Hematopoietic cells are often exposed to transient hypoxia as they develop and migrate between blood and tissues. We tested the hypothesis that hypoxia-then-reoxygenation represent a stress for hematopoietic progenitor cells. Here we report that reoxygenation-generated oxidative stress induced senescence, tested as staining for SA-β-galactosidase (SA-β-gal), of bone marrow progenitor cells. Reoxygenation induced significant DNA damage and inhibited colony formation in lineage-depleted bone marrow cells enriched for progenitor cells. These reoxygenated cells exhibited a prolonged G0/G1 accumulation without significant apoptosis after 24 h of treatments. Reoxygenated bone marrow progenitor cells expressed SA-β-gal and senescence-associated proteins p53 and p21WAF1. Reoxygenated Fanc−/− progenitor cells, which underwent significant apoptosis and senescence, tested as staining for SA-β-gal, also expressed p16INK4A. Suppression of apoptosis by the pan-caspase inhibitor benzylxycarbonyl-VAD-fluoromethyl ketone dramatically increased senescent Fanc−/− progenitor cells. Senescence induction, tested as staining for SA-β-gal, in reoxygenated progenitor cells was closely correlated with extent of DNA damage and phosphorylation of ATM at Ser-1981 and p53 at Ser-15. Moreover, inhibition of ATM signaling reduced SA-β-gal positivity but increased apoptosis of reoxygenated progenitor cells. Thus, these results suggest that the ATM/p53/p21 pathway influences cell fate decision between apoptosis and senescence in reoxygenated hematopoietic progenitor cells.

The ATMP53/p21 Pathway Influences Cell Fate Decision between Apoptosis and Senescence in Reoxygenated Hematopoietic Progenitor Cells*

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The abbreviations used are: NAC, N-acetyl-L-cysteine; 2-AP, 2-amino purine; ATM, ataxia-telangiectasia mutated; siATM, small interfering ATM; HSC, hematopoietic stem cell; FA, Fanconi anemia; siRNA, small interfering RNA; β-gal, β-galactosidase; BM, bone marrow; Lin−, lineage-negative; LSK, Lin−Sca-1−c−kit+; WT, wild type; Z, benzylxycarbonyl; FMK, fluoromethyl ketone; FITC, fluorescein isothiocyanate; CFC, colony-forming cell; PE, phycoerythrin; APC, allophycocyanin.

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Reoxygenation Induces Hematopoietic Cell Senescence

Fig. 1. Reoxygenation-generated oxidative stress induces DNA damage and growth inhibition in BM progenitor cells. A, KSL cells were incubated in 20% O₂ (control) or first subjected to hypoxia (1% O₂) for 4 h then reoxygenation (Reoxy) (20%). The cells were then incubated in 20% O₂ for 48 h. The NAC cultures (Reoxy+NAC) were the same as the reoxygenated cells except that the medium contained NAC (1 mM). A, representative images of the comet assays used to analyze DNA strand breaks. Numbers below the images are DNA damage quantified by determining the comet tail movement (increasing values represent increasing amounts of DNA damage). The mean tail moment of the WT cells without treatment (Control) is expressed as 100%. For each treatment, 30 cells were scored for tail moment from random sampling. Data reflect means ± S.D. of three independent experiments. B, untreated (Control) or reoxygenated WT and Fanc−−/− BM KSL cells were evaluated for CFC activity at day 7. Data represent the number (mean ± S.D.) of total number of colonies from three independent experiments. *, statistical significance between paired samples at p < 0.05. C, untreated (Control) or reoxygenated WT and Fanc−−/− BM Lin− cells were stained with lineage marker antibodies (biotin-conjugated) along with Sca-1-PE and c-kit-APC antibodies and then with annexin V. Percentages of apoptosis in the KSL population were analyzed by flow cytometry. D, untreated (Control) or reoxygenated WT and Fanc−−/− BM Lin− cells were analyzed for cell cycle distribution at 24 h after reoxygenation. Shown are representative flow cytometric presentations of three independent experiments. Numbers in plots indicate percent of cells in G₁, S, G₂₅, M phases. E, untreated (Control) or reoxygenated WT and Fanc−−/− BM cells were transplanted into sublethal irradiated NOD/SCID mice along with 1 × 10⁶ irradiated competitor cells. BM cells from the transplanted mice were stained with H2kb-PE (for donor-derived cells) and H2kd-FITC (for recipient-derived cells) antibodies (Pharmingen) and analyzed by flow cytometry to detect donor-derived hematopoietic progenitors.

Apoptosis Assay—Aliquots of 1 × 10⁶ BM Lin− cells were stained with Sca-1-PE and c-kit-APC antibodies followed by annexin V staining. These experiments also included PE and APC isotype controls, and Fcγ receptor- and scavenger receptor-negative controls. Apoptosis was therefore analyzed in different populations of Lin− cells by flow cytometry.

