 h after treatment with various doses of NA. B: The mRNA levels of p73β, p21 and Bax were examined by Northern blotting at 24 h.

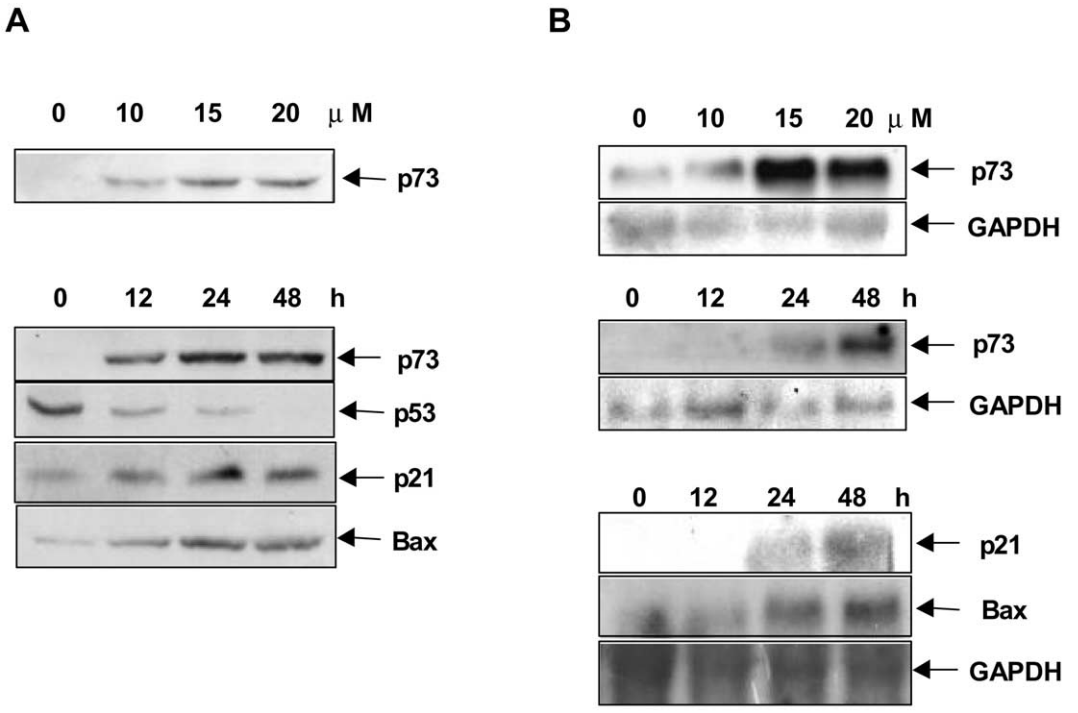

Fig. 3. Expression of p73β and its downstream genes is increased during NA-induced apoptosis in MCF7 cells. A: Protein levels of p73β, p21, Bax and p53 were examined by Western blot analysis at the indicated time points after treatment with 15 μM NA or at 24 h after treatment with various doses of NA. B: Northern blot analyses of p73β, p21 and Bax were performed as a parallel set.

