Sublethal Doses of an Anti-erbB2 Antibody Leads to Death by Apoptosis in Cardiomyocytes Sensitized by Low Prosenescent Doses of Epirubicin: The Protective Role of Dexrazoxane

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ABSTRACT

The cardiotoxic synergism resulting from the sequential treatment with anthracyclines and trastuzumab has been attributed to the trastuzumab-induced loss of the erbB2-related functions that serve as a salvage pathway against the damaging effects of anthracyclines. Cellular senescence is a novel mechanism of cardiotoxicity induced by subapoptotic doses of anthracyclines. After having identified prosenescent and proapoptotic doses of epirubicin and rat MAb c-erbB2/Her-2/neu Ab-9 clone B10 (B10), an anti-erbB2 monoclonal antibody, we investigated the effects of the sequential treatment with prosenescent doses of both drugs on H9c2 cells and neonatal rat cardiomyocytes pretreated with or without the cardioprotective agent dexrazoxane. Cells were analyzed by senescence-associated β-galactosidase staining, single-stranded DNA, annexin/propidium double staining, F-actin, and mitochondrial transmembrane potential. ErbB2 expression levels, AKT activation, and the effects of the inhibition of nicotinamide adenine dinucleotide phosphate oxidase [NAD(P)H oxidase] and phosphoinositide-3-OH kinase (PI3K) were also assessed. Data demonstrate that 1) the toxic effects of epirubicin mainly occur through NAD(P)H oxidase activation; 2) the erbB2 overexpression induced by epirubicin is a redox-sensitive mechanism largely dependent on NAD(P)H oxidase; 3) the loss of erbB2-related functions caused by B10 determines marginal cellular changes in untreated cells, but causes massive death by apoptosis in cells previously exposed to a prosenescent dose of epirubicin, 4) dexrazoxane promotes survival pathways, as demonstrated by the activation of Akt and the PI3K-dependent erbB2 overexpression; and 5) it also prevents epirubicin-induced senescence and renders epirubicin-treated cells more resistant to treatment with B10. Data underline the importance of NAD(P)H oxidase in epirubicin-induced cardiotoxicity and shed new light on the protective mechanisms of dexrazoxane.

Epidermal growth factor receptor erbB2 is a transmembrane glycoprotein receptor with tyrosine kinase activity. Trastuzumab (Damen et al., 2008) is an anti-erbB2 humanized monoclonal antibody. When added to chemotherapy in patients with erbB2 overexpressing breast cancer it leads to a significant gain in survival (Slamon et al., 2001). However, the gain in survival occurs at the cost of an increased risk of hypokinetic cardiomyopathy and heart failure (Bria et al., 2008). Trastuzumab-related cardiotoxicity is not associated with severe structural changes and is often reversible after discontinuing therapy (Ewer and Ewer, 2008). However, clinical studies have shown that, if women have been co-treated or pretreated with anthracyclines, both the incidence and severity of myocardial dysfunction remarkably increase (Slamon et al., 2001; Tan-Chiu et al., 2005). It has been hypothesized that while activating cardiomyocyte stress pathways, anthracyclines also activate survival pathways, the most important of which is the neuregulin/erbB2 system (Gabrielson et al., 2007; Pentassuglia et al., 2009). At low doses of anthracyclines, the survival pathway effects over-
whelm the toxic effects, and cardiac dysfunction does not appear, at least for several years. By inhibiting the erbB2 receptor, trastuzumab induces a second stress that impairs this survival pathway, creating an unbalance in favor of the toxic effects induced by anthracyclines. Thus, Ewer recently concluded that trastuzumab-related cardiac dysfunction must be viewed as an anthracycline-related problem, rather than a trastuzumab-related problem (Ewer and Tan-Chiu, 2007). Accordingly, preventing low-dose anthracycline-induced cardiotoxicity may represent a rational approach to protecting cells from further stress provoked by exposure to an anti-erbB2 antibody.

