Disruption of actin microfilaments by cytochalasin D leads to activation of p53

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Abstract Activation of p53 plays a central role in the cell's response to various stress signals. We investigated whether p53 is activated upon disruption of actin microfilaments, caused by cytochalasin D (CD). We show that treatment with CD leads to accumulation of p53 in the cells and activation of p53-dependent transcription. Treatment with CD led to arrest of G1-to-S transition in cells retaining wild-type p53, while cells with inactivated p53 showed partial rescue from it. CD also induces apoptosis in p53+/+, but not in p53−/− cells. The obtained data suggest that disruption of the actin microfilaments activates p53-dependent pathways.

Key words: p53; Cytochalasin D; Actin microfilament; Growth arrest; Apoptosis

1. Introduction

p53 plays a critical role in the control of cell growth [1–3]. Being one of the key components of the cell cycle control network, p53 governs the cell's reaction to multiple stress signals such as DNA damage [4,5], inhibition of nucleic acid synthesis [6,7], hypoxia [8] or overexpression of oncogenes [9]. p53 has the capacity to bind to specific DNA sequences and activate transcription of the genes whose promoters contain such p53-responsive elements, so in response to various stress signals p53 accumulates and enhances transcription of these genes [1]. The p53 target genes are capable of facilitating either G1 arrest (*waf1, gadd45*) or apoptosis (*bax* and a set of redox-related *PIG* genes) in the cell affected by the stress conditions [10–14].

Among the conditions that result in activation of p53 are disruption of microtubules and detachment of the cells from the substrate [15,16]. These findings suggest that cytoskeleton integrity, being vital to normal cell functioning, may also be monitored by p53-dependent safeguard mechanisms. This, in turn, allowed us to suggest that disruption of another cytoskeleton system – the actin microfilament network – might also lead to activation of p53.

In this study we have investigated activation of p53 upon treatment of cells with cytochalasin D (CD), which binds to the barbed ends of actin filaments and inhibits both the dissociation and the association of actin monomers at that end. This leads to almost complete disorganization of the actin network and accumulation of numerous foci of short actin filaments in the cells [17]. For this purpose several experimental models were used. To study the induction of p53 we used primary and immortalized fibroblasts retaining wild type (wt) p53. Using two independent reporter genes we examined the effect of CD on the transcriptional activation ability of p53. To address the effect of p53 on growth and survival of the cells treated with CD, we employed two distinct cell systems. For induction of p53-dependent growth arrest we used immortalized fibroblast lines with different states of endogenous p53. Finally, to confirm that the activated p53 is capable of inducing apoptosis, we used a murine fibroblast line which is highly sensitive to p53-dependent apoptosis. We report here that disruption of the actin microfilaments with a specific drug activates p53 in the cells and may lead to p53-dependent growth arrest or apoptosis.

2. Materials and methods

2.1. Cells

The cells used were: (i) normal rat embryo fibroblasts (REF) at passages 2–4 in vitro; (ii) a spontaneously immortalized REF52 rat fibroblast cell line with functional wt p53 and its derivatives; (iii) the REF52/GSE22 subline expressing genetic suppressor element #22 (GSE22), a short fragment of rat p53 cDNA corresponding to the C-terminal fragment of p53 which inhibits p53 function via a dominant negative mechanism [18]; and (iv) REF52/mdm-2/2, overexpressing exogenous murine *mdm*-2 proto-oncogene. The *mdm*-2 product is capable of binding to p53 and suppressing its transcriptional activation potential [19]. (v) For the chloramphenicol acetyltransferase (CAT) assay, we used the REF52-CAT subline, constitutively expressing CAT under the control of the p53-responsive promoter from the *waf1* gene [20]. (vi) Murine wt-p53-positive immortalized fibroblasts (line 12(1)ConA) expressing bacterial β-galactosidase (β-gal) governed by p53-responsive promoter [21] (kindly provided by Dr. M.V. Chernov, Cleveland Clinic Foundation, Cleveland, OH, USA); (vii) immortalized murine fibroblasts (line 10(1)) which has a homozygous deletion of the p53 gene [22]; (viii) *E1A+ras*-transformed mouse embryo fibroblasts (MEF) from p53+/+ and p53−/− mice. The p53+/+ cell line C8 is highly susceptible to apoptosis caused by various DNA damaging agents while its p53−/− counterpart (A4) is highly resistant to such treatments [23].