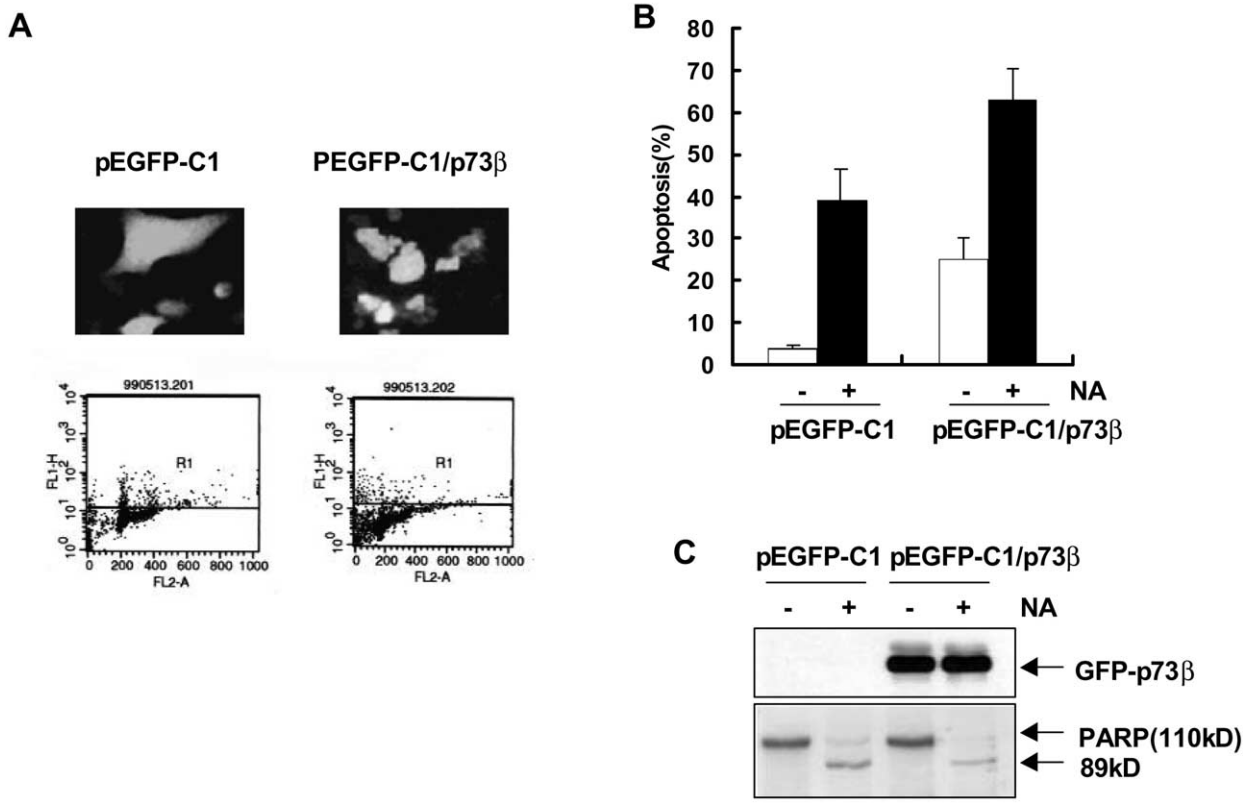

Fig. 4. Overexpression of p73β accelerates NA-induced apoptosis. A: HeLa cells expressing GFP or GFP-p73β were fixed at 48 h post-transfection, stained with propidium iodide and analyzed by FACS. GFP-expressing cells were gated using an arbitrary line (R1), and the DNA content of the sub-G1 fraction was analyzed in cells. B: After transfection of GFP or GFP-p73β into HeLa, cells were treated with NA for 24 h. Apoptosis was determined by the rounded-up morphology of GFP-positive cells under a fluorescence microscope. C: Transfection of p73β was monitored by Western blot analysis using a monoclonal antibody against GFP. Cleavage of PARP in cells treated with NA was also examined by Western blotting.

NA-induced apoptosis, HeLa cells were transiently transfected with an expression plasmid encoding GFP-tagged human p73β. We examined these GFP-positive cells under a fluorescence microscope without fixation to assess the rate of apoptosis in transfected cells. As shown in Fig. 4A (upper panel), GFP-p73β was localized at the nucleus. The nuclei of cells expressing GFP-p73β were apoptotically fragmented. A large number of cells expressing p73β underwent apoptosis, as reflected by the rounding and shrinkage morphology of dying cells. When GFP-p73β-transfected cells were monitored by FACS, over 30% of cells were observed in the sub-G1 phase (Fig. 4A, lower panel). These results indicate that an increase in p73β, which induces downstream gene expression, is sufficient to trigger the apoptosis of HeLa cells.

We examined whether p73β contributes to cell death induced by NA (Fig. 4B). HeLa cells were transfected with GFP-p73β for 36 h and incubated in the absence or presence of NA for 12 h. Apoptosis was determined by counting of rounded or cleaved cells among the cells expressing GFP. The death of GFP-expressing cells was monitored by FACS analysis. As illustrated in Fig. 4B, NA-induced apoptosis was substantially accelerated in cells containing p73β. This apoptosis was confirmed by the cleavage pattern of PARP (Fig. 4C). Our results demonstrate that functionally active p73β plays a key role as a positive regulator during NA-induced apoptosis.

