Doxorubicin induces senescence or apoptosis in rat neonatal cardiomyocytes by regulating the expression levels of the telomere binding factors 1 and 2

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Spallarossa P, Altieri P, Aloi C, Garibaldi S, Barisone C, Ghigiotti G, Fugazza G, Barsotti A, Brunelli C. Doxorubicin induces senescence or apoptosis in rat neonatal cardiomyocytes by regulating the expression levels of the telomere binding factors 1 and 2. Am J Physiol Heart Circ Physiol 297: H2169–H2181, 2009. First published October 2, 2009; doi:10.1152/ajpheart.00068.2009.—Low or high doses of doxorubicin induce either senescence or apoptosis, respectively, in cardiomyocytes. The mechanism by which different doses of doxorubicin may induce different stress-response cellular programs is not well understood. A recent study showed that the level of telomere dysfunction may induce senescence or apoptosis. We investigated the pathways to both apoptosis and senescence in neonatal rat cardiomyocytes and in H9c2 cells exposed to a single pulsed incubation with low or high doses of doxorubicin. High-dose doxorubicin strongly reduces TRF2 expression while enhancing TRF1 expression, and it determines early apoptosis. Low-dose doxorubicin induces downregulation of both TRF2 and TRF1, and it also increases the senescence-associated-β-galactosidase activity, downregulates the checkpoint kinase Chk2, induces chromosomal abnormalities, and alters the cell cycle. The involvement of TRF1 and TRF2 with apoptosis and senescence was assessed by short interfering RNA interference. The cells maintain telomere dysfunction and a senescent phenotype over time and undergo late death. The increase in the phase >4N and the presence of micronuclei and anaphase bridges indicate that cells die by mitotic catastrophe. p38 modulates TRF2 expression, whereas JNK and cytoplasmic p53 regulate TRF1. Pretreatment with specific inhibitors of MAPKs and p53 may either attenuate the damage induced by doxorubicin or shift the cellular response to stress from senescence to apoptosis. In conclusion, various doses of doxorubicin induce differential regulation of TRF1 and TRF2 through p53 and MAPK, which is responsible for inducing either early apoptosis or senescence and late death due to mitotic catastrophe.

p53; mitogen-activated protein kinases; anthracyclines

THE CLINICAL USE OF ANTHRACYCLINES in anti-cancer treatment is limited by their adverse cardiotoxic effects, which include cardiomyopathy and heart failure (22). At high doses, cardiotoxicity often occurs within a few months, whereas low doses rarely cause deterioration of ventricular function in the first year after therapy (58). However, there is now increasing evidence that, several years after therapy, one of three patients treated with low-dose anthracyclines develops hypokinetic cardiomyopathy (21). A number of mechanisms have been proposed to explain anthracycline cardiotoxicity. Although the most accredited hypothesis states that anthracyclines induce myocyte loss through oxidative stress and apoptotic cell death (3, 48), there is controversy whether apoptosis contributes to late onset cardiotoxicity induced by low doses of doxorubicin (4). Maejima et al. (34) recently showed that when cultured neonatal rat cardiomyocytes are exposed to low concentrations of doxorubicin, the cells do not enter apoptotic program but exhibit a senescence-like phenotype. The hallmark of cellular senescence is the cell cycle arrest that is accompanied by important changes in many aspects of cell morphology (20, 38). Senescence is the result of changes in the expression of many proteins that regulates cell cycle, cytoskeletal function, and cellular architecture and causes impairment of cell functions, including the regenerative capacity (19, 30, 53, 57).

Studies performed on tumor cells indicate that low doses of doxorubicin, as well as several anti-cancer agents, induce mitotic catastrophe, a phenomenon that is characterized by chromosomal abnormalities and abnormal mitosis that leads to late cell death. It also has been shown that cells that ultimately die of mitotic catastrophe initially show a senescence-like phenotype (18). Therefore, the induction of senescence has been proposed as a novel mechanism of cardiotoxicity induced by low doses of doxorubicin (34).

A number of studies suggest that telomere dysfunction plays a main role in the stress-induced senescence program and in apoptosis (29). Telomeres are specialized, repetitive, noncoding sequences of DNA bound by several proteins, including telomere binding factors 1 and 2 (TRF1 and TRF2), which play a crucial role in telomere biology and govern chromosomal stability. The TRF1 multiprotein complex regulates telomere length and specifically affects mitotic progression, whereas TRF2 is critical for maintaining the telomere t-loop “end-capping” structure, whose function is to prevent chromosome end-to-end fusion and chromosome abnormalities (50, 55).