The cells were maintained in DMEM medium (all chemicals were from Sigma, USA unless otherwise indicated) supplemented with 10% fetal calf serum (Gibco-BRL, UK).

The doubling time of all the REF52 sublines, 12(1)ConA and 10(1) cell lines was approximately 24 h; that of the C8 and A4 cell lines was approximately 18 h.

2.2. CD treatment

CD was added to the culture medium at final concentrations of 0.25–0.4 μm/ml 1–3 h after seeding. These doses are considered small,

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Abbreviations: REF, rat embryo fibroblasts; MEF, murine embryo fibroblasts; CD, cytochalasin D; wt, wild type; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; 5-BrdU, 5-bromodeoxyuridine; β-gal, β-galactosidase

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and their effect is completely reversible [24]. The duration of treatment is indicated for indivudal experiments.

2.3. Actin staining

Cells were seeded onto glass coverslips at 10^4 cell/cm^2 and treated with 0.25 μg/ml CD for 72 h or left untreated. The coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 in PBS. For staining, cells were incubated with TRITC-labelled phalloidin in a moist chamber.

2.4. Western blotting

Cells (10^7) were washed twice with cold PBS and lysed in 0.5 ml buffer containing 20 mM HEPES (pH 7.6), 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF and 100 μg/ml aprotinin. Nuclei obtained by low-speed centrifugation were shaken for 45–60 min in the same buffer supplemented with 500 mM NaCl. After centrifugation at 10 000 rpm for 10 min, nuclear extracts were separated by SDS-PAGE. Proteins were transferred onto ECL membranes, probed with p53-specific monoclonal antibody PAb421, and developed using ECL Western blotting kit (Amersham, UK).

2.5. CAT assay

The CAT assay was performed as described [19]. Quantitative measurements of CAT activity were done by densitometry using Ultroscan XL (LKB, Sweden).

2.6. Analysis of 5-BrdU incorporation

Cells were seeded onto glass coverslips at 10^5 cells/cm^2 and treated with 0.25 μg/ml CD for 72 h or left untreated. At the end of treatment 5-bromodeoxyuridine (5-BrdU) (Serva, USA) was added to the medium at a final concentration of 10 mM for 5 h. The cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 in buffer M (50 mM imidazole, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, pH 6.8), containing 4% polyethylene glycol M_r 40 000, and hydrolyzed with 4 N HCl. The cells were subsequently incubated with anti-5-BrdU antibodies and TRITC-conjugated anti-mouse IgG secondary antibodies (Chemicon, USA). To visualize all nuclei, the cells were counterstained with DNA-specific Hoechst dye #33258 (Polysciences, USA).

2.7. β-Galactosidase staining

β-Gal staining was performed as described [20].

2.8. Apoptotic DNA ladder assay

The cells ($1.5–3\times10^6$) were scraped into centrifuge tubes, washed with PBS and pelleted by centrifugation. Cell pellets were then suspended for 30 min on ice in 200 μl of lysis buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.2% Triton X-100). After centrifugation the supernatant was collected, treated with proteinase K (0.1 mg/ml for 1.5 h at 37°C) and extracted with phenol/chloroform and chloroform. The DNA was precipitated by adding ammonium acetate (final concentration 2 M) and ethanol, treated with RNase A (final concentration 0.1 mg/ml) for 1.5 h and separated by electrophoresis in 1% agarose.