The most accredited hypothesis for explaining anthracycline cardiotoxicity is that anthracyclines induce myocyte loss through oxidative stress and apoptotic cell death (Menna et al., 2008). It has recently been shown in cardiac myocytes that when anthracyclines are used at subapoptotic doses, they induce stress-induced premature senescence (SIPS) (Maejima et al., 2008). Senescence is a fundamental cellular program that contributes to the physiology of living tissues, the aging process, and diseases. The hallmark of cellular senescence is cell cycle arrest accompanied by morphological and structural changes including flattened and enlarged cell shapes and cytoskeleton remodeling. These structural changes are associated with the inability to divide and the reduction of telomere length that may result in late death (Ben-Porath and Weinberg, 2004).

It has been shown that pretreatment with dexrazoxane (ICRF-187) (Malisza and Hasinoff, 1996), an antioxidant and iron-chelating agent, is effective in preventing many features of anthracycline cardiotoxicity (Spallarossa et al., 2006; van Dalen et al., 2008). However, there are no data on whether dexrazoxane also prevents senescence in cardiac muscle cells.

Aims of the present study were, first, to characterize the cell damage induced by an anti-erbB2 monoclonal antibody in myocardial rat cells previously exposed to subapoptotic, pro-senescent concentrations of epirubicin. Second, to assess whether pretreatment with dexrazoxane is able to prevent epirubicin-induced senescence-like phenotype, and to investigate the mechanisms and signal transduction pathways through which dexrazoxane exerts cardioprotective effects. Third, to assess whether cells that had been protected by dexrazoxane toward epirubicin are more resistant against the cardiotoxic effects of an anti-erbB2 monoclonal antibody.

We performed experiments using H9c2 cardiomyoblasts and neonatal cardiomyocytes that represent an extensively used model for studying myocardial toxicity induced by anthracyclines and anti-erbB2 drugs (Spallarossa et al., 2004, 2006; Pugatsch et al., 2006; Salvatorelli et al., 2006; Maejima et al., 2008; Gordon et al., 2009). Although postmitotic adult cardiomyocytes are the most functionally significant cell type in the heart, numerous studies have shown that the heart also contains a pool of progenitor cells and a population of immature, dividing myocytes that are considered the link between progenitor cells and differentiated cardiomyocytes (Chen et al., 2007). Thus, in the normal heart there is a turnover of cardiomyocytes involving the death and the generation of new cardiomyocytes. Because of the enhanced sensitivity of dividing cardiomyocytes to anthracyclines, it has been suggested that anthracyclines may inhibit the regenerative capacity of the heart and, through this mechanism, impair the self-repairing potential of the heart that ultimately leads to late events (Konorev et al., 2008). Neonatal rat cardiomyocytes share some characteristics with the population of replicating cells that are present in the adult heart, and that are reminiscent of a fetal/neonatal phenotype (Chen et al., 2007). Thus, our experimental model may be considered a convenient indicator of what might happen to these cardio-regenerative cells when the heart is exposed to anthracyclines and an anti-erbB2 antibody.

Materials and Methods

To carry out the study we chose to use epirubicin and B10, because epirubicin is becoming the most used anthracycline in the treatment of breast cancer, and B10 is a rat-anti-erbB2 monoclonal antibody whose activity has been found to biologically resemble that of trastuzumab, the humanized antibody that is used in the clinical setting (Pugatsch et al., 2006). All materials, unless otherwise stated, were supplied by Sigma-Aldrich (Poole, United Kingdom).

Cell and Culture Conditions. H9c2 rat heart-derived embryonic myocytes (American Type Culture Collection, Manassas, VA) were cultured as described previously (Spallarossa et al., 2006). Neonatal ventricular myocytes from 2-day-old Sprague-Dawley rats were purified and cultured as described previously (Antos et al., 2003). Cells were always used at less than 70% of confluence. They were incubated with various doses of epirubicin for 3 h or with various doses of rat MAb c-ErbB2/Her-2/neu Ab-9 (B10) (NeoMarkers, Fremont, CA) for 24 h to evaluate senescence and apoptosis, and to choose the pro-senescent subapoptotic dose of each drug that was most suited to perform the study.