It is believed that telomeric dysfunction beyond a certain limit triggers a DNA damage response mediated by p53, a tumor suppressor protein, and by mitogen-activated protein kinases (MAPKs) (23, 47). MAPKs are highly conserved serine/threonine kinases that are activated in response to a wide variety of stimuli and play a role in numerous cell functions including survival, growth, and proliferation (54, 61, 62). Both p53 and MAPKs are involved in doxorubicin-induced cardiotoxicity (31, 49). The signal transduction pathways of doxorubicin-induced senescence and the mechanism by which different doses of the same stressing agent may induce either apoptosis or senescence and mitotic catastrophe are still poorly understood.

In the present study we investigated the cellular response to stress that occurs in rat neonatal cardiomyocytes exposed to a pulsed incubation with apoptotic and subapoptotic concentrations of doxorubicin. Our results confirm that subapoptotic

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doses of doxorubicin induce senescence, and, for the first time, we have demonstrated the following: 1) cardiomyocytes with a senescence-like phenotype undergo late cardiac death by mitotic catastrophe; 2) various doses of doxorubicin induce a differential regulation of TRF1 and TRF2 that is associated with the induction of either apoptosis or senescence; 3) p53 and MAPK activation is not only downstream to telomere dysfunction, as suggested by previous literature, but is also a doxorubin-induced signal that affects TRF1 and TRF2 expression; and 4) increases in p53 expression levels, as well as the cytoplasmic accumulation of p53, which are the result of low doses of doxorubicin, downregulate TRF1 and play an active role in inducing senescence, whereas the inhibition of p53 results in a transition of the cellular response to doxorubicin treatment from senescence to apoptosis.

**MATERIALS AND METHODS**

All materials, unless otherwise indicated, were supplied by Sigma-Aldrich (Poole, UK).

**Cell and Culture Conditions**

Ventricular myocytes from 2-day-old Sprague-Dawley rats were purchased (Lonza) and cultured as described (2). H9c2 rat heart-derived embryonic myocytes (American Type Culture Collection) were cultured as previously described (49). Cells were always used at <70% of confluence.

Cells were preincubated for 1 h with or without the ERK1/2 pathway inhibitor PD-98059 (50 μM; Calbiochem), the JNK inhibitor SP-600125 (20 μM), or the p38 MAPK inhibitor SB-203580 (3 μM), and with the p53 inhibitor pifithrin-α (PFT; 5 μM) (31). They were then incubated with or without different doses of doxorubicin for 3 h (14) and analyzed at the time indicated for each experiment. To evaluate MAPK phosphorylation, we incubated cells with various doses of doxorubicin for 20 min. Since both PFT and the MAPK inhibitors were dissolved in 0.1% dimethyl sulfoxide, an equivalent amount of vehicle was added to both the control and the drug-treated samples when the experiments were performed with these inhibitors.

**Reverse Transcriptase-Polymerase Chain Reaction**

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the previously described procedure (49). The quantity of mRNA was normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Amplification of the cDNA by PCR was performed using the primers shown in Table 1. The PCR products were quantified by a gel documentation system with analysis software (Syngene).

**Immunoblotting**

Immunoblotting was performed using the previously described procedure (49). After various treatments, cells were processed to determine the levels of TRF2 (clone 4S794.15; Imgenex), TRF1 (C-19; Santa Cruz Biotechnology), p53 (clone C6A5; BioVision), α-tubulin (B-7; Santa Cruz Biotechnology), the phosphorylated MAPK p-p38 (D-8; Santa Cruz Biotechnology), p-ERK (E-4; Santa Cruz Biotechnology), and p-JNK (G-7; Santa Cruz Biotechnology). The quantity of protein was normalized for GAPDH (0411; Santa Cruz Biotechnology).

**Senescence-Associated β-Galactosidase Activity**

Cells were stained for β-galactosidase activity as described by Dimri et al. (16). The number of senescence-associated β-galactosidase staining (SA-β-gal)-positive cells was determined in 100 randomly chosen low-power fields (×100) and expressed as a percentage of all counted cells (35).

**Annexin V-FITC/Propidium Iodide Staining**

Cells were labeled with annexin V-FITC and propidium iodide (A/PI), and 100 randomly selected fields were counted using a fluorescence microscope (48). The number of stained cells was normalized to the total number of cells as counted by phase-contrast microscopy of the same field. Images from independent fields were captured and processed using MetaMorph software (Universal Imaging, Downingtown, PA).