3. Result

3.1. Cytochalasin D-induced disruption of actin cytoskeleton causes increased transcriptional activity of p53

In control REF52 cells numerous thick straight bundles of actin filaments were present. The comparatively low doses of CD that we used disrupted these structures, and in CD-treated cells actin was accumulated in multiple aster-like aggregates of short filaments. The actin structures in REF52/GSE22 and REF52/mdm-2/2 cells were similar to those found in REF52 cells (Fig. 1).

To test whether p53 accumulates in the cells upon application of CD, we performed Western blot analysis of nuclear lysates of the cells. As demonstrated in Fig. 2a, primary REFs as well as immortalized REF52 fibroblasts showed increased

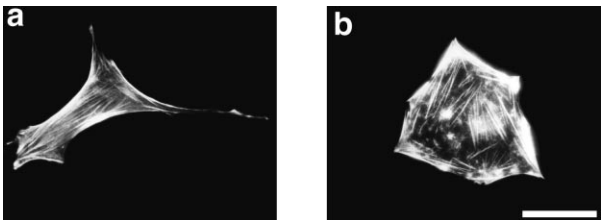

Fig. 1. Control (a) and CD-treated (b) REF52 cells stained for actin. CD transforms bundles of actin filaments into actin aggregates distributed irregularly within the cell. Bar, 50 μm.

intracellular content of wt p53 after treatment with CD for 24 and 48 h.

To investigate directly whether increased amounts of p53 correlate with its higher function activity, we tested transcriptional activation by p53 of two reporter genes governed by p53-responsive promoters. Treatment of REF52-CAT cells bearing the CAT reporter construct with CD at concentrations of 0.2–1 μg/ml resulted in an approximately six-fold activation of the enzyme (Fig. 2b). CD applied at concentrations of 0.4–0.8 μg/ml produced maximal activation of p53.

We also used the β-gal reporter system which allowed us to monitor p53 activity in individual cells. 12(1)ConA cells were

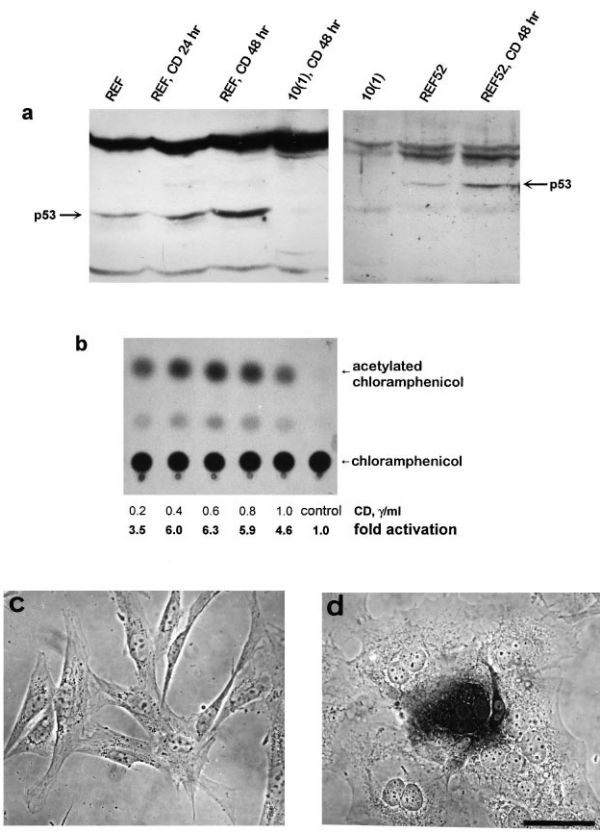

Fig. 2. CD causes activation of p53. a: Western blot of nuclear lysates shows accumulation of p53 in REF and REF52 cells after treatment with CD at 1 μg/ml for 24 and 48 h. In p53-deficient 10(1) cells no protein was detected in either control or CD-treated cultures. b: CAT assay of REF52-CAT cells expressing p53-responsible reporter. The cells were treated with the drug at the indicated concentrations for 36 h. c, d: Control (c) and CD-treated (d) 12(1)ConA cells stained for β-gal reporter activity. c: no visible staining. d: two β-gal-positive cells in the center of the cluster. The majority of the cells contain two nuclei. Bar, 50 μm.

treated with 0.4 μg/ml CD for 72 h, and then stained for β-gal activity. CD treatment led to an overall increase in staining (51.3% of the cells stained positively compared to 2.4% in control), although the degree of staining varied considerably between individual cells (Fig. 2c,d).

3.2. CD treatment leads to inhibition of S-phase entry

For further demonstration of enhanced p53 activity upon CD treatment we tested the cellular effects of p53 activation. For this purpose, we monitored the incorporation of 5-BrdU into cellular DNA in the unsynchronized REF52 cell line and in its sublines REF52/GSE22 and REF52/mdm-2/2. Untreated cells did not differ significantly in their ability to enter S phase – approximately 50% of the cells of all the lines stained positively for 5-BrdU after incubation of 2-day-old cultures with 5-BrdU for 5 h. Treatment with CD caused a reduction in 5-BrdU-positive cell number in all REF52 sublines. The effect of the drug was seen within 24 h and reached its peak at 72 h when S-phase entry in REF52 cells was practically blocked. Upon similar treatment the sublines with blocked p53 function continued cycling, although more slowly compared to the control (Fig. 3a). After 72 h of CD treatment the cells often formed additional nuclei as a result of CD-blocked cytokinesis. However, we did not observe any correlation between the cell's ability to enter S phase and the number of nuclei. Interestingly, the percentage of 5-BrdU-positive cells after CD treatment was two-fold higher in REF52/mdm-2/2 than in REF52/GSE22 cell line (Fig. 3a). Recent evidence reveals interaction between mdm-2 and Rb proteins leading to inhibition of Rb growth regulatory function [25]. In this light our

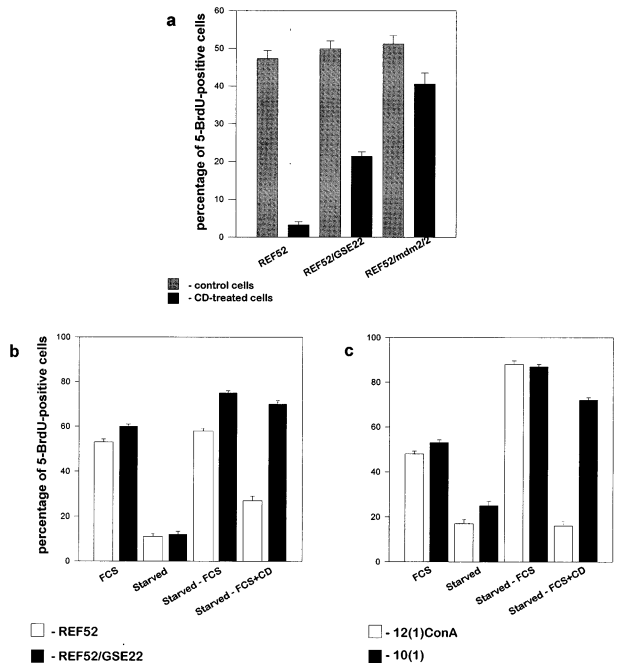

Fig. 3. CD leads to p53-dependent growth arrest in cells. a: Histogram showing the percentage of 5-BrdU-positive REF52 (p53+/+), REF52/GSE22 and REF52/mdm-2/2 (blocked p53 function) cells in control cultures (gray) and after treatment with CD for 72 h (black). For each point, more than 500 cells were scored. b, c: Histogram showing the percentage of 5-BrdU-positive cells in synchronized culture of REF52 and REF52/GSE22 (c) and 12(1)ConA (p53+/+) and 10(1) (p53−/−) (d) after treatment with CD for 15 h.

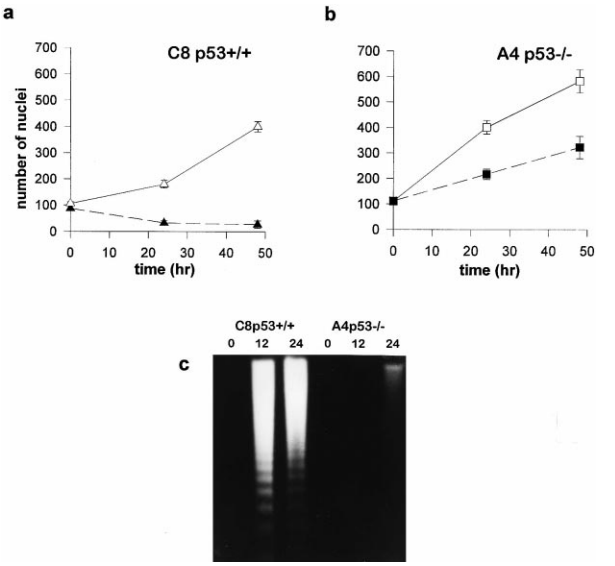

Fig. 4. CD leads to p52-dependent apoptosis. Growth and apoptosis rates of C8 (p53+/+) (a) and A4 (p53−/−) (b) cells in control (open squares/triangles) and during CD treatment (filled squares/triangles). Cell quantitation was done by counting the nuclei of the cells attached to the coverslips. The nuclei were stained with fluorescent Hoechst dye #33258. In the course of the CD treatment, p53−/− cells continue to proliferate, although the rate of proliferation decreases. The number of p53+/+ cells decreases rapidly. c: Electrophoresis of low molecular weight DNA isolated from C8 p53+/+ (lanes 1–3) and A4 p53−/− cells (lanes 4–6). Numbers indicate the duration of the CD treatment (hours). The apoptotic DNA ladder is visible in the C8 p53+/+ cells growing in the presence of CD for 12 and 24 h (lanes 2 and 3) while only traces of degraded DNA are present in A4 p53−/− cells after CD treatment for 24 h.

results may be interpreted as a cumulative effect of inactivating both p53 and Rb by mdm-2.

To determine if G1-to-S transition might be arrested in CD-treated cells, we analyzed the incorporation of BrdU into synchronized cell cultures treated with CD. The cells were synchronized by 48 h serum withdrawal, after which, in 1 h after addition of the serum to the cultures, CD and BrdU were added to the cells, and the cultures were incubated further for 15 h.

Fifteen hours is less than the doubling time for the cell lines we used (approximately 24 h), and the majority of the cells (95% of the REF52 and REF52/GSE22 and 90% for 12(1)ConA and 10(1)) remained mononuclear. When CD was added to the synchronized cultures, p53+/+ cells in both pairs (REF52 and 12(1)ConA lines) did not enter S phase during the 15 h of the treatment, while the cells lacking p53 activity (REF52/GSE22 and 10(1) lines) were not arrested and commenced DNA synthesis (Fig. 3b,c). These results allowed us to suggest that p53 was activated shortly after addition of CD to the cultures, and that the p53-mediated cell cycle arrest occurred immediately thereafter, in the G1 phase.

3.3. Induction of apoptosis by CD is dependent on the p53 state

Another known effect of p53 activation is induction of apoptosis. However, the choice between p53-dependent cell cycle arrest and apoptosis depends largely on the cell type. To check whether treatment with CD might in fact induce p53-dependent apoptosis, we used MEFs transformed with E1A plus *ras* oncogenes, which undergo apoptosis rather

than growth arrest upon activation of p53 [23]. p53−/− MEFs transformed with the same oncogenes served as a negative control.

In untreated cells of both lines actin filaments were scarce and relatively thin. CD caused complete degradation of the filaments and accumulation of the small actin aggregates in the cytoplasm.

The control p53+/+ C8 and p53−/− A4 cell lines had similar growth characteristics; however, upon application of CD the difference became dramatic. Since CD leads to the appearance of multinuclear cells in culture, we monitored the number of nuclei of the cells attached to the substrate per unit area, rather than the cell number. In the course of the treatment the C8 p53+/+ cells readily underwent apoptosis, and by 48 h the number of nuclei remaining on the substrate constituted 6.3% of their number at 0 h (Fig. 4a). Most of those nuclei were stained picnotically. The number of nuclei of the A4 p53−/− cells decreased from 100% at 0 h to 55.4% at 48 h (Fig. 4b). By 48 h most of the p53−/− cells contained 2–4 nuclei, and they retained their size and shape, showing no signs of apoptosis.

Apoptosis is usually accompanied by internucleosomal cleavage of genomic DNA. After treatment with CD, the C8 p53+/+ cells contained large amounts of low molecular weight DNA migrating on agarose gel as a characteristic apoptotic ladder. The DNA fragmentation was seen as early as 12 h after application of the drug. In contrast, the A4 p53−/− cells contained only traces of degraded DNA after CD treatment for 24 h (Fig. 4c).

4. Discussion

In this work we have shown that in response to CD cells accumulate functionally active p53 and consequently undergo cell cycle arrest or apoptosis. The cells with deleted p53, or where the p53 function is blocked by either introduction of a dominant negative GSE or overexpression of mdm-2, failed to undergo growth arrest and formed additional nuclei in the presence of CD. We also showed that cells expressing endogenous wt p53, which are highly prone to p53-mediated apoptosis, react to CD by undergoing apoptosis. In contrast, similar p53−/− cells did not die by apoptosis, but became multinuclear.

Earlier studies reported that low doses of cytochalasin induce cell cycle arrest in G0/G1 but not in G2 phase [26–28]. In this work we showed that this arrest is mediated by p53. When the cells were synchronized in G0 by serum withdrawal and then treated with CD for 15 h, cells lacking functional p53 or with p53 lost through mutations passed G1 phase and commenced DNA synthesis. In contrast, the cells containing wt p53 were arrested in G1 and did not enter S phase.

CD impairs the cell's ability to undergo cytokinesis, yet the mitotic spindle remains intact. This means essentially normal segregation of chromosomes and filial nucleus formation. Therefore, treatment of the cells with CD results in formation of multinuclear cells in culture [26,29,30]. In our recent study we have shown that spontaneously derived or colcemid-induced micronuclei cause activation of p53 with strong dependence on their number [31]. Considering this the appearance of additional nuclei in CD-treated cells seems a plausible candidate for the signal triggering p53 activation. However, 15-h incubation with CD was not long enough for most cells to complete the cycle and pass through mitosis, so the majority (90–95%) of cells stayed mononuclear. Thus, this experiment allowed us to conclude that the CD-induced p53 activation is independent of the nucleus number.

Therefore, it is likely that activation of p53 in CD-treated cultures is due to some consequence of actin cytoskeleton disintegration in both mononuclear and multinuclear cells. In particular, disruption of actin filaments by CD results in a decrease of cortical tension in the cell that might be accompanied by a decrease in DNA synthesis [32]. Another possibility is that p53 activation is a result of shortening of focal adhesions associated with poorer cell spreading and attachment. This effect of low doses of CD is well known [24,33]. The latter hypothesis is consistent with the observation that detachment from the substrate causes apoptosis in the C8 p53+/+ cell line [16], which was also used by us.

Breaking the actin filament network with a specific drug causes p53 activation and consequent growth arrest or apoptosis. On the whole, our experiments stress the importance of the actin cytoskeleton in regulation of cell proliferation.

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