Experimental Design. Cells were pretreated for 3 h with or without dexrazoxane (20 μM) (Novartis Pharma, Basel, Switzerland), then they were treated for 3 h with or without epirubicin, and successively they were incubated with or without B10 for 24 h. To evaluate the mechanisms of cardioprotection and survival signaling pathways, cells were also exposed to the following pretreatments: N-acetylcyesteine (NAC, 10 mM) for 90 min; the NAD/P(H) oxidase inhibitor, diphenyleneiodonium (DPI, 20 μM) for 90 min; the phosphoinositide-3-OH kinase (PI3K) pathway inhibitor, LY294002 (10 μM) for 30 min. At the end of the treatments, cells were analyzed for apoptosis (single-stranded DNA, ssDNA), annexin V-fluorescein isothiocyanate (FITC)/propidium iodide staining (AV/PI), and mitochondrial transmembrane potential. AV/PI staining was also analyzed 24 h after the end of the treatments to assess both early and late apoptosis. Senescence was evaluated 24 h after the end of the treatments because the rate of senescent cells before this time point was too low to allow statistical analysis, whereas, for longer culture times, untreated neonatal cardiomyocytes spontaneously underwent replicative senescence. F-actin was assessed 24 h after the end of the experiments.

SA-β-Gal Staining. Cells were stained for β-gal activity as described previously (Dimri et al., 1995). The number of senescence-associated β-galactosidase (SA-β-gal)-positive cells was determined in 100 randomly chosen low-power fields (∗100) and expressed as a percentage of all counted cells.

Detection and Quantitation of Apoptosis by ssDNA Antibodies. Cell monolayers grown on glass coverslips were exposed to agents as described earlier so as to detect apoptosis by identifying ssDNA (Tauszig-Delamasre et al., 2007), then they were fixed in 100% methanol at −20°C for 24 h. To induce DNA denaturation in situ, cells were heated to 100°C in PBS containing 5 mM MgCl₂ for 5 min, then immersed in ice-cold water for 10 min. After incubation with 40% fetal bovine serum in PBS on ice for 15 min, cells were incubated with a monoclonal antibody to ssDNA (10 μg/ml, Apostain F7-26; MedSystem Diagnostics, Vienna, Austria) for 30 min at room temperature, then washed twice in PBS. The last two steps were performed using the improved biotin–streptavidin-amplified detec-
respectively.

Annexin V-FITC/Propidium Iodide Staining. Cells were labeled with Annexin V-FITC and propidium iodide (Spallarossa et al., 2004), and 100 randomly selected fields were counted by use of a fluorescence microscope. The number of stained cells was normalized to the total number of cells as counted by phase microscopy of the same field. Images from independent fields were counted under a fluorescence microscope.

Determination of Mitochondrial Transmembrane Potential. Mitochondrial transmembrane potential was determined by use of the MitoCapture Apoptosis Detection Kit (Furukawa et al., 2002). This kit provides a fluorescence-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial membrane potential. It uses the MitoCapture Reagent, a cationic dye that fluoresces differently in healthy cells and in apoptotic cells. In healthy cells, the MitoCapture Reagent accumulates and aggregates in the mitochondria, emitting a bright red fluorescence. In apoptotic cells, this reagent cannot aggregate in the mitochondria because of the altered mitochondrial membrane potential, and thus it remains in the cytoplasm in its monomeric form and emits green fluorescence. The fluorescent signals can be detected easily by fluorescent microscopy using appropriate filters.

F-Actin Detection. Cells were fixed, permeabilized, and labeled simultaneously in PBS containing 50 µg/ml lysopalmityl phosphatidylcholine, 3.7% formaldehyde, and 5 units/ml of fluorescent phalloidin (A-12379 Alexa Fluor 488 phalloidin; Invitrogen, Carlsbad, CA). Cells were rapidly washed three times with PBS and were viewed by fluorescent microscopy. To quantify the fluorescence and to measure cell area, image analysis was performed by 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. The video image was generated by a charge-coupled device camera connected through a frame grabber to a computer. Single images were digitized for image analysis at 256 gray levels. Imported data were quantitatively analyzed by Q500MC Software-Qwin (Leica). The single cells were randomly selected by the operators by using the cursor, and positive areas were estimated automatically. Constant optical threshold and filter combination were used.