**Trypan Blue Exclusion**

Trypan blue exclusion was used to determine the viability of cardiomyocytes. After treatments as described above, cells were trypsinized and incubated with 0.4% trypan blue dye for 2 min and observed with the use of a hemocytometer under a light microscope. Cells that were able to exclude the stain were considered viable, and the percentage of non-blue cells over total cell number was used as an index of viability.

**Cell Immunofluorescence**

TRF1, TRF2, and p53 expression were all documented by immunofluorescence as described by Verzola et al. (56). Cardiomyocytes were grown on chamber slides to subconfluence and incubated for 3 h in the presence or absence of doxorubicin in cells that had been pretreated with or without MAPK inhibitors, after which they were analyzed. Subsequently, the cells were washed with cold PBS and fixed in 2% paraformaldehyde for 10 min. After an overnight incubation with anti-TRF1, anti-TRF2, or anti-p53 antibodies at 4°C, cells were washed extensively with PBS and exposed to biotinylated conjugated secondary antibody (Vector) for 30 min. After being washed, cells were incubated with FITC-streptavidin (Sigma) for 30 min. Slides were observed under a fluorescence microscope. Images from independent fields were captured and processed using the MetaMorph software.

**Flow Cytometric Analysis**

**Nuclear DNA content.** Trypsinized and floating cells were pooled, washed twice with PBS, and resuspended in 400 μl of hypotonic

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**Table 1. PCR primer sequences, PCR product size, and GenBank accession number**

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR Primer Sequences, 5'-3' Forward and Reverse</th>
<th>PCR Product Size, bp</th>
<th>GenBank Accession No.</th>
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<td>RNU65656</td>
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<tr>
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<td>146</td>
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<td>Bcl-2</td>
<td>ACGCCAGGAGAATCAGAGAACG</td>
<td>308</td>
<td>NM_016993</td>
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</tbody>
</table>

TRF1 and TRF2, telomere binding factors 1 and 2.
solution labeled with PI [5 μg/ml PI, 0.1% (wt/vol) Na-citrate, and 0.1% Triton X-100 in sterile water]. Cells were incubated on ice for 30 min until DNA content analysis. 

PI labeling. Trypsinized and floating cells were pooled, washed twice with PBS, and suspended in PI solution. All test cells were monitored by fluorescence-activated cell sorting (FACS; Becton-Dickinson), and data were analyzed using CellQuest software (Becton Dickinson).

Chromosome Analysis

Twenty-four hours after treatment with doxorubicin, cells were exposed to colcemid (0.04 μg/ml) for 90 min at 37°C and to hypotonic treatment (0.075 M KCl) for 15 min at room temperature. Cells were fixed in a methanol and acetic acid (3:1 by volume) mixture for 15 min then washed three times in the fixative. The slides were air-dried and stained with Giemsa for analysis (37).

F-Actin Detection

Cardiomyocytes growing on slides were fixed, permeabilized, and labeled simultaneously in PBS containing 50 μg/ml lysopalmitylphosphatidylcholine, 3.7% formaldehyde, and 5 U/ml fluorescent phalloxin (A-12379 Alexa488 phalloidin; Molecular Probes). Cells were rapidly washed three times with PBS and viewed by fluorescent microscopy. To quantify the fluorescence and to measure cells area, we performed image analysis using the Leica Q500 MC image analysis system (Leica, Cambridge, UK). Three hundred cells were randomly analyzed for each sample, and the optical density of the signals was quantitated by a computer. Single images were digitized for image analysis at 256 gray levels. Imported data were quantitatively analyzed using Q500MC Software-Qwin (Leica). The single cells were randomly selected by the operators by using the cursor, and then positive areas were automatically estimated. Constant optical threshold and filter combination were used.

TRF1 and TRF2 siRNA Transfection

ON-TARGETplus SMARTpool short interfering RNAs (siRNA), for silencing the expression of target genes TRF1 and TRF2, and ON-TARGETplus Non-targeting Pool as a control were purchased from Dharmacon. All transfections were carried out using the manufacturer’s protocol with DharmaFECT1 transfection reagent (Dharmacon). Briefly, cardiomyocytes were trypsinized, counted, and plated at a density of 10^6 cells/cm². After 24 h, cells were transfected with 10, 50, or 100 nM of SMARTpool siRNA or control siRNA using DharmaFECT1 reagent and analyzed after 24, 48, or 72 h with A/PI staining, SA-β-gal activity, and Western blot analysis.

Statistical Analysis

Data are means ± SE of four independent experiments. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni post hoc test and Wilcoxon signed rank test when appropriate.