3.3. *p73β induction is mediated by E2F-1 during NA-induced apoptosis*

We investigated the molecular mechanism of p73β induction during NA-stimulated apoptosis. Two distinct mechanisms activating p73 have been demonstrated so far, specifically via c-abl-dependent tyrosine phosphorylation after exposure of cells to γ-irradiation or by transcription factor E2F-1 following T cell receptor hypermethylation. Our data showed that p73β was transcriptionally induced upon treatment with NA. Therefore, the possibility that E2F-1 is responsible for p73β expression during NA-stimulated apoptosis was examined. We measured E2F-1 protein levels after exposure of HeLa cells to NA. The amount of E2F-1 protein in cells was not affected by NA (Fig. 5A).

E2F-1 activity is modulated by the negative regulator, pRB. In normal conditions, E2F-1 is sequestered into an inactive form by pRB. After stimulation, pRB is phosphorylated by cdks, following which E2F-1 is activated by release from pRB. Accordingly, we examined whether E2F-1 was activated by release from pRB, following treatment with NA. Total cell extracts were subjected to immunoprecipitation with pRB antibody, and immunoprecipitates were immunoblotted with E2F-1 antibody. The amount of E2F-1 that bound to pRB was significantly reduced in the presence of NA (Fig. 5A, middle panel). Thus, it is possible that E2F-1 activated by release from pRB induces p73β expression during NA-induced apoptosis. To confirm that E2F-1 triggers endogenous p73β expression, HeLa cells were transfected with this transcription factor and p73β protein levels were examined. Levels of p73β were augmented by the ectopic expression of E2F-1 (Fig. 5B). Examination of p73β protein levels after transfection with pRB revealed that expression was maintained at a basal level. Our results indicate that p73β induced by NA is mediated by E2F-1 at the transcriptional level.