Immunoblotting. Immunoblotting was performed by use of the previously described procedure (Spallarossa et al., 2006). All antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Thirty hours after the beginning of the treatments, cells were processed to determine the levels of p16INK4A using p16(N-20) and erbB2 using erbB2 antibody C-18. Phosphorylated Akt (pAkt) levels were evaluated at each time point indicated in the experiment. After incubation in horseradish peroxidase secondary antibody, blots were visualized with enhanced chemiluminescence substrate (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and films were quantified by densitometry with an image analyzer system (Syngene, Frederick, MD). Filters were stripped and reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or total Akt antibodies to normalize the amounts of erbB2 and of phosphorylated Akt, respectively.

Statistical Analysis. Data are reported as mean ± S.E.M. of four independent experiments. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni’s post hoc test.

Results

After having performed preliminary tests on H9c2 cells, we carried out the study on neonatal rat cardiomyocytes and obtained similar results. Herein, we report the data that were collected from the experiments on neonatal cardiomyocytes. We first performed a dose-effect curve to test the effects of various doses of epirubicin and B10 on senescence and apoptosis. To assess apoptosis, we chose the ssDNA technique, which stains condensed chromatin by using antibodies against ssDNA and is considered a definite marker of apoptosis. To assess senescence, we evaluated SA-β-gal activity and p16INK4A tumor suppressor protein expression levels (Dimri et al., 1995; Sharpless and DePinho, 2004) which are two widely recognized indicators of cellular senescence. SA-β-gal activity is a manifestation of residual lysosomal activity at suboptimal pH (pH 6), which becomes detectable because of the increased lysosomal content on senescent cells (Gerland et al., 2003).

At low doses, epirubicin and B10 induced a senescence-like phenotype, whereas at high doses they led to apoptosis. In comparison with doxorubicin, the equicardiotoxic doses of epirubicin were 2.6-fold higher. Senescent cells showed enlarged volume and flattened morphology, and they seemed vacuolated (Fig. 1).

Because our aim was to create an experimental model in which the two agents would exert mild cardiotoxic effects when used separately, we chose a concentration of 0.3 µM for epirubicin and 1 mg/ml for B10 to carry out the study, because these are the highest concentrations that can be used without inducing apoptosis. Both epirubicin and B10 induced senescence at these concentrations. The study was designed so that cells would have to be pretreated with or without dexrazoxane, incubated with epirubicin, B10, or epirubicin followed by B10, and then analyzed by several techniques. In an attempt to investigate whether the mechanism of action of dexrazoxane is related to its antioxidant properties, we also repeated several experiments using the NAC antioxidant and the NAD(P)H inhibitor, DPI, instead of dexrazoxane.

We observed that the sequential incubation with epirubicin, followed by B10, produced a dramatic change in the cellular response program to the stress, which shifted from a senescence-like phenotype induced by epirubicin alone (30% of cells were SA-β-gal positive without signs of apoptosis) to massive death by apoptosis (65% of cells were ssDNA positive) (Fig. 2). B10 alone produced a very small increase in the number of SA-β-gal-positive cells. We also found that the effects of dexrazoxane pretreatment differed in the various treatment groups: dexrazoxane lowered the increase in SA-β-gal activity in epirubicin-treated cells, it resulted in a small increase in SA-β-gal activity in solvent control-treated cells, it exerted a mild proapoptotic effect on B10 treated cells, and, most interestingly, it halved the ssDNA positivity rate in cells that had been exposed to epirubicin followed by B10. We evaluated p16INK4A levels as a second marker of senescence and found that the increase in p16INK4A protein levels in the various treatment groups was similar to the increase in SA-β-gal activity (Fig. 3). The effects of pretreatment with NAC were exactly the same as the effects of dexrazoxane in all treatment groups. Pretreatment with DPI produced the same effects as both dexrazoxane and NAC did in cells that had been treated with epirubicin alone. Conversely, pretreatment with DPI had no effect on solvent control-treated cells, whereas it determined a small increase in the number of senescent cells in B10-treated cells and abolished the apoptotic effect of the sequential treatment with epirubicin and B10.
We also used MitoCapture to determine whether epirubicin or B10 induce a decrease in the mitochondrial membrane potential. In healthy cells the cationic dye accumulates in the mitochondria, producing a bright red fluorescence, whereas in cells with altered mitochondrial membrane potential the dye remains in its monomeric form in the cytoplasm, leading to green fluorescence. We calculated the ratio of red to green staining to quantify the results. Fluorescence analysis showed that neither epirubicin nor B10 produced significant changes in the red-to-green fluorescence ratio, whereas the sequential application of both produced a severe mitochondrial perturbation that was counteracted in part by the pretreatment with dexrazoxane (Fig. 4).

Phalloidin staining for F-actin confirmed that in these experimental conditions neither B10 nor epirubicin alone caused severe damage to cardiomyocytes, but that they did increase cell size, a typical pattern of the senescence phenotype, and that they also increased both the length and the density of the cytoplasmic actin fibers. Cytoskeletal components are key regulators of cellular architecture. The enhanced actin fibers may be interpreted functionally as a frame structure that is needed to support the enlarged cells. Epirubicin, but not B10, also caused a mild myofibrillar disarray. Conversely, when B10 was administered after epirubicin, it led to disruption of the actin fibers, thus confirming the presence of apoptosis because cleavage of F-actin fibers is an early indicator of apoptosis (Brown et al., 1997). It is noteworthy that this phenomenon was almost completely abolished when cells were pretreated with dexrazoxane before exposure to epirubicin (Fig. 5).

We then analyzed cells for AV/PI. It is known that translocation of the phospholipid phosphatidylserine membrane from the inner to the outer leaflet of the plasma membrane is one of the earliest signs of apoptosis and that it precedes other apoptotic processes such as the loss of plasma membrane integrity. Consequently, when cells are stained with AV (a Ca2+-dependent phospholipid-binding protein with high affinity for phosphatidylserine) and PI (a membrane-impermeable DNA staining), the early-stage apoptosis is characterized by an AV(+)/PI(−) cell population, whereas in late-stage apoptosis many cells become AV(+)/PI(+). Recent studies indicate that the early loss of plasma membrane integrity, which is characterized by AV(+)/PI(+), is often associated with the induction of a senescent-like phenotype in cells treated with subapoptotic doses of anthracyclines (Eom et al., 2005). Analysis of AV/PI double staining at 24 h revealed the following: B10 has very little effect on AV/PI double staining; epirubicin induces the early loss of membrane integrity in almost 30% of cells, which is not typical of apoptotic cardiac damage and cannot be interpreted as necrosis, because of the loss of plasma membrane integrity that occurs in necrosis is preceded by the loss of mitochondrial potential. The exposure to B10 after epirubicin determines the typical features of early-stage apoptosis in more than 50% of cells. Again this phenomenon was partially attenuated when cells were pretreated with dexrazoxane before

![Figure 1](https://example.com/fig1.jpg)  
**Fig. 1.** Dose-dependent effects of epirubicin and B10 on apoptosis and senescence. Bar graph showing the percentages of ssDNA and SA-β-gal-positive cells after treatment with various doses of epirubicin, doxorubicin (a) or B10 (b). c, representative photographs illustrating the morphological changes and SA-β-gal activity in cardiomyocytes with stress-induced premature senescence. In this picture, cells were treated with and without 0.3 μM epirubicin, stained for β-gal activity, and counterstained with Giemsa solution (magnification, ×400). *, p < 0.05 versus untreated cells.
exposure to epirubicin (Fig. 6). Figure 7 shows the effects of various treatments on erbB2 expression. Treatment with epirubicin increased the expression levels of erbB2 by 80%. Treatment with dexrazoxane, NAC, or DPI significantly attenuated such epirubicin-induced increase of erbB2 expression. In contrast, treatment with dexrazoxane or NAC, but not with DPI, induced an increase of approximately 200% of the expression level of erbB2 in cells that had been exposed to epirubicin. Treatment with B10 reduced the expression levels of erbB2 by 70%. Neither pretreatment with epirubicin or dexrazoxane, nor pretreatment with dexrazoxane followed by epirubicin influenced the effects of B10 on erbB2 expression.

Both in vivo and ex vivo studies indicate that survival factors or transforming events such as erbB2 overexpression/activation activate Akt in a PI3K-dependent manner (Zhou et al., 2000; Kanakry et al., 2007; Tokunaga et al., 2007). In Figure 3, p16INK4A protein levels. Bar graph showing the p16INK4A protein levels in cells pretreated with or without dexrazoxane and then incubated with epirubicin, B10, or epirubicin followed by B10. The amount of p16INK4A was normalized to GAPDH. Note that increases in p16INK4A are parallel to those observed in SA-β-gal activity. *, p < 0.05 versus untreated cells; §, p < 0.05 versus Epi; #, p < 0.05 versus Epi+B10.
keeping with those studies, we found that treatments that up-regulate erbB2 expression, such as epirubicin or dexrazoxane, also increase Akt phosphorylation levels, whereas pretreatment with dexrazoxane, which prevents the epirubicin-induced erbB2 up-regulation, also prevents epirubicin-induced Akt activation. We then investigated whether PI3K is involved in the regulation of erbB2 expression. By using the PI3K inhibitor, LY294002, we observed that PI3K is involved in the overexpression of erbB2 induced by dexrazoxane or by the sequential administration of dexrazoxane and epirubicin, but not in the overexpression induced by epirubicin alone (Fig. 8).

Discussion

Data show that both B10 and epirubicin at high doses induce apoptosis in cardiac myocytes, but at low doses they induce SIPS. This result is in agreement with previous studies demonstrating that cells exposed to stress may respond by entering either senescence or apoptosis, and that the type of cell response to stress depends on cell type and intensity of the stress (Eom et al., 2005; Maejima et al., 2008).

Epirubicin Cardiotoxicity Mainly Occurs through NAD(P)H Oxidase. Compared with doxorubicin, the equicardiotoxic doses of epirubicin were 2.6-fold higher. This result is similar to clinical studies that showed that the ratio is approximately 2:1 (Ewer and Benjamin, 2000). Improved glucuronidation and systemic elimination of epirubicin are believed to decrease cardiotoxicity in cancer patients by diminishing the deleterious interactions of epirubicin with cardiomyocytes. However, our own data, as well as those of others, show that epirubicin is less cardiotoxic than doxorubicin, even in cultured cardiomyocytes. Salvatorelli et al. (2006) showed that epirubicin, unlike doxorubicin, undergoes sequestration in cytoplasmic acidic organelles and therefore cannot reach the mitochondrial reductases that convert it to ROS and generate smaller amounts of toxic alcohol secondary metabolites. This is why epirubicin was thought to be a much better partner than doxorubicin for the association with other toxic drugs like anti-erbB2 antibodies.

Epirubicin, however, is not devoid of a ROS-mediated toxic effect. Our study suggests that the ROS-mediated toxic effect of epirubicin mainly occurs through NAD(P)H oxidase, a plasma membrane-associated enzyme.

Prosenescent Doses of Epirubicin Induce Apoptosis When erbB2-Related Functions Are Impaired. Even though epirubicin does not induce severe cell damage at the doses we tested, as suggested by the preserved mitochondrial transmembrane potential, it may act synergistically with the cardiotoxic effects of anti-erbB2 antibodies. In fact, our results indicate that erbB2 plays a key regulatory role in de-
deciding the program of cell response to stress. Cardiomyocytes that have been exposed to pulsed incubation with low doses of epirubicin show the activation of a survival pathway characterized by erbB2 overexpression and Akt phosphorylation and exhibit SIPS for as long as the erbB2 receptor is active. However, the myocytes undergo early and massive death by apoptosis if they are successively incubated with B10 the anti-erbB2 antibody that internalizes the erbB2 receptor, thus blocking this survival pathway. We used B10 at a concentration that, by itself, produces only minimal cellular damage, as documented not only by the low rate of SA-β-gal positive cells and the lack of apoptosis, but also by the maintenance of the mitochondrial transmembrane potential and F-actin fiber organization. Thus, it stands to reason that the erbB2-related functions gain importance when cells get stressed, and can make the difference when cells are exposed to stresses of mild intensity, such as low doses of epirubicin, because they prevent apoptosis at the cost of a moderate rate of senescence.

Chuang et al. (2007) demonstrated that erbB2 gene expression in human breast cancer cells is repressed by manganese superoxide dismutase, a primary antioxidant enzyme. Our study confirms the involvement of a redox-sensitive mechanism in the regulation of erbB2 and demonstrates that the erbB2 overexpression induced by epirubicin largely depends on NAD(P)H oxidase. In fact, we observed that DPI is superior to NAC or dexrazoxane at preventing epirubicin-induced erbB2 overexpression, most likely because DPI abrogates the major source of ROS, whereas these antioxidants have to passively cross the cell membrane and can only partially quench the ROS generated by NAD(P)H oxidase. Thus, NAD(P)H oxidase plays a dual role in treatment with epirubicin. On the one hand, it is a major contributor to the cardiotoxic effects of epirubicin, whereas, on the other, it triggers the activation of survival pathways.

Our data concerning the effects of epirubicin on Akt are in line with a recent study (Gabrielson et al., 2007) showing that doxorubicin treatment in rats results in an increase in Akt phosphorylation. However, our results are in contrast with Li et al. (2006), who reported that Akt activation remained unchanged in mice after a single treatment with anthracyclines, and with Xiang et al. (2009), who showed that the cardiomyocytes treated with doxorubicin reveal significantly decreased Akt activation. We hypothesize that such discrepancies in phospho-Akt modulation may be related to doses and to timing of evaluation. The increase in phospho-Akt suggests an initial survival response to injury, whereas its down-regulation could be associated with a more severe pattern of cardiotoxicity.

**Autophagic Changes in Cardiomyocytes.** The aim of this study was to assess the synergistic effects of epirubicin and B10 and not to evaluate the fate of epirubicin- or
B10-induced senescent cells. However, the increase in SA-β-gal activity together with the appearance of vacuolated cells suggests that the abnormal autophagy may be involved in the development of a senescent-like phenotype. Three types of autophagic disorders have been described in the context of heart failure (Takemura et al., 2006): 1) excessive autophagy, as occurs with high doses of anthracyclines (Lu et al., 2009); 2) decompensated autophagy, if autophagy fails because of too much stress-induced overload; 3) dysfunctional autophagy, if the stress induces dysfunction of the autophagic process.

We speculate that dysfunctional autophagy dependent on imperfect autophagic turnover due to lipofuscin accumulation (Terman et al., 2004) might be involved in SIPS.

Dexrazoxane Renders Cardiomyocytes More Resistant to the Sequential Administration of Epirubicin and B10. Using dexrazoxane, we obtained a number of novel results. First, pretreatment with dexrazoxane can prevent the prosenescence effects of low-dose epirubicin. This is a very interesting finding in the light of the growing importance ascribed to the cardiotoxic effects of low-dose anthracyclines. Second, cells that have been protected by dexrazoxane against the toxic effects of low doses of epirubicin are much more resistant to the stress induced by the subsequent incubation with B10. This result confirms the hypothesis that toxicity of an anti-erbB2 agent could be significantly attenuated by preventing the sensitizing effect of anthracyclines. Third, if incubation with dexrazoxane is not followed by any treatment, there is a mild, yet significant increase in the rate of SIPS coupled with an up-regulation of erbB2 expression, which, unlike what is observed after epirubicin, is not mediated by NAD(P)H oxidase and oxidative stress, but occurs via PI3K. We also found that dexrazoxane activates Akt. Dexrazoxane is thought to exert cardioprotective effects through its iron-chelating metabolite that decrease the formation of ROS. However, many ROS scavengers or iron chelators are ineffective in preventing Dox cardiotoxicity. Our findings suggest that the cardioprotective effect of dexrazoxane also occurs through iron chelation-independent mechanisms that include the activation of PI3K/Akt and erbB2 survival pathways. Fourth, if incubation of cells with dexrazoxane or NAC is followed by exposure to B10, the subsequent loss of the erbB2 function results in a shift from SIPS to apoptosis, which, however, involves a limited number of cells. Our interpretation of the two latter points is as follows. First, we must bear in mind that some degree of oxidative stress is
necessary for cell biology. For example, several gene transcription factors require transient oxidation for their function and, most importantly, small quantities of ROS are needed for cell proliferation (Halliwell et al., 2000). It follows that antioxidants exert protective effects if cells are exposed to enhanced oxidative stress, but that they may provoke damage if they are not. Data also suggest that the erbB2-related survival pathway might play an important role not only in anthracycline cardiotoxicity, but also in other conditions of cell suffering, such as antioxidant-induced senescence. This could explain why the blockade of the erbB2 receptor in cells having mild signs of senescence after pretreatment with dexrazoxane produces mild enhancement of the apoptosis rate. This finding is in contrast with the results of a recent study showing that pretreatment with antioxidants was able to prevent the anti-erbB2-induced cell death in their experimental model.

Conclusions

Many women suffer from myocardial dysfunction after having been treated with anthracycline-trastuzumab regi-

Fig. 7. Changes in erbB2 expression levels after various treatments. Quantization of data shows that B10 down-regulates, whereas epirubicin up-regulates erbB2 expression as assessed by Western blot analysis performed 24 h later. Note that dexrazoxane and NAC alone increase the erbB2 expression levels, whereas pretreatment with these drugs attenuates the epirubicin-induced erbB2 overexpression. The NAD(P)H oxidase inhibitor, DPI, alone does not affect erbB2 expression, even though it abolishes the epirubicin-induced erbB2 overexpression. Data demonstrate that the epirubicin-induced erbB2 overexpression occurs through ROS-generating NAD(P)H oxidase activation. The amount of erbB2 was normalized to GAPDH. *, p < 0.05 versus untreated cells; †, p < 0.05 versus Epi; ‡, p < 0.05 versus Dex; †, p < 0.05 versus NAC.

Fig. 8. PI3K/Akt activation is part of the survival pathway induced by dexrazoxane. Data also suggest that if cells are protected by dexrazoxane against the epirubicin-induced damage, epirubicin does not induce erbB2 overexpression and Akt activation. a, time-curve analysis showing the modulation of Akt phosphorylation (pAkt) by dexrazoxane and epirubicin and the sequential treatment with dexrazoxane and epirubicin; the value of pAkt was normalized to Akt. b, bar graph showing that pretreatment with Ly294002, a specific PI3K inhibitor, prevents the dexrazoxane-induced erbB2 up-regulation, but not the up-regulation induced by epirubicin. AU, arbitrary units; LY, LY294002. Amount of erbB2 was normalized to GAPDH. †, p < 0.05 versus Epi; †, p < 0.05 versus Epi + Dex; ‡, p < 0.05 versus Epi.

vent cell death induced by an anti-erbB2 antibody (Gordon et al., 2009). It must be highlighted that, although we used the anti-erbB2 antibody, B10, at a concentration that does not induce cell damage, Gordon et al. (2009) blocked the erbB2 by using the C-18 antibody at a high enough concentration to induce cell death. Because it has recently been found that erbB2 regulates many antioxidant defense genes and that cell death induced by the erbB2 antibody occurs through ROS-generating processes (Timolati et al., 2006), it is not surprising that pretreatment with antioxidants was able to prevent the anti-erbB2-induced cell death in their experimental model.
mens, and many others who have been treated with anthra-
cyclines either do not receive trastuzumab at all, or receive
overly low doses of it because of the risk of cardiac toxicity.
Our study represents the pathophysiological experimental
background that is needed to plan clinical trials aimed at
evaluating whether pretreatment with dexrazoxane during
anthracycline chemotherapy can prevent left ventricular
dysfunction, which may arise later on during treatment with
trastuzumab.

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