RESULTS

Low and High Doses of Doxorubicin Have Different Effects on Plasma Membrane Integrity and Bax-α/Bcl-2 Protein Ratio

Twenty-four and forty-eight hours after having been exposed to doxorubicin for 3 h, A/PI double staining revealed the typical pattern of early-stage apoptosis [A(+)/PI(-)] and late-stage apoptosis [A(+)/PI(+)] only in cells that had been exposed to 1 μM doxorubicin (Fig. 1, A and B). After

Fig. 1. Low-dose doxorubicin induces nonapoptotic cardiac damage. A: representative photographs of annexin-V (A)/propidium iodide (PI) staining of neonatal rat cardiomyocytes treated with 2 different doses (0.1 or 1 μM) of doxorubicin. ct, Control. B: percentage of A and/or PI-positive (+) cells at the indicated doses and time points (magnification, ×200). C: mRNA Bax and Bcl-2 expression 6 h after treatment with 0.1 or 1 μM doxorubicin. Histograms illustrate the mRNA Bax/Bcl-2 expression ratio. *P < 0.05 vs. ct.
treatment with 0.1 μM doxorubicin, we observed that the percentage of A(+) cells was lower, and that 86% of the A(+) cells were PI(+) at 24 h, suggesting that the integrity of the plasma membrane was precociously lost. As a further apoptosis index, we measured the Bax-Bcl-2 ratio, which increased threefold after treatment with 1 μM doxorubicin, whereas it remained unchanged in cells treated with 0.1 μM doxorubicin (Fig. 1C). Enhancement of the ratio indicates that cells underwent apoptosis. Thus these results suggest that 0.1 μM doxorubicin induces nonapoptotic cardiac damage.

Low Doses of Doxorubicin Induce Abnormal Mitosis Associated With a Senescent-Like Phenotype and Alterations of Cytoskeletal Protein Levels

Senescent cells can be identified by the expression of enzymatic SA-β-gal activity at pH 6.0 and by an enlarged, flattened phenotype (16). Forty-eight hours after having been exposed to doxorubicin for 3 h, SA-β-gal activity expression was 34, 20, and 8% in cells treated with 0.1, 0.05, and 0.01 μM doxorubicin, respectively, whereas it remained unchanged in cells treated with higher doses (Fig. 2A). Cells treated with 0.1 μM
doxorubicin showed enlarged volume, flattened morphology, and the appearance of vacuolated cells (Fig. 2B). Some cells also contained several nuclei of unequal size, including micronuclei (Fig. 2B), whereas nuclei staining documented the presence of anaphase bridges (Fig. 2C).

Cells treated with 1 μM doxorubicin exhibited condensed nuclei, which are characteristic of apoptotic cells, (Fig. 2B). Cell viability, determined by trypan blue exclusion analysis, was maintained for the first 4 days after exposure to 0.1 μM doxorubicin, whereas it decreased in a dose-dependent manner after exposure to 1 and 10 μM doxorubicin (Fig. 2D).

We then examined how pulsed incubation with doxorubicin affects cytoskeletal remodeling. Phalloidin staining showed that at a dose of 0.1 μM, doxorubicin increased cell size and both the length and density of the cytoplasmic actin fibers, whereas at a dose of 1 μM, it led to the disruption of the actin fibers (Fig. 2E). Western blot analysis showed a 300% increase in actin levels and a 50% reduction in ER fibers (Fig. 2). Flow cytometric analysis was used to assess changes in DNA content. At 48 h, cells treated with 1 μM doxorubicin demonstrated an increase in the sub-G1 phase and a decrease in the G2/M phase, whereas cells treated with 0.1 μM doxorubicin showed an increase in the S phase and in the hyperploid (>4N DNA) cell population (Fig. 3A). In 0.1 μM doxorubicin-treated cells, we also observed both a downregulation of the expression of the DNA damage-induced checkpoint kinase Chk2, which normally prevents the induction of mitosis, and polyploid metaphases with several chromosomal abnormalities, including dicentric chromosome, end-to-end fusion, and ring and string configuration (Fig. 3, B and C). These findings indicate the presence of mitotic catastrophe.

**Doxorubicin Regulates Expression of TRF1 and TRF2**

Doxorubicin at 0.1 μM decreased protein and mRNA levels of TRF1 and TRF2, whereas at 1 μM, it reduced TRF2 and enhanced TRF1 expression (Fig. 4, A and B). These results were confirmed by fluorescence analysis (Fig. 4C).

