Heart Failure

Juvenile Exposure to Anthracyclines Impairs Cardiac Progenitor Cell Function and Vascularization Resulting in Greater Susceptibility to Stress-Induced Myocardial Injury in Adult Mice

Chengqun Huang, MD; Xiaoxue Zhang, MD; Jennifer M. Ramil, BS; Shivaji Rikka, MS; Lucy Kim, BS; Youngil Lee, PhD; Natalie A. Gude, BS; Patricia A. Thistlethwaite, MD; Mark A. Sussman, PhD; Roberta A. Gottlieb, MD; Åsa B. Gustafsson, PhD

Background—The anthracycline doxorubicin is an effective chemotherapeutic agent used to treat pediatric cancers but is associated with cardiotoxicity that can manifest many years after the initial exposure. To date, very little is known about the mechanism of this late-onset cardiotoxicity.

Methods and Results—To understand this problem, we developed a pediatric model of late-onset doxorubicin-induced cardiotoxicity in which juvenile mice were exposed to doxorubicin, using a cumulative dose that did not induce acute cardiotoxicity. These mice developed normally and had no obvious cardiac abnormalities as adults. However, evaluation of the vasculature revealed that juvenile doxorubicin exposure impaired vascular development, resulting in abnormal vascular architecture in the hearts with less branching and decreased capillary density. Both physiological and pathological stress induced late-onset cardiotoxicity in the adult doxorubicin-treated mice. Moreover, adult mice subjected to myocardial infarction developed rapid heart failure, which correlated with a failure to increase capillary density in the injured area. Progenitor cells participate in regeneration and blood vessel formation after a myocardial infarction, but doxorubicin-treated mice had fewer progenitor cells in the infarct border zone. Interestingly, doxorubicin treatment reduced proliferation and differentiation of the progenitor cells into cells of cardiac lineages.

Conclusions—Our data suggest that anthracycline treatment impairs vascular development as well as progenitor cell function in the young heart, resulting in an adult heart that is more susceptible to stress. (Circulation. 2010;121:675-683.)

Key Words: anthracyclines ■ angiogenesis ■ heart failure ■ myocardial infarction ■ progenitor cells ■ vasculature

Anthracyclines are very effective antineoplastic agents with a broad antitumor spectrum, including many solid tumors and leukemias. Unfortunately, their clinical use is limited by progressive and dose-related cardiotoxicity that may not manifest itself until many years after treatment. Three distinct types of anthracycline-induced cardiotoxicity have been described: acute, early-onset, and late-onset cardiotoxicity. Acute anthracycline-induced cardiotoxicity occurs typically within the first week of treatment and is usually reversible on discontinuation of treatment. Early-onset cardiotoxicity usually presents within a year after completion of treatment, and most patients who develop significant cardiotoxicity have a chronic dilated cardiomyopathy. Late-onset cardiotoxicity is characterized by a latent period during which cardiac function appears normal and the patient is asymptomatic. This type of cardiotoxicity can manifest as much as 15 years after treatment and is characterized by progressive left ventricular dysfunction leading to irreversible congestive heart failure. Heart failure is often precipitated by events such as exercise, pregnancy, and acute viral infection. This latent cardiac toxicity presents a particularly challenging dilemma when pediatric cancers are treated. The only known risk factors for late-onset cardiotoxicity are cumulative anthracycline dose and younger age at the time of treatment. The frequency of cardiotoxic effects has been reported to be as high as 57% among survivors of childhood cancer, and a survey by the Pediatric Cardiomyopathy Registry shows that >15% of all adult patients with cardiomyopathy were previously treated for cancer during childhood or adolescence.

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Because anthracyclines are such effective anticancer drugs, their mechanisms of action have been under intense investi-
gation for many years. However, the mechanisms underlying the cardiotoxic effects are still not fully understood, and multiple mechanisms have been proposed to explain the actions of anthracyclines. These include reactive oxygen species production, apoptosis, disruption in DNA replication, and transcription.9,10 In contrast, very little is known about the underlying mechanisms of late-onset cardiotoxicity, and no animal model has been developed that recapitulates this clinical scenario.

In this study, we developed a juvenile mouse model of anthracycline-mediated late-onset cardiotoxicity. Juvenile mice exposed to modest doses of doxorubicin develop normally and have no obvious cardiac abnormalities as adults. However, these hearts have abnormal vasculature and a reduced number of progenitor cells, which correlated with an increased sensitivity to physiological and pathological stimulus.

Methods

Animals and Experimental Protocol
FVB/N mice were injected at a postnatal age of 5, 10, 15, and 20 days after birth. Hematoxylin–eosin staining of heart sections prepared from 21-day-old mice. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining of heart sections prepared from 21-day-old mice (n=4). Pos. indicates positive. In contrast, very little is known about the underlying mechanisms of late-onset cardiotoxicity, and no animal model has been developed that recapitulates this clinical scenario.

In this study, we developed a juvenile mouse model of anthracycline-mediated late-onset cardiotoxicity. Juvenile mice exposed to modest doses of doxorubicin develop normally and have no obvious cardiac abnormalities as adults. However, these hearts have abnormal vasculature and a reduced number of progenitor cells, which correlated with an increased sensitivity to physiological and pathological stimulus.

Swimming Protocol
Eight- to 12-week old saline-treated (n=10) and doxorubicin-treated (n=10) mice swam together in a glass tank in 34°C water with a light current to prevent floating. To get accustomed to swimming, the mice initially swim for 15 minutes per day. Sessions were gradually increased to 90 minutes twice a day. After 1 week, mice were swimming for 90 minutes twice a day for 14 days separated by a 2-hour rest period.11 The total swimming time was 21 days. One group of saline-injected (n=10) and doxorubicin-injected (n=10) mice was used as sedentary controls. Twenty-four hours after completion of the 21-day swimming regimen, mice were evaluated for cardiac function and then euthanized for tissue harvest.

In Vivo Coronary Artery Ligation and Infarct Size Measurement
Coronary occlusion was performed as described previously.12 Briefly, 8- to 12-week-old saline-treated (n=29) and doxorubicin-treated (n=37) mice were anesthetized with isoflurane, intubated, and ventilated. Pressure-controlled ventilation (Harvard Apparatus) was maintained at 9 cm H2O throughout the procedure. An 8-0 silk suture was placed around the proximal left coronary artery and then ligated. The suture was left in place, and the animal was immediately closed up. Sham-operated mice had a suture placed around the left anterior descending artery that was not tightened. Only mice that

Table. Measurement of Cardiac Function In Vivo (n=15)

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<th>Saline</th>
<th>Doxorubicin</th>
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<tr>
<td>Heart rate</td>
<td>303±96</td>
<td>283±87</td>
<td>0.58</td>
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<tr>
<td>Developed pressure</td>
<td>93.1±14</td>
<td>87.9±13</td>
<td>0.32</td>
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<td>dP/dT max</td>
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<tr>
<td>dP/dT min</td>
<td>−3813±858</td>
<td>−3528±690</td>
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Figure 1. Establishment of a juvenile mouse model of late-onset anthracycline-mediated cardiotoxicity. A, Mouse pups were injected with saline or doxorubicin (DOX) at 5, 10, 15, and 20 days after birth. B, Hematoxylin–eosin staining of heart sections prepared from 21-day-old mice. C, Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining of heart sections prepared from 21-day-old mice (n=4). Pos. indicates positive. D, Hematoxylin–eosin (H & E) staining of adult heart sections. E, Electron micrographs of adult hearts from saline- and doxorubicin-treated mice.

Figure 2. Assessment of blood flow and vascular structure in adult mouse hearts exposed to juvenile saline or doxorubicin (DOX) treatments. A, Measurement of blood flow in adult hearts with the use of fluorescent microspheres (n=6). B, Microfil polymer injection to visualize coronary branching in hearts. C, Representative CD31 staining of adult heart sections at day 60 and quantification of capillary density at 11, 21, 40, and 60 days after birth (n=4). D, Western analysis of VEGF expression in heart. E, Quantification of VEGF in heart lysates (n=4).
survived the procedure were used for experiments. To measure infarct size, mice were euthanized after 24 hours, and the hearts were harvested. The ventricles were cut into 1-mm sections and stained in 1% triphenyltetrazolium chloride.13

Isolation of Cardiac Progenitor Cells
Isolation of c-kit–positive progenitor cells was done with the use of a protocol adapted from Beltrami et al.14 Briefly, mouse hearts from 7-day-old mouse pups were minced and digested with Collagenase II in J-MEM (supplemented with HEPES, glutamine, taurine, and insulin). The digested heart pieces were filtered through a 30-m filter and incubated with anti-CD117–conjugated Miltenyi Biotec Microbeads. C-kit–positive cells were isolated by passing cells over a Miltenyi Biotec MiniMACS sorting column. The cells were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F12 (ratio 1:1), basic fibroblast growth factor (10 ng/mL), epidermal growth factor (20 ng/mL), leukemia inhibitory factor (10 ng/mL), HEPES (5 mmol/L), and insulin-transferrin-selenite for up to 4 passages.

Statistical Analysis
All values are expressed as mean±SD. Time, treatments, and procedures were approximately normally distributed and analyzed with the use of ANOVA followed by t tests. Two-factor repeated-measures ANOVA was utilized for mouse weights and Masson trichrome staining. All other variables were analyzed with parametric testing (Student t test). Survival rate was analyzed by the Kaplan-Meier method with the log-rank test. P<0.05 was considered significant. In the figure legends, n is equal to the sample size per group. All tests were performed with the use of GraphPad Prism 5. Detailed experimental protocols are described in the online-only Data Supplement.

Results
To study late-onset cardiotoxicity, mice were injected with either saline or doxorubicin at 5, 10, 15, and 20 days after birth (Figure 1A). No acute cardiotoxicity was observed and myocytes appeared normal (Figure 1B) and there was no increase in apoptosis (Figure 1C) immediately after completion of the injections. Adult hearts from saline- and doxorubicin-treated mice appeared morphologically normal (Figure 1D). Doxorubicin-mediated cardiotoxicity is characterized by disorganized myofibrils, increased vacuolization, and swelling of organelles.15 However, electron microscopy revealed no degenerative changes suggestive of doxorubicin-mediated toxicity in cardiac myocytes (Figure 1E). Doxorubicin treatment resulted in slower weight gain in pups compared with saline treatment, but this difference diminished as the mice matured (Figure I in the online-only Data Supplement). There were no cardiac hypertrophy (Figure II in the online-only Data Supplement) and no difference in cardiac function in doxorubicin-treated mice in adulthood compared with saline-treated mice (Table).

Because doxorubicin has potent antiangiogenic effects,16 we investigated whether doxorubicin treatment affected vascular development in the juvenile heart. Measurement of blood flow and visualization of the coronary branching pattern revealed that juvenile doxorubicin exposure resulted in reduced blood flow (Figure 2A) and reduced coronary branching (Figure 2B) in the adult heart. Although major vessels in doxorubicin-treated hearts appeared to have narrower lumen than saline-treated hearts, examination of the vessels revealed no differences in thickness and composition (Figure IIIA and IIIB in the online-only Data Supplement). In a time course study, capillary density was unaffected in the young animal during (at day 11) and immediately after completion (at day 21) of the doxorubicin injections. However, at day 40 and 60, capillary densities were significantly reduced in the doxorubicin-treated animals (Figure 2C). The vascular endothelial growth factor (VEGF) is involved in development of vessels and stimulates recruitment and pro-
liferation of endothelial cells.\textsuperscript{17} Interestingly, myocardial VEGF expression was significantly reduced in the heart at day 21 and remained reduced into adulthood (Figure 2D and 2E), suggesting that juvenile doxorubicin exposure might affect vascular development by interfering with VEGF signaling. VEGF levels in the plasma were not different between the saline- and doxorubicin-treated animals at any time point (Figure IV in the online-only Data Supplement).

Sufficient vasculature is important for the heart to adapt to increased workload such as exercise. To investigate whether the doxorubicin-exposed mice were more sensitive to exercise, we subjected saline- and doxorubicin-treated adult mice to endurance swimming. After 21 days of swimming, hearts of doxorubicin-injected mice displayed cardiac hypertrophy (Figure 3A) and dilation of the left ventricle (Figure 3B). In contrast, saline-treated mice did not display any significant hypertrophy or dilatation of the ventricle after the swimming, confirming that cardiac hypertrophy was attributable to the combination of doxorubicin treatment and exercise. Hematoxylin-eosin staining revealed many areas of myofibril disarray in doxorubicin-exposed hearts after swimming (Figure 3C). In addition, hearts from doxorubicin-treated mice had increased fibrosis after swimming (Figure 3D). Hemodynamic analysis of mice showed that the ventricular end-diastolic pressure was reduced in swimming saline-treated mice, whereas it was increased after swimming in doxorubicin-treated mice (Figure 3F). These data demonstrate that modest doses of doxorubicin in young mice result in decreased capability of the hearts to adapt to increased workload in adulthood.

Moreover, neovascularization in the border zone after myocardial infarction (MI) is an important beneficial response that limits the development of left ventricular remodeling and deterioration to heart failure.\textsuperscript{18} To investigate whether juvenile doxorubicin exposure impaired the neovascularization response, adult saline- and doxorubicin-treated mice were subjected to MI by permanent ligation of the anterior descending coronary artery. Interestingly, doxorubicin-treated mice were highly sensitive to MI, and the survival rate of doxorubicin-treated mice was only \( \approx 25\% \) compared with \( \approx 80\% \) for the saline-treated mice (Figure 4A). Doxorubicin-exposed mice had larger (but not significant) infarct size (80.3\%) than saline-treated mice (71.3\%) (Figure 4B and 4C). More importantly, doxorubicin-exposed mice had significantly larger area at risk, suggesting differences in the vasculature (Figure 4D). Doxorubicin-treated mice also had extensive fibrosis compared with saline-treated mice after MI (Figure 4E). Even though there was no increase in fibrosis under nonstressed conditions between the saline- and doxorubicin-treated hearts (data not shown), the amount of fibrosis was significantly increased in the remote (nonrisk) area in the doxorubicin-treated heart. In addition, measurement of capillary density in the infarct border zone 4 days after MI showed that doxorubicin-exposed mice had reduced neovascularization after MI compared with saline-treated mice (Figure 5A). In addition, the abundance of staining for \( \alpha \)-smooth muscle actin was attenuated in doxorubicin-treated hearts in the border zone (Figure 5B). Visualization of the vasculature after MI confirmed that doxorubicin-treated hearts had reduced vessel formation in the border zone (Figure 5C). These results suggest that doxorubicin treatment affects vascular development in the juvenile heart and puts the adult heart at greater risk for ischemic injury.

MI is associated with migration of progenitor cells into the damaged area, where they participate in regeneration and blood vessel formation.\textsuperscript{19–21} Immunostaining of adult saline-treated heart sections for the presence of cells positive for the common stem cell marker c-kit\textsuperscript{22} revealed small c-kit\textsuperscript{+} cells with high nucleus/cytoplasm ratio in the border zone of the infarct by 4 days after MI (Figure 6). Interestingly, there were significantly fewer c-kit\textsuperscript{+} cells in the border zone of animals that had been exposed to doxorubicin as pups (Figure 6C). We also investigated whether the MI stimulated differentiation of the cardiac progenitor cells into cells of different lineages, as has been reported previously.\textsuperscript{23–26} At 7 days after MI, 63\% and 44\% of c-kit\textsuperscript{+} cells stained positive for endothelial cell lineage markers Flk-1 and CD31, respectively, in saline-treated hearts. Importantly, only 29\% and 21\% of the c-kit\textsuperscript{+} cells stained positive for Flk-1 and CD31 in doxorubicin-treated hearts (Figure V in the online-only Data Supplement). In saline-treated hearts, 53\% of the c-kit\textsuperscript{+} cells were positive for GATA-4, and 62\% were positive for MEF2C, suggesting that these cells are committed to a myocyte lineage (Figure VI in the online-only Data Supplement). In contrast, only 32\% and 38\% of doxorubicin-exposed c-kit\textsuperscript{+} cells expressed GATA-4 and MEF2C, respectively. There was no significant difference in c-kit\textsuperscript{+} cells...
that were positive for smooth muscle cell marker α-smooth muscle actin (Figure VII in the online-only Data Supplement). Although the c-kit+ cells may not be directly committed to myogenesis in our model, the data suggest that juvenile doxorubicin exposure affects differentiation of cardiac progenitor cells into myocytes and endothelial cells but not into smooth muscle cells.

In contrast to the adult heart, the juvenile heart contains a large population of cardiac progenitor cells. To investigate whether doxorubicin treatment had an effect on cardiac progenitor cells in the juvenile heart, heart sections from mouse pups at 12 days of age were stained for the presence of c-kit. We found that doxorubicin-exposed mice had significantly fewer c-kit+ cells (Figure 7A and 7B), suggesting that doxorubicin might be harmful to cardiac progenitor cells. Hearts from saline-injected mice contained many clusters of c-kit+ cells, which are thought to reflect intense expansion of progenitor cells. In contrast, hearts of doxorubicin-injected pups had fewer clusters of cells positive for c-kit, suggesting that doxorubicin might inhibit proliferation of cardiac progenitor cells. The cell cycle inhibitor p16INK4a was significantly upregulated in c-kit+ cells in doxorubicin-exposed hearts at 12 days of age (Figure 7C and 7D). Cardiac progenitor cells isolated from hearts of doxorubicin-treated mice also incorporated less BrdU into the DNA in vitro compared with saline (Figure 7E), consistent with reduced proliferation. This suggests that juvenile doxorubicin treatment might have permanently reduced the number of resident progenitor cells in these hearts.

To further investigate the effect of doxorubicin treatment on proliferation of cardiac progenitor cells, we isolated c-kit+ cells from mouse hearts and treated them with doxorubicin for 72 hours. Treatment of cardiac progenitor cells with 10 or 100 nmol/L doxorubicin attenuated proliferation by 40% and 50%, respectively (Figure 8A). In parallel experiments, doxorubicin did not induce cell death at these concentrations, confirming that the reduced number of cells was not due to cell death (Figure 8B). Inhibition of proliferation was also confirmed by BrdU incorporation studies, in which fewer doxorubicin-treated cells incorporated BrdU into the DNA (Figure 8C). Although an equal number of cells were plated before treatment, wells containing cells treated with saline always had significantly more cells after 72 hours compared with doxorubicin-treated cells (Figure XIII in the online-only Data Supplement). Doxorubicin treatment also reduced telomerase activity (Figure 8D) and induced expression of the senescence marker p16INK4a (Figure 8E and 8F), suggesting that doxorubicin induces senescence in cardiac progenitor cells.

**Discussion**

Several laboratories have identified resident progenitor cells in the heart that have the capacity to differentiate into the various cardiac cells both in vitro and in vivo. These cells are thought to exist in the heart to facilitate growth during adolescence and to provide a mechanism for minor repair. Senescence and death of cardiac progenitor cells correlate with development of heart failure. That children are at higher risk for late-onset cardiotoxicity than adults could be explained by the fact that the heart is still growing
and very likely contains more cardiac progenitor cells than the adult myocardium. C-kit–positive cells are present in juvenile human hearts, and it was reported that c-kit–positive cells were more abundant in children with congenital heart disease and that this increase correlated with an increase in apoptotic myocytes. Thus, stress to the young human heart appears to induce mobilization of progenitor cells to the damaged myocardium. Our studies are currently limited to the mouse, but it will be important to verify our findings in human hearts.

Progenitor cells migrate into the border zone after MI, where they participate in myocardial regeneration, blood vessel formation, and remodeling. We found a reduction in the number of c-kit+ progenitor cells in juvenile mouse hearts after doxorubicin treatment, which correlated with fewer c-kit+ cells in the border zone of an infarct in the adults compared with saline-treated mice. These mice were more susceptible to MI, with greater infarcts and increased mortality. Interestingly, mice heterozygous for a defective c-kit receptor had a reduced number of c-kit+ progenitor cells in the infarcted heart, which also correlated with increased injury and mortality after MI. Clearly, functional c-kit+ progenitor cells are important for repairing a heart in response to stress such as MI. Our findings suggest that juvenile doxorubicin treatment has permanently reduced the number of cardiac progenitor cells and reduced their differentiation in response to injury. Thus, a minor ischemic event that would cause minimal or no damage in a healthy person with functional progenitor cells might result in more significant damage in a person with fewer progenitor cells migrating to and differentiating into cardiac cells at the site of injury. Accumulation of such stresses over time may be one of the factors that predispose children exposed to anthracyclines to late-onset cardiomyopathy. Interestingly, studies have reported that survivors of Hodgkin lymphoma were at increased risk of an infarction and had an increased risk of dying from it. These studies also found that the risks were greater in patients treated with anthracyclines when young than in patients who had been treated at older ages.

Doxorubicin induces senescence by reducing telomerase activity in many different cell types. Telomerase activity is also essential for proliferation of pluripotent stem cells and for certain tissue-specific self-renewing stem cells. Neural stem cells isolated from telomerase-deficient mice were incapable of expansion in vitro, suggesting that telomere function is important for proliferation of stem cells. Interestingly, these mice had a reduced number of resident neuronal stem cells that had impaired proliferation. Heart biopsies from old patients with heart failure showed that
c-kit+ progenitor cells had undergone senescence and substantial telomeric shortening, suggesting that these cells can no longer compensate for lost myocytes and therefore may exacerbate the development of heart failure.32 Thus, telomerase in cycling progenitor cells counters progressive shortening of the telomeres and promotes growth and survival of progenitor cells.

In this study, doxorubicin exposure had long-lasting effects on vascular development, resulting in a vascular tree with fewer ramifications, thereby limiting the capacity to respond to increased demand. This is not simply a developmental defect but also a reflection of limited progenitor capacity because neovascularization after MI was also impaired. Similarly, c-kit mutant mice had reduced angiogenesis after MI.34 Neovascularization in the border zone after an infarction is an important process to prevent the development of left ventricular remodeling and deterioration to heart failure. Our results suggest that juvenile doxorubicin treatment particularly affects the microvasculature and implies that childhood doxorubicin exposure puts the adult heart at greater risk for ischemic injury due to a barely adequate blood supply. Moreover, defective c-kit+ cells can affect the myofibroblast repair response after MI, which is important for stabilization of the scar.31 Because we observed a reduction in the formation of α-smooth muscle actin–positive cells in the border zone after MI in the doxorubicin-treated mice, it is possible that doxorubicin exposure has affected the function of the myofibroblasts.

Occasionally, the doxorubicin-treated mice died of cardiac rupture. This is likely attributable to insufficient angiogenesis, which is essential for the repair process and function of the fibroblasts. After an infarction, wound healing ensues, a complex process that involves inflammation, new tissue formation, and remodeling. A defect in infarct healing and remodeling can cause cardiac rupture.42 Moreover, endothelial progenitor cells are recruited to ischemic regions, improving neovascularization,43 and it is likely that doxorubicin reduces the pools of other progenitor cells, including endothelial progenitor cells. It is also possible that doxorubicin exposure has impaired the heart’s ability to produce and secrete homing factors that will attract progenitor cells to the site of injury or has made the heart a hostile environment that prevents implantation of circulating progenitor cells. This has important implications not only for anthracycline cardiomyopathy but for other childhood exposures that may affect progenitor cell pools.

Our model provides new insight into the mechanism of late-onset cardiotoxicity in mice. However, the similarity and relevance of this model to anthracycline-induced cardiomyopathy in humans are currently unknown and need to be determined. In addition, because the focus of this study was on late-onset cardiotoxicity, we limited this study to the heart, and we did not examine other tissues. Our findings suggest that antecedent doxorubicin exposure has lasting effects on the progenitor cells that participate in cardiac repair and neovascularization. Progenitor cells have therapeutic poten-
tial, and exogenously administered stem cells have been shown to successfully engraft and improve left ventricular function in animal models of MI. Thus, it will be important to explore whether replacing or protecting the cardiac progenitor cells will ameliorate late-onset anthracycline-induced cardiotoxicity.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Anthracyclines such as doxorubicin are effective chemotherapeutic agents used for treatment of many cancers. Unfortunately, their clinical use is limited by the risk of severe cardiotoxicity and heart failure that may not manifest until years later. Anthracyclines are of special concern in pediatric oncology because the youngest children are at the greatest risk of developing late-onset cardiotoxicity. In this study, we present the first evidence that anthracycline exposure in juvenile mice reduces the size of the cardiac progenitor pool, impairs their ability to differentiate into cells of cardiac and vascular lineages, and adversely affects vascular development in the heart. Despite normal development and hemodynamic function as adults, these mice develop rapid heart failure in response to physiological or pathological stress. This animal model recapitulates many of the features of late-onset anthracycline toxicity in humans and explains why younger children, who have more dividing progenitor cells, are more vulnerable. Our findings suggest that anthracycline-mediated effects on the progenitor cells might underlie the reduced ability to accomplish physiological hypertrophy as well as cardiac repair and neovascularization. Injection of stem cell mobilizing factors has been shown to enhance migration of bone marrow cells to the heart and to attenuate acute doxorubicin cardiotoxicity. The therapeutic potential of progenitor cells has been demonstrated in animal models in which successful engraftment of progenitor cells improved left ventricular function after myocardial infarction. If progenitor cells are required for neovascularization accompanying physiological hypertrophy or repair, then stem cell mobilization or replacement may ameliorate late-onset heart failure due to anthracyclines.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Isolation of adult mouse myocytes

Adult mouse myocytes were isolated as previously described with minor modification 1. Hearts were rapidly excised and cannulated via the aorta and connected to the perfusion apparatus. Hearts were perfused with calcium-free media containing Joklik-modified minimal essential medium (MEM), HEPES (10 mM), taurine (30 mM), DL-carnitine (2 mM), creatine (2 mM), 2,3 butanedione monoxime (BDM) (10 mM), and Liberase Blendzyme (Roche) and 20 μM calcium chloride at a rate of 3 ml/min for 15 min. After perfusion, atria were removed and ventricles cut into small pieces and triturated with a transfer pipette in Liberase Blendzyme solution. Myocytes were washed twice with heart medium containing 1% bovine serum albumin (BSA) and calcium chloride (20 μM). Calcium concentration were increased to 1.2 mM in 5-min intervals before the myocytes were plated on dishes coated with 10 μg/L laminin for 2 hrs.

Assessment of Cardiac Function

Hemodynamic function was performed in 15 animals per group (saline vs. doxorubicin) under non-stressed conditions at 8-12 weeks of age, 11 saline-injected (sedentary n=5, swimming n=6), and 13 doxorubicin-exposed mice (sedentary n=8, swimming n=5). Cardiac function was assessed in vivo in anesthetized mice by inserting a Millar microtip catheter into the carotid artery. Baseline functional measurements including heart rate (HR) and developed pressure (DP) were determined.

Microsphere Blood Flow Measurements

The measurement of blood flow has been described previously 2. Briefly, 8 mice (saline n=4 and DOX n=4) were initially anesthetized with a mixture of ketamine (0.07 mg/g) and xylazine (8
µg/g). Anesthesia was maintained throughout the experiment with 0.5% isoflurane balanced with 1 L/min of oxygen. 18,000 fluorescent microspheres (10 µm size, Invitrogen) were injected into the left ventricle at a flow rate of 200 µl/min for 15 sec. At the same time of microsphere injection, a reference blood sample was withdrawn from the femoral artery at 0.2 ml/min. Blood and heart samples were collected and digested with 4N KOH for 48 hours according to the manufacture’s protocol (Invitrogen). To elute the dye, the hydrolysate containing the digested tissue/microsphere was filtered with 3 µm membrane and placed in 1 ml of cellosolve acetate for 8 hours. Fluorescence in each sample was measured in a 96-well plate reader. Relative tissue blood flow rate was calculated using following formula:

\[ F_i = \frac{(I_i)(R)}{I_{ref}} \]

Where \( F_i \) is blood flow in the sample, \( I_i \) and \( I_{ref} \) are the intensity of fluorescence in sample and reference blood, \( R \) is the withdrawal rate of reference blood sample.

**Microfil Injection and Coronary Cast**

The microfil injection was based on the protocol described by Kaneko et al. 3. Corrosion casts were prepared and processed according to protocols in Batson’s No. 17 plastic replica and corrosion kit (Polysciences Inc.). The hearts (saline n=3 and DOX n=3) were placed into PBS overnight and then dehydrated in serial ethanol and cleared in methyl salicylate.

**Western Blotting**

Hearts from 8 mice (saline n=4 and DOX n=4) were harvested at days 21, 40, and 60 after birth. Hearts were homogenized in ice-cold Lysis Buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and complete protease inhibitor cocktail (Roche). Lysates were incubated on ice for 30 min and then cleared by centrifugation at 20,000 x g for 20 min. The protein concentration of the supernatants were determined by the Coomassie Blue
binding assay (Pierce Chemical Co) with BSA standards. Equal amounts of proteins were loaded and separated on SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting using monoclonal antibodies against VEGF (Santa Cruz Biotech.) and tubulin (Sigma). VEGF was normalized against tubulin and data are presented as fold decrease compared to saline control.

**ELISA**

Blood was collected from mice upon sacrifice and then spun at 2500 x g for 20 min to obtain the serum. A sandwich ELISA was performed on the serum samples using Quantitative Mouse ELISA Kit (100-230-VEM, Alpha Diagnostic International, San Antonio, TX). The serum was incubated overnight in microtiter wells to allow binding to the immobilized VEGF antibody. A second VEGF antibody conjugated to biotin was added for 60 minutes followed by 4 washes. Next, a streptavidin horseradish peroxidase conjugate was added for 30 minutes followed by 5 washes. The levels of VEGF were measured by obtaining readings at OD_{450\text{nm}}. Values were plotted against a standard curve of purified recombinant mouse VEGF.

**Histological Analysis and Immunostaining**

Hearts were fixed for histological analysis in 4% formaldehyde, embedded in paraffin and sectioned. 4 μm thick sections were stained with hematoxylin and eosin (H & E) or Masson's trichrome according to standard protocols. For immunostaining, tissue sections were rehydrated and then microwaved on 50% power for 15 min in 10mM citrate buffer (pH 6.0) for antigen retrieval. After endogenous tissue peroxidase activity was quenched in 3% H_{2}O_{2} for 20 min, samples were blocked with TN buffer (TNB) supplied in amplification kit (Perkin/Elmer) and incubated with primary antibodies overnight at 4° C (c-Kit: R&D; p16: Santa Cruz Biotechnology; Tropomyosin, Sigma; α-smooth muscle actin and CD31; Abcam: Flk1: Invitrogen; GATA4: Santa Cruz; MEF2C: Aviva).
Sections were washed 5 times for 5 min before incubation of secondary antibody diluted in TNB. Tyramide was used to amplify the c-kit signal according to the manufacturer’s protocol. Endothelial cells stained with anti-CD31 were detected with Vectastain ABC kit peroxidase. Tissue sections and cells were observed through a Nikon TE300 fluorescence microscope (Nikon) equipped with a cooled CCD camera (Orca-ER, Hamamatsu). 7 fields per heart were assessed in every experiment, selecting epi/mid myocardium and endo/mid myocardium for 3 quadrants plus mid myocardium for septum. For MI experiments, 7 fields were assessed in the border zone of the infarct.

**Transmission Electron Microscopy**

Hearts were fixed *in vivo* in 4% paraformaldehyde + 1.5% glutaraldehyde in 0.1M cacodylate buffer. The hearts were removed, each sliced longitudinally into 2 pieces for continued immersion fixation in the same fixative for 6hrs on ice. The left and right ventricles were then sliced into long strips for further overnight fixation in 3% glutaraldehyde in 0.1M cacodylate overnight at 4°C. The tissue pieces were washed in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer and dehydrated in a graded ethanol series. The tissue was then treated with propylene oxide as the transition solvent before embedding in Epon/Araldite (Electron Microscopy Sciences (Hatfield PA). Thick sections were cut and stained with toluidine blue for general tissue assessment. Thin sections (60nm) were cut with a diamond knife (Diatome, Hatfield PA), mounted on copper slot grids coated with parlodion and subsequently stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR). Images were documented using a Megaview III ccd camera (Olympus Soft Imaging Solutions, Lakewood CO). Images were then handled in Adobe Photoshop.
MTT assay

An equal number of cells were plated in a 96 well plate one day prior to treatment with vehicle or doxorubicin. Proliferation was assessed by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide (MTT) to formazan by viable cells. Cells were incubated with MTT solution (5 mg/mL in PBS) for 4 h, the formazan (blue crystals) were solubilized in 10% SDS, 0.01 N HCl o/n, and MTT reduction was measured the next day by absorbance at 570 nm in a plate reader. Absorbance values that were lower than control cells indicate a reduction in the rate of proliferation.

TUNEL Staining and Cell Death Assay

TUNEL staining to label fragmented DNA in heart sections was performed using the In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. Nuclei were counterstained with Hoechst 33342 (Molecular Probes). TUNEL positive nuclei were counted in 10 randomly selected fields of each condition and expressed as a percentage of the total number of nuclei. Cells were treated with vehicle or doxorubicin for 72 h and cell death was measured by Trypan blue dye uptake using a bright-field microscopy. Living cells exclude the dye, whereas dead cells take up the blue dye. Each condition was set up in triplicate wells and a minimum of 300 cells/well were counted.

BrdU Pulse Chase Experiment

C-kit positive cells were isolated from 12 day old mice which had received injections of saline (n=4) or 1 mg/kg doxorubicin (n=4) at day 5 and 10. The cells were plated overnight and then labeled with 10 μM BrdU (Sigma) for 6 h. Alternatively, cultured c-kit+ cells (passage 2-3) isolated from untreated mice were treated with saline or 100 nM doxorubicin for 24 h before pulse-labeling with BrdU. After 6 h, the cells were washed to remove unincorporated BrdU, and then fixed in cold 70% ethanol for 30 min. Cells were incubated in 2 N HCl for 20 min, washed in 0.1 M sodium borate (pH
8.5) for 2 min, followed by a wash in PBS for 5 min. Cells were stained with anti-BrdU (Sigma) and with a secondary Alexa 594 antibody (invitrogen). Nuclei were counterstained with Hoechst 33342.

**Telomerase Activity Assay**

Telomerase activity was measured in extracts prepared from cultured progenitor cells treated with 100 nM DOX for 72 h and 96 h. Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP) using the *Telo*TAGGG PCR ELISAPLUS kit (Roche Molecular Biochemicals) according to the manufacturer’s instruction.
**Figure S1.** Weight of mice after injections with saline or doxorubicin (n=10).
Figure S2

A. HW/BW ratio of non-stressed mice (n=10).

B. Cross sectional measurement of myocytes.

C. Bright field images of myocytes isolated from adult Saline and DOX hearts.

Figure S2. A. HW/BW ratio of non-stressed mice (n=10). B. Cross sectional measurement of myocytes. C. Bright field images of myocytes isolated from adult Saline and DOX hearts.
Figure S3. A. Representative H & E staining of vessels in heart sections prepared from adult mice that were treated with saline or DOX as juveniles. There were no obvious differences in the structure of vessels. B. Transmission electron micrograph of vessel in adult saline and DOX hearts.
Figure S4. Plasma levels of VEGF in saline and DOX treated mice were determined by ELISA (n=4).
Figure S5. Staining of CPCs for markers of endothelial cell lineage. A. Co-localization of c-kit+ and Flk1 or CD31 in heart sections from saline and DOX mice 7 days after the myocardial infarction. B. Quantitation of c-kit+ cells positive for Flk1 or CD31 (n=3).
Figure S6. Staining of CPCs for markers of myocyte cell lineage. **A.** Co-localization of c-kit+ and GATA4 or MEF2C in heart sections from saline and DOX mice 7 days after the myocardial infarction. **B.** Quantitation of c-kit+ cells positive for GATA4 or MEF2C (n=3).
Figure S7. Staining of CPCs for markers of smooth muscle cell lineage. 

A. Co-localization of c-kit+ and α-smooth muscle actin (α-SMA) in heart sections from saline and DOX mice 7 days after the myocardial infarction. 

B. Quantitation of c-kit+ cells positive for α-SMA (n=3).
Figure S8. An equal number of cells were plated on 35 mm plates, treated with 100 nM doxorubicin for 72 h, and then counted on a hematocytometer (n=4).
SUPPLEMENTAL REFERENCES