We additionally investigated the effects of pRB on NA-in-

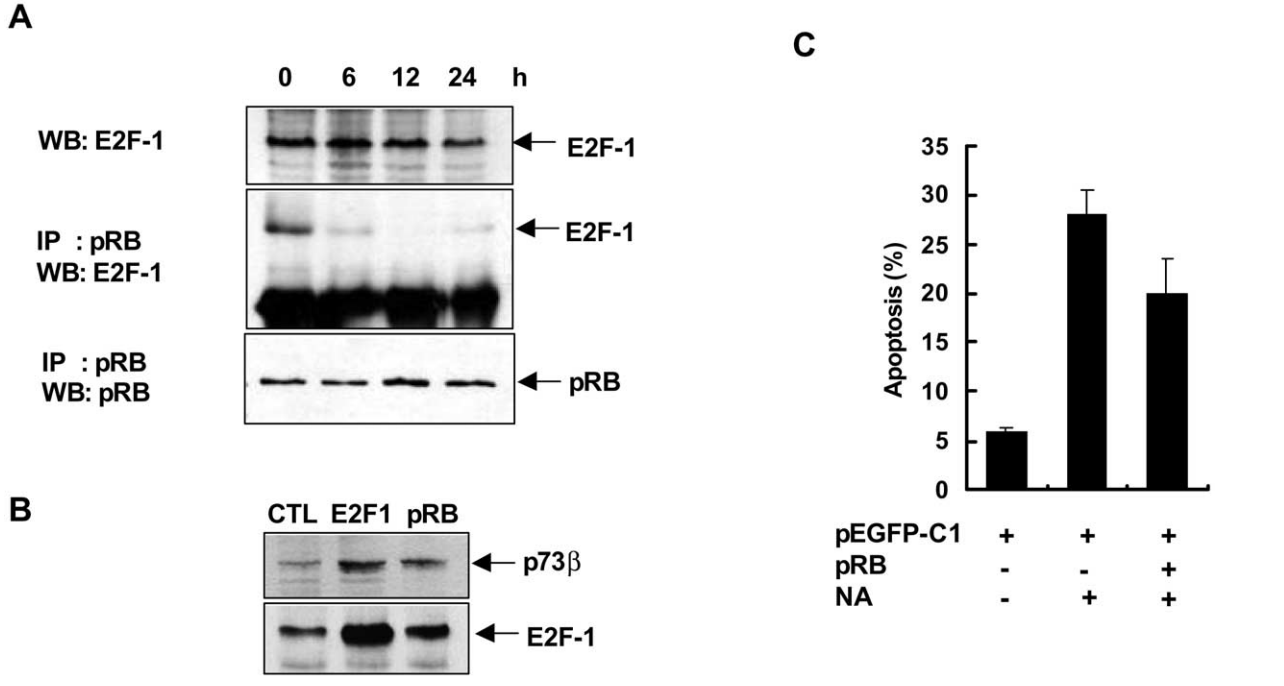

Fig. 5. p73β expression is mediated by E2F-1 during NA-induced apoptosis. A: Expression of E2F-1 was examined by Western blot analysis after treatment with NA (upper panel). Total cell lysates (200 μg) were immunoprecipitated with pRB antibody and the amount of E2F-1 bound to pRB was examined by Western blotting using E2F-1 antibody (middle panel). The same blot was reprobed with an antibody against pRB (lower panel). B: HeLa cells were transfected with E2F-1- or pRB-expressing vector for 48 h, and levels of p73β were monitored by Western blotting. C: Cells were co-transfected with pRB and the GFP-expressing vector. After 48 h, cells were treated with 15 μM NA for 12 h, and apoptosis was determined by observing morphological changes in GFP-expressing cells under a fluorescence microscope.

duced apoptosis in HeLa cells. Overexpression of pRB resulted in a decrease in apoptosis, indicating that the protein protects cells from p73β-mediated death. Expression of pRB alone did not affect cell death (Fig. 5C). Together with the data discussed above, these results suggest that p73β is transcriptionally induced by E2F-1 and functions as a positive regulator of apoptosis via the induction of downstream gene expression during NA-stimulated cell death.

4. Discussion

p73 is a member of the p53 tumor suppressor protein family, which is implicated in development, cellular differentiation, apoptosis and tumor suppression [31]. However, the type of stimuli inducing p73 and the mechanism of regulation of activity are yet to be identified. In this study, we demonstrate that p73β is induced during NA-caused apoptosis of HeLa cells. In turn, p73β mediates in NA-induced apoptosis by transactivating the downstream genes, p21 and bax. Furthermore, p73β induction is mediated by the transcription factor, E2F-1. Overexpression of pRB (a negative regulator of E2F-1) inhibits p73β-induced apoptosis.

Numerous studies show data confirming p53-independent apoptosis, which is sometimes accomplished with p21 induction [8–10]. Since p73 is a structural and functional homolog of p53, the former protein may replace the latter as a tumor suppressor. However, accumulating data report that the functions of these two proteins are not fully interchangeable, since p73 is not induced by various apoptotic stimuli activating p53 [26,32,33]. We observed p53-independent p21 induction during NA-induced apoptosis in breast carcinoma, HeLa cells, and hepatocarcinoma (data not shown). The molecular mechanism of p53-independent p21 expression during NA-induced apoptosis remains to be clarified. In this study, we examined the possibility that p73β is responsible for p53-independent p21 induction and apoptosis by NA in HeLa cells as well as MCF7 cells. Our results confirm that p73β is one of the factors involved in NA-stimulated apoptosis, although the induction of this protein alone is evidently not sufficient to entirely account for the apoptotic effects of NA. Previously, we reported that ceramide-induced apoptosis was accompanied by p53-independent p21 induction and that conversely, p21 potentiated ceramide-induced apoptosis in human hepatocarcinoma cells [34,35]. However, ceramide does not induce p73β expression (data not shown). Therefore, although p53 may be substituted by p73 in our system, it is evident that all p53-independent p21 induction mechanisms do not involve p73.

Despite the structural similarities between p73β and p53, the former protein was not activated by many p53-inducing stimuli. Several stimuli have been shown to induce p73. Cisplatin and γ-irradiation activate p73 via a post-transcription mechanism [26] and through c-abl-mediated tyrosine phosphorylation, respectively [32,33]. A number of recent studies reveal that p73 is induced by E2F-1 and contributes to the apoptotic effects of this transcription factor [36,37]. Moreover, p73 was reported to be induced by oncogenes, which were also mediated by E2F-1 [38]. Data from this study show that p73β is induced through E2F-1, which is activated upon release from pRB during NA-induced apoptosis. We tested the possibility of tyrosine phosphorylation of p73β during apoptosis. However, p73β phosphorylation was not detected in our system, indicating that the c-abl activation pathway is not involved in p73β induction during NA-induced cell death. p73 has several splicing variants, which have different effects on apoptosis. Since only p73β was investigated, we cannot exclude the possibility that other splicing variants are additionally involved in apoptosis triggered by NA.

In summary, p73β is induced through the E2F-1 pathway, which in turn transactivates the downstream genes, p21 and bax, during NA-induced apoptosis. Based on these results, we suggest that NA is one of the apoptotic stimuli that induce p73 along with cisplatin and γ-irradiation.

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