To analyze the influence of TRF1 and TRF2 levels on cellular response, we selectively silenced TRF1 or TRF2 using the siRNA transfection technique. By performing transfection with various doses of siRNA targeting TRF2, we obtained a dose-dependent downregulation of the TRF2 protein levels (Fig. 5A). The 10 nM siRNA induced 18% knockdown of TRF2 protein that was associated with senescent morphology changes, including SA-β-gal positivity, marked increase in cell size, flattened morphology, and early PI positivity, whereas the 100 nM siRNA induced a 55% knockdown of the TRF2 protein that was associated with apoptosis. siRNA at a dose of 50 nM produced a 35% knockdown of TRF2 protein that was associated with the presence of both senescent and apoptotic cells (Fig. 5C), which is likely the result of the various levels of TRF2 silencing in each cell. By performing transfection with 100 nM siRNA targeting TRF1, we obtained a 60% knockdown of the TRF1 protein (Fig. 5B). As shown in Fig. 5C, the transfection with siRNA targeting TRF1 did not alter the cells.

**Doxorubicin Regulates TRF1 and TRF2 Expression Through MAPK- and p53-Mediated Pathways**

We observed that 0.1, 1, and 10 μM doxorubicin induced dose-dependent phosphorylation of p38 and JNK, whereas ERK 1/2 was only activated by 10 μM doxorubicin. We found no changes in total MAPK protein levels (Fig. 6A). Using specific inhibitors, we analyzed the role played by p38, JNK, and p53 in mediating the effect of doxorubicin on TRF1 and TRF2 expres-
Pretreatment with the p38 inhibitor SB-203580 attenuated the downregulation of TRF2 induced by both 0.1 and 1 μM doxorubicin but did not modify the effects of doxorubicin on the TRF1 expression. Conversely, pretreatment with the JNK inhibitor SP-600125 did not influence the effects of doxorubicin on the expression of TRF2 but blunted both the 1 μM doxorubicin-induced TRF1 upregulation and the 0.1 μM doxorubicin-induced TRF1 downregulation. Pretreatment with the p53 inhibitor PFT did not influence the effects of doxorubicin on the expression of TRF2 but did modulate the effects on TRF1 expression. PFT abolished the downregulation of TRF1 in cells incubated with 0.1 μM doxorubicin and increased the TRF1 upregulation in cells incubated with 1 μM doxorubicin.

We then analyzed whether pretreatment with these inhibitors influenced the effects of doxorubicin on the number of SA-β-gal and A(+) cells (Fig. 6, C and D). We observed that in cells exposed to 1 μM doxorubicin, pretreatment with SB-203580 drastically reduced the number of A(+) cells, specifically the number of cells in late apoptosis characterized by A(+) / PI(−) double positivity, whereas pretreatment with SP-600125 or PFT did not produce any effects. When used 0.1 μM doxorubicin, we found that pretreatment with SB-203580, SP-600125, and PFT reduced the number of SA-β-gal cells from 34% to 7, 18, and 16% respectively. Pretreatment with SB-203580 decreased the number of A(+) / PI(+) cells, whereas pretreatment with SP-600125 and PFT not only increased the number of damaged cells but also induced the appearance of the A(+) / PI(−) cell population. These results indicate that when doxorubicin is used at subapoptotic doses, pretreatment with SP-600125 or PFT switches the cell response to the stress from senescence to apoptosis. Since pretreatment with PFT and SP-600125 on cells treated with 0.1 μM doxorubicin produced similar effects on TRF1 expression, such as senescence-like phenotype and A(+) / PI(−) staining, this prompted us to investigate the relationship between p53 and JNK. We observed that the 0.1 μM doxorubicin-induced expression of p53 is JNK dependent (Fig. 7A). Fluorescence microscopy confirmed these results and showed that p53 accumulation occurs in the cytoplasm and not in the nucleus, where it is considered a marker of p53-mediated apoptosis (Fig. 7B). This result further confirms that the 0.1 μM doxorubicin dose is not apoptotic.

Together, these data prove that TRF1 and TRF2 are at the center of the pathways that lead to senescence or apoptosis in response to doxorubicin. Figure 8 shows the putative signaling pathways involved in doxorubicin-induced senescence or apoptosis.

Effects of a Single Pulsed Incubation With Low-Dose Doxorubicin Are Still Detectable 21 Days Later

Neonatal rat cardiomyocytes cannot be cultured at length; therefore, in an attempt to examine the long-term effects (21 days) of a 3-h pulsed exposure to low-dose doxorubicin, we used H9c2 cardiomyocytes. We repeated all the experiments described in the previous paragraphs and obtained the same results that we had found with neonatal rat cardiomyocytes. FACS analysis showed that the percentage of PI(+) cell population increased from 8 to 58% at day 6 and decreased to 53% at day 15 and to 24% at day...
21, a percentage that was three times the basal value (Fig. 9A). Both cell size (Fig. 9, A and B) and the percentage of SA-β-gal-positive cells (Fig. 9C) increased following doxorubicin treatment and peaked at day 6, still remaining above the basal values at day 21. TRF1 and TRF2 protein expression remarkably decreased at day 6 and was still lower than the basal value at day 21 (Fig. 9D). No significant differences were observed in cells incubated without doxorubicin. These experiments show that brief exposure to low-dose doxorubicin induces cell damage that lasts over time.

