See related Commentary on page 1586

The American Journal of Pathology, Vol. 176, No. 4, April 2010

DOI: 10.2353/ajpath.2010.091071

Cardiovascular, Pulmonary and Renal Pathology

Adriamycin Nephropathy

A Failure of Endothelial Progenitor Cell-Induced Repair

Kaoru Yasuda,* Hyeong-Cheon Park,* Brian Ratliff,* Francesco Addabbo,*† Antonis K. Hatzopoulos,‡ Praveen Chander,§ and Michael S. Goligorsky*

From the Renal Research Institute, Division of Nephrology, Departments of Medicine and Pharmacology, and the Department of Pathology,§ New York Medical College, Valhalla, New York; the Department of Pharmacology,† University of Bari, Italy; and the Department of Medicine, Division of Cardiovascular Medicine and the Department of Cell & Developmental Biology,‡ Vanderbilt University, Nashville, Tennessee*

Adriamycin-associated nephropathy (AAN) remains poorly understood. We hypothesized that adriamycin affects endothelial progenitor cells (EPCs), leading to impaired regeneration. We analyzed renal hematopoietic stem cells (HSCs) and EPCs in mice with AAN and examined the potential contribution of adoptive transfer of intact EPCs to the repair processes. FACS analyses revealed that populations of HSCs and EPCs were scarcely represented in control kidneys and did not change numerically in kidneys obtained from mice with AAN. The observed defect in engraftment was attributable to the decreased viability and increased senescence of EPCs. Adoptive transfer of intact EPCs improved proteinuria and renal function, with a threefold decrease in mortality. Infusion of EPCs to adriamycin-treated mice reduced plasma levels of interleukin-1α and -β and granulocyte-colony stimulating factor as well as increased the level of vascular endothelial growth factor with concomitant improvement of vascular density and reduction of apoptosis. An additional mechanism of tissue repair is proposed based on tunneling nanotube formation between EPCs and endothelial cells exposed to adriamycin, leading to the multiple rounds of exchange between EPCs and mature cells. In conclusion, AAN is associated with development of EPC incompetence; adoptive transfer of intact EPCs blunts morphological and functional manifestations of AAN; and the proposed mechanisms of repair by EPCs include direct incorporation into blood vessels, paracrine signaling, and tunneling nanotube renewal of mitochondrial pool in endothelial cells. ***(Am J Pathol 2010, 176:1685–1695; DOI: 10.2353/ajpath.2010.091071)***

Molecular pharmacology profile of anthracycline antibiotic Adriamycin (Doxorubicin) includes inhibition of nucleic acid synthesis and cytochrome *c* oxidase, intercalation of DNA, and generation of reactive oxygen species, which account not only for its oncolytic effects but also for the depression of the bone marrow and development of cardiomyopathy and nephropathy.[1–3] While cardiotoxicity is a major limiting factor in the use of this chemotherapeutic agent, adriamycin-associated nephropathy (AAN) contributes significantly to its toxicologic profile. Toxicity of anthracyclines in general is poorly understood.[2] AAN has been variably attributed to complement activation, increased production of reactive oxygen species, reduction in heparan sulfate and increased heparanase expression in glomeruli, and dysregulation of renin-angiotensin system,[4,5] as well as activation of p38 MAP kinase and TGF-beta1/Smad signaling,[6] among other proposed mechanisms. It is instructive that the kidney-resident side population cells, capable of multilineage differentiation, as well as the main population cells (devoid of side-population cells) adoptively transferred to mice with AAN resulted in the reduction of proteinuria.[7] These studies raised a legitimate question whether adriamycin affects not only the bone marrow hematopoietic stem cells (HSCs), but also bone marrow–derived and

Supported in part by National Institutes of Health grants DK54602, DK052783, and DK45462 (to M.S.G.) and Westchester Artificial Kidney Foundation.

Accepted for publication December 10, 2009.

K.Y. and H.-C.P. contributed equally to this study.

Current address for H.-C.P.: Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea; for F.A.: Department of Pharmacology, University of Bari, Italy.

Address reprint requests to Michael S. Goligorsky, M.D., Ph.D., Renal Research Institute, Division of Nephrology, Departments of Medicine and Pharmacology, New York Medical College, Valhalla, NY 10595. E-mail: Michael_goligorsky@nymc.edu.

renal-resident stem and/or endothelial progenitor cells and whether this injury may provide explanation for the progressive nature of AAN. Here, we analyzed quantitatively and qualitatively stem and endothelial progenitor cells, (consensually characterized as HSCs based on the co-expression of surface markers CD150 and CD117 [c-Kit] or endothelial progenitor cells [EPCs] based on the co-expression of surface markers CD34 and Flk-1 with or without CD45 expression) present in the kidneys of mice with AAN and examined the potential contribution of adoptive transfer of intact endothelial progenitor cells to the repair processes.

Materials and Methods

Animals and Induction of AAN

All animal protocols were conducted in accord with the National Institutes of Health guidelines and were approved by the Institutional Animal Care Committee. Male 8- to 12-week-old BALB/c mice (Jackson Labs, Bar Harbor, ME) were housed under 12-hour light:dark cycle, fed a regular chow, and received water *ad lib*. Animals received tail vein injection of 10.2 mg/kg adriamycin to induce AAN. An additional group of mice was treated with EPCs by adoptive transfer of approximately 5×10^5 cultured EPCs injected into the circulation via the tail vein on the day 5 postadriamycin. Nontreated mice received an injection of normal saline. On days 0, 4, and 10 and at 2 and 3 weeks, mice were placed in metabolic cages for 24-hour urine collection. Mice were sacrificed at 3 weeks after adriamycin injection. On the day of sacrifice, after Ketamin/Xylazine anesthesia, blood was obtained through left ventricular puncture and animals were perfused with normal saline followed by perfusion-fixation with 4% paraformaldehyde for morphological studies. Alternatively, fixation step was omitted and mice were used for stem cell isolation, as detailed below.

Cell Isolation from the Kidney and FACS Analysis

For stem cell isolation, single-cell suspension was prepared from the whole kidney. Kidneys from each experimental group were placed in 2 ml of ice-cold RPMI 1640 (Invitrogen, Carlsbad, CA) and minced using a sterile scalpel. Digestion of the tissue was performed in collagenase II (Invitrogen) solution (1 mg/ml of RPMI 1640) for 30 minutes at 37°C in 5% CO_2. Cell suspensions were passed through a 35-μm nylon sieve. Repeated digestions were performed until microscopic evaluation showed a suspension of single cells. Finally, cells were washed in PBS-BSA 1% (w/v), counted, and kept on ice in the dark.

FACS analysis was performed to quantify the dynamics of EPCs and HSCs in AAN model. For this analysis, 1×10^6 cells from the single-cell suspensions were incubated with specified primary antibodies for 1 hour at 4°C in the dark. The following antibodies were used for incubation: FITC-conjugated anti-mouse CD34, PE-conjugated anti-mouse Flk-1, PE-conjugated anti-mouse CD150, FITC-conjugated anti-mouse CD117 (c-Kit; BD Pharmingen, San Diego, CA). After each incubation step, cells were washed with PBS-BSA 1% (w/v) and finally fixed in 1% paraformaldehyde. Data were acquired using a FACScan cytometer equipped with a 488-nm argon laser and a 635-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The set-up of FACScan was performed using unstained cells. For quantification of EPCs and HSCs, the number of CD34/Flk-1 and CD150/c-Kit double-positive cells within the monocytic cell population was counted.

Preparation of EPCs and Cell Culture

To isolate bone marrow mononuclear cells, cells were obtained by flushing the tibias and femurs of BALB/c mice with PBS and density gradient centrifugation with Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) was performed. Bone marrow mononuclear cells were cultured in Mouse Endothelial Progenitor Cell Culture Serum-Free Media (Celprogen, San Pedro, CA) on dishes coated with 10 μg/ml pronectin (Sigma, St Louis, MO). After 3 days in culture, nonadherent cells were removed, and medium exchanged every 2 days. Thus prepared cells were further characterized to ensure the purity of EPC population (>95% of cells are labeled by these markers) by (1) uptake of DiI-labeled acetylated low-density lipoprotein (2.4 μg/ml of Dil-Actylated-LDL, Biomedical Technologies, Inc., Stoughton, MA), and (2) Lectin binding (25 μg/ml of Fluorescein-*Ulex Europeus* Lectin, Biomeda Corp., Forster City, CA). Colony-forming unit assay was performed according to the previously described protocol.[8] Briefly, 1×10^5 bone marrow mononuclear cells were plated on pronectin-coated dishes and 2 weeks later colonies (>50 cells) were counted. Cells were also stained for the expression of CD31. In some *in vitro* experiments, mouse embryonic EPCs, previously established and characterized,[9] were used.

To detect apoptotic and necrotic cells, FACS analysis using fluorescein isothiocyanate-Val-Ala-Asp (OMe)-fluoromethylketone (FITC-VAD-FMK, Calbiochem, La Jolla, CA) and 7-Aminoactinomycin D (7-AAD, Invitrogen) was performed. Detection of cell senescence was accomplished by staining for senescence-associated β galactosidase (SA-β-gal).

Morphological Analyses

Kidneys were collected from mice at 3 weeks after adriamycin injection for morphological analysis. Midcoronal kidney sections were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections (4 μm thick) were stained with hematoxylin and eosin, periodic acid-Schiff, and Masson trichrome and examined by a nephropathologist blinded to the origin of individual preparations. Semiquantitative grading of injury, designed to evaluate the degree of glomerular injury (segmental sclerosis, podocyte hypertrophy, and proliferation) and tubulointerstitial injury (tubular casts, debris, necrosis, and