Clonogenic Progenitor Cell Assays and BM Transplantation—BM LSK cells were subjected to hypoxia-reoxygenation with or without 2-AP and cultured in a 35-mm tissue culture dish in 4 ml of semisolid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/ml stem cell factor, 10 ng/ml interleukin-3, 100 ng/ml granulocyte-colony-stimulating factor, and 4 units/ml erythropoietin. Colonies (colony-forming cells (CFCs)) were counted on day 7. To evaluate the effect of reoxygenation on the repopulation ability of the BM progenitor cells, we used a NOD/SCID repopulation assay. NOD/SCID mice (Jackson Laboratories) were handled under sterile conditions and maintained under microisolators. WT or Fanc−−/− BM (2 × 10⁶) cells were transplanted by tail vein injection into sublethal irradiated (3.5 Gy) 8-week-old mice along with 5 × 10⁵ competitor cells. BM cells from the transplanted mice were stained with H2kb-PE (for donor-derived cells) and H2kd-FITC (for recipient-derived cells) antibodies (Pharmingen) and analyzed by flow cytometry to detect donor-derived hematopoietic progenitors.

SRNA and Assays for DNA Damage and Senescence—The siRNA oligonucleotides targeting nucleotides 8111–8131 of mouse ATM mRNA (GeneBank™ sequence accession number NM007499; GGT-GACTATAAAATCATTTAA) were cloned in the pSM2c retroviral vector (Open Biosystems). Infected cells were selected for puromycin resistance. The generation of DNA strand breaks in control and reoxygenated BM LSK cells was assessed by the single cell gel electrophoresis (comet) assay.
were subjected to reoxygenation for 48 h and stained for SA-β-gal. Sca-1+ KSL cells were incubated in the presence of the pan-caspase inhibitor Z-VAD-FMK (100 μM) and stained for SA-β-gal. Values represent mean ± S.D. of three experiments. *, statistical significance between paired samples at *p* < 0.05.

**RESULTS AND DISCUSSION**

**Reoxygenation-generated Oxidative Stress Induces DNA Damage and Inhibits Colony Formation in BM Progenitor Cells**—Because reoxygenation represents oxidative stress to the cell and oxidative stress induces DNA damage (2, 4, 6), we first sought to determine whether hypoxia-thenreoxygenation caused DNA damage in BM progenitor cells. Analysis of DNA strand breaks by comet assay revealed that there was increased accumulation of DNA damage in reoxygenated Lin-Sca-1+ c-kit+ (KSL) BM cells compared with untreated counterparts, respectively (Fig. 1A). The Fancc-deficient mice have a profound defect in the hematopoietic stem and progenitor cell compartment, and FA HSCs and progenitors have been shown to be hypersensitive to a variety of stresses including oxidative stress (8, 12, 13). Consistent with these observations, reoxygenated Fancc−/− KSL cells induced significant (3.8-fold) more DNA strand breakage than reoxygenated WT KSL cells (Fig. 1A). Treatment of reoxygenated WT or Fancc−/− KSL cells with the anti-oxidant NAC completely abrogated the effect (Fig. 1A), suggesting that the DNA damage was generated by oxidative stress. The number of CFUs derived from both reoxygenated WT and Fancc−/− BM progenitors was significantly decreased compared with their untreated counterparts, and NAC completely restored the progenitor activity of these reoxygenated KSL cells (Fig. 1B).

**Reoxygenated BM Progenitor Cells Undergo Growth Arrest and Have Reduced BM Repopulating Ability**—While reoxygenation induced more apoptosis in Fancc−/− BM KSL cells than in WT KSL cells shortly after exposure to high oxygen, apoptotic cells decreased thereafter (Fig. 1C). In addition, the extent of the increase was not as significant as reoxygenation-generated DNA damage at 24 h post-reoxygenation (compare Fig. 1A). Actually, apoptosis decreased to basal level 48 h post-reoxygenation (Fig. 1C). Thus, apoptosis is not the major consequence of the DNA damage. We therefore evaluated the cell cycle profile of these BM cells. Reoxygenated BM cells clearly exhibited a prolonged G0 + G1 accumulation (Fig. 1D), suggesting that there might exist an overactivated G0/G1 checkpoint in these BM progenitor cells. The marrow repopulating ability of reoxygenated progenitors was assessed by transplanting equal numbers of either untreated (control) or reoxygenated progenitors into sublethally irradiated NOD/SCID recipients. Engraftment was evaluated 4 weeks after transplantation by flow cytometric determination of donor-derived cells (H2kb+) in BM cell suspensions of the bone marrow harvested from recipient animals. The bone marrow of animals that received transplants of reoxygenated WT or Fancc−/− cells showed 2- or 3-fold lower engraftment than the untreated counterparts, respectively (Fig. 1E). Consistent with the observations of others (10), Fancc deficiency impairs the repopulating ability of Fancc−/− BM progenitors.

**Cell Fate Choice between Senescence, Tested as Staining for SA-β-gal, and Apoptosis in Reoxygenated BM Progenitor Cells**—Because reoxygenated BM progenitor cells underwent G0/G1 arrest, we reasoned that stress-induced senescence might be one fate of these cells. To examine this possibility, we stained WT and Fancc−/− BM KSL cells for SA-β-galactosidase, a biomarker for senescence (14). Nearly 20% of the reoxygenated WT KSL cells and more than 40% of the reoxygenated Fancc−/− KSL cells stained positive for SA-β-galactosidase activity after 48 h of reoxygenation (Fig. 2A).

Because we observed reoxygenation-induced apoptosis, especially in Fancc−/− BM progenitor cells, we asked whether blockage of apoptosis would increase senescence, tested as staining for SA-β-gal, in these reoxygenated BM cells. Indeed, when these reoxygenated BM KSL cells were incubated in the presence of the pan-caspase inhibitor Z-VAD-FMK, more than 60% of the Fancc−/− cells entered senescence, tested as staining for SA-β-gal, compared with ~40% without the apoptotic inhibitor (Fig. 2B). Therefore, reoxygenated Fancc−/− BM progenitor cells blocked for apoptosis may be prone to developing senescence.
Reoxygenation-induced Senescence, Tested as Staining for SA-β-gal, in BM Progenitor Cells Involves the ATM/p53/p21WAF1 Pathway—Because reoxygenation induces DNA damage and subsequent p53 activation, which is dependent on the ATM kinase (4, 5), we asked whether reoxygenation-induced senescence, tested as staining for SA-β-gal, in BM progenitor cells involves the ATM/p53/p21WAF1 pathway. We examined the reoxygenation-induced phosphorylation of ATM (Ser-1981)
and p53 (Ser-15) and expression of p21 in BM KSL cells. ATM autophosphorylation at Ser-1981 activates the kinase and is largely responsible for phosphorylating p53 at Ser-15 in response to DNA damage (15, 16). We found ~20% each of ATM<sup>Ser-1981</sup>- and p53<sup>Ser-15</sup>-positive (Fig. 3, A and B) in reoxygenated WT BM KSL cells. In reoxygenated Fancc<sup>-/-</sup> BM KSL cells, the intensity and percentages of cells stained positive for ATM<sup>Ser-1981</sup> and p53<sup>Ser-15</sup> increased significantly (Fig. 3, A and B). We also found that higher levels of p21-positive (22%) stained cells were present in reoxygenated WT BM KSL cells compared with untreated WT cells. The percentage of p21-positive cells increased to 66% in those FA BM KSL cells (Fig. 3, A and B).

To provide evidence that there is a link between activation of the ATM/p53/p21 pathway and senescence, we wanted to know whether blockage of ATM signaling inhibited reoxygenation-induced senescence, tested as staining for SA-β-gal, in BM progenitor cells. It is known that inhibition of ATM can relieve senescent cell cycle arrest (17). We used both siRNA and the kinase inhibitor 2-AP, which has been shown to suppress ATM inhibition of ATM activity increased apoptosis in reoxygenated WT cells but did not have detectable effect on Fancc<sup>-/-</sup> BM KSL cells (Fig. 3, F). Expression of siATM or 2-AP treatment reduced p16 expression in reoxygenated WT cells but did not have detectable effect on Fancc<sup>-/-</sup> BM KSL cells (Fig. 3, F), indicating that reoxygenation-induced p16<sup>INK4A</sup> expression in Fancc<sup>-/-</sup> BM KSL cells was independent of ATM activity. However, when we correlated ATM inhibition and p16<sup>INK4A</sup> expression with SA-β-gal activity, we found that reoxygenated WT BM KSL cells treated with 2-AP and siATM were almost devoid of SA-β-gal-positive cells (decreased from 14.6% to 2.5 and 3.3%, respectively; Fig. 3, D). In contrast, inhibition of ATM activity did not significantly reduced SA-β-gal staining in reoxygenated Fancc<sup>-/-</sup> BM KSL cells, which expressed high levels of p16<sup>INK4A</sup> (Fig. 3, F).

In summary, we have shown that 1) reoxygenation-generated oxidative stress induced DNA damage and G<sub>0</sub>/G<sub>1</sub> arrest in BM progenitor cells without significant apoptosis; 2) reoxygenation induced senescence, tested as staining for SA-β-gal, in BM progenitor cells; 3) induction of senescence, tested as staining for