**DISCUSSION**

**Different Doses of Doxorubicin Induce Senescence or Apoptosis Through Different Modulation of TRF1 and TRF2 Expression Levels**

This study, which investigated the physiological bases of doxorubicin-induced senescence, was conceived moving from two lines of evidence. The first arises from the study by Eom et al. (18), who found that chronic exposure of hepatoma cells...
to high doses of doxorubicin induced apoptosis, whereas lower doses induced senescence and late death by mitotic catastrophe, and from the study by Maejima et al. (34), who were the first to demonstrate that low doses of doxorubicin induced senescence in neonatal cardiomyocytes. The second line of evidence is represented by the study of Lechel et al. (29), who demonstrated that low and high levels of TRF2 inhibition are respectively associated with senescence and apoptosis. The data of the present study indicate that different doses of doxorubicin affect the expression of the telomeric binding proteins TRF1 and TRF2 differently and that these, in turn, decide the type of response to the stress, i.e., senescence or apoptosis. Our data also show that TRF2 plays a prominent role in deciding the type of cellular response. Using the siRNA technique, we have confirmed that high levels of TRF2 downregulation induce apoptosis and low levels of TRF2 downregulation induce senescence and apoptosis. The

Fig. 6. A: effects of exposure to various doses of doxorubicin on MAPKs. Total cell lysates were analyzed after 20 min of exposure to doxorubicin by Western blotting (top), using antibodies specific for phosphorylated (Ph) and total forms of MAPK. Bar graph (bottom) shows values for Ph-MAPK normalized to the amount of total enzyme and expressed as the relative increase above control value, which was set at 100. B–D: effects of the p38 MAPK inhibitor SB-203580 (SB), the JNK inhibitor SP-600125 (SP), and the p53 inhibitor pifithrin-α (PFT) on TRF1 and TRF2 protein expression levels (B), the percentage of SA-β-gal active positive cells (C), and the percentage of A/PI-positive cells (D). Dox, doxorubicin. Western blot analysis was performed 24 h after treatment; SA-β-gal activity and A/PI staining were assessed 48 h after treatment. *P < 0.05 vs. ct. $P < 0.05 vs. 0.1 μM doxorubicin. ‡P < 0.05 vs. 1 μM doxorubicin. #P < 0.05 vs. SB.

Fig. 7. Effects of 0.1 μM doxorubicin on p53 protein expression levels evaluated by Western blot analysis (A) and immunofluorescence (B) after pretreatment with or without SB or SP. Analyses were performed 24 h after treatment. *P < 0.05 vs. ct. $P < 0.05 vs. 0.1 μM doxorubicin.
lation induce senescence, and we also found that the selective knockdown of TRF1 induces neither senescence nor apoptosis. These results are in agreement with a number of studies finding that a dominant negative allele of human TRF1 does not affect the growth and viability of a variety of primary and transformed human cells (12, 25, 26, 55).

We documented that if doxorubicin induces high levels of TRF2 downregulation, apoptosis occurs regardless of the level of TRF1 expression. Conversely, if the stress induces a moderate level of TRF2 downregulation, as it does with 0.1 μM doxorubicin, TRF1 is crucial for deciding cell response. A consensual reduction of TRF1 expression induces senescence; otherwise, apoptosis occurs.

This finding is in line with previous studies demonstrating that in particular cellular conditions, TRF1 can affect cell cycle progression and is an important signal for cell survival. Lu et al. (33) observed that TRF1 inhibition suppresses the ability of the mitotic kinase NIMA to induce premature mitotic entry and apoptosis. Kishi et al. (27) found that TRF1 overexpression can induce apoptosis in cells with short telomeres but not in those containing long telomeres. The similarity between our and Kishi’s data may be better appreciated by keeping in mind that short telomeres and TRF2 downregulation have similar effects on cell biology (24).

Doxorubicin-induced senescent cardiomyocytes show morphologically flattened and enlarged cell shapes associated with cytoskeleton remodeling that is characterized by a significant increase in the level of actin protein with clearly visible polymerized F-actin filaments crossing the whole cell and by a decrease in tubulin levels. Cytoskeletal components are key regulators of cellular architecture. Functionally, the enhanced actin fibers may construct a frame structure that is needed to support the enlarged cells (1, 9). Cytoskeletal components also are involved in cellular processes such as cell differentiation and cell cycle regulation and are essential mediators of the cell signaling pathways that regulate senescence (38, 39). The reduction of α-tubulin levels in senescent cells has been viewed as a process that impairs cellular microtubule integrity and may disrupt the normal mitotic processes (51).

Some structural alterations of senescent cells are specific for the cell type. For example, whereas our data and those of Alexander et al. (1) document an association between the senescent phenotype and overexpression of actin in cardiomyocytes and human osteosarcoma cells, Nishio and Inoue (39) documented a reduction in the levels of actin protein in senescent fibroblasts. Differently from neonatal cardiomyocytes that do not express vimentin (28, 42), senescent fibroblasts show an extraordinary production of vimentin (38) that, by anchoring cytoplasmic p53, prevents nuclear import of p53 and p53-dependent apoptosis (39).

We found that a pulsed, brief incubation with doxorubicin at 1 μM induced apoptosis through a p53-independent pathway. This result is in agreement with previous studies that employed similar doses of doxorubicin (60) but contrasts with other studies that used higher concentrations of doxorubicin and/or for a longer amount of time, leading to the discovery that apoptosis occurs through a p53-dependent pathway (11, 46). Accordingly, we think that the role of p53 in doxorubicin-induced apoptosis in cardiac muscle cells may vary depending on the concentration and duration of treatment.

We also found that doxorubicin at 0.1 μM induces a senescent phenotype, accompanied by an increase in the p53 levels that accumulate in the cytoplasm. Many studies have demonstrated that cytoplasmic p53 may contribute to apoptosis through transcription-independent mechanisms by amplifying the transcription-dependent apoptotic signals triggered by nuclear p53 (46). However, there also are studies that suggest a protective role for cytoplasmic p53. In agreement with the above-mentioned study by Nishio and Inoue (39), Qu et al. (43) also demonstrated that p53 cytoplasmic accumulation induced by endoplasmic reticulum stress prevents p53 stabilization and p53-mediated apoptosis upon DNA damage.

Nithipongvanitch et al. (40) found nuclear, cytoplasmic, and mitochondrial accumulation of p53 following acute doxorubicin treatment and demonstrated that the loss of mitochondrial p53 renders mitochondrial DNA more susceptible to oxidative stress, thus suggesting that mitochondrial p53 accumulation may participate in mitochondrial DNA repair as a rapid adaptive response to oxidative stress in cardiomyocytes.

Our data show that p53 plays an active, protective role. In fact, if the incubation of low, prosenescent doses of doxorubicin was preceded by pretreatment with PFT, the p53 inhibitor, then cells underwent apoptosis rather than showing a senescence phenotype. This finding is in agreement with a study by Elmore et al. (17), who found that breast tumor cells that are acutely exposed to doxorubicin exhibit an increase in p53 activity and a dramatic increase in β-galactosidase. They also found that inactivation of wild-type p53 results in a transition of the cellular response to doxorubicin treatment from replicative senescence to delayed apoptosis (17).

Both the literature as well as our own experimental data collectively suggest that the following mechanisms may be involved in doxorubicin-induced senescence. Doxorubicin induces TRF2 downregulation, which causes telomere uncapping and ultimately cell death. At low doses, doxorubicin also induces TRF1 knockdown. Although TRF1 inhibition does not play a crucial role in unstressed cells, it is of great importance in cells treated with low doses of doxorubicin, because it prevents premature mitotic entry and apoptosis. p53 plays a key role in downregulating TRF1 in cells treated with low doses of doxorubicin. If p53 activity is inhibited by PFT, the exposure to low doses of doxorubicin produces TRF1 overexpression; thus the cellular response program to doxorubicin shifts from senescence to apoptosis. How p53 regulates TRF1
expression is an open question. We hypothesize that p53 may function in the cytoplasm by relaying a negative regulator of TRF1 transcription.

The present study shows that p53 and MAPK activation is a doxorubicin-induced signaling that regulates TRF1 and TRF2 expression: p38 mediates the effects on TRF2, whereas JNK regulates TRF1 through p53 activation at low doses of doxorubicin and in a p53-independent manner at high doses of the drug. Previous studies have shown that p38 and p53 are senescence-executing molecules that are activated by telomere dysfunction (23, 25). We do not believe that the previous observations conflict with our present results, since the point of MAPK and p53 activation can be either upstream or downstream from telomere dysfunction. This is in agreement with the study by Iwasa et al. (23), who found that p38 is activated by H$_2$O$_2$ and that p38 activation persists after H$_2$O$_2$ removal. It may be argued that doxorubicin directly induces MAPK and p53 activation, which determines telomere dysfunction, which in turn increases their activation.

**Cells With Doxorubicin-Induced Senescent Like Phenotype May Die of Mitotic Catastrophe**

In an attempt to better understand the mechanism involved in doxorubicin-induced late cardiac toxicity, we chose to expose cells to a pulsed, short-term incubation with doxorubicin, because it has been shown that prolonged treatment is lethal even if the doses of the drug are low (18). We observed that whereas 1 μM doxorubicin led to rapid death through apoptosis, 0.1 μM doxorubicin induced a senescent-like phenotype and caused death through mitotic catastrophe of a lower number of treated cells. In addition, we found that a relevant number of surviving myocytes had a senescent phenotype that was documented 21 days after treatment by the increase of cell volume, the presence of SA-β-gal activity, and the downregulation of TRF2 and TRF1 expression.

Mitotic catastrophe is a mode of death slower than apoptosis that results from DNA damage coupled with altered functions of the various checkpoint mechanisms whose role is to arrest progression into mitosis until repair (7). In the present study we...
observed the typical biochemical and morphological features of mitotic catastrophe, which include the presence of multinucleated cells with micronuclei and/or nuclei of unequal size (likely arising through abnormal mitosis), anaphase bridges, changes in DNA content (>4N), and evidence of chromosomal abnormalities (18, 29, 59). In agreement with Castedo et al. (8), who found that the doxorubicin-induced inhibition of Chk2 facilitates the induction of mitotic catastrophe, we observed a downregulation of the Chk2, a cell cycle checkpoint that governs the order and timing of cell cycle transition to ensure completion of one cellular event before the commencement of another and therefore acts as a negative regulator of mitotic catastrophe.

It is well-known that polyploidy and chromosomal aberrations are the consequence of TRF2 downregulation. In fact, TRF2 downregulation determines the molecular disassembly of the telomere complex and causes the deprotection of chromosome ends, which then become the substrate of repair activities, resulting in covalent fusion of two chromosomes and the appearance of anaphase bridges, which must be torn away to allow the mitosis to proceed. As a result, each daughter cell will inherit a chromosome with a double-strand break at one end that will be fused to another uncapped end, perpetuating a cycle of breakage-fusion-bridge that may lead to cell death (13). If cells do not escape from this cycle, cell death may occur even much later on and several mitoses after the application of the stress (32).

Do Telomere Dysfunction and Senescence Play a Role in Doxorubicin-Induced Heart Failure Cardiomyopathy?

Until a few years ago, the prevailing belief was that the heart is a terminally differentiated organ whose response to pathological loads or cell death can only be accomplished by hypertrophy of differentiated cells. In the past few years, a number of studies have demonstrated that the heart contains a pool of stem cells that are able to create new myocytes (5, 36, 41).

More recently, it has been shown that the heart is characterized by a heterogeneous population of myocytes and that small, immature dividing myocytes can be found together with the old, hypertrophied nonreplicating ones (10). There is currently increasing evidence of a slow, on-going turnover of cardiomyocytes in the normal heart involving the death of cardiomyocytes and the generation of new cardiomyocytes (6, 19, 53). A number of studies have demonstrated that the heart contains a pool of resident progenitor cells (36) and a population of small, immature dividing p16INK4a-negative myocytes with long telomeres (45) that have been considered the link between progenitor cells and more differentiating, nonreplicating cardiomyocytes. Conceptually, heart failure may now be considered a myocyte-deficiency disease in which pump dysfunction supervenes when cell loss is not compensated by hypertrophy of the existing myocytes and regeneration of new myocytes. It must be kept in mind that we are already well aware of a model in which the impaired function of regenerative cells contributes to myocardial dysfunction, i.e., aging. This is a process in which the impaired function of progenitor cells significantly contributes to the dysregulation of endogenous repair mechanisms and explains the increased severity of cardiovascular pathophysiology that is observed in the geriatric population (15, 19, 44, 52).

Neonatal rat cardiomyocytes share some characteristics with the population of small, immature replicating cells that are present in the adult heart and that are reminiscent of a fetal/neonatal phenotype (10). Thus our experimental model may be considered a convenient indicator of what might happen to these young and cardioregenerative cells when the heart is exposed to doxorubicin. Current research in the field of cardioregenerative medicine is mainly focused on ways to stimulate and strengthen the regenerative capacity of progenitor cells in response to stress. Future research also should look into the possibility of protecting progenitor cells themselves from dangerous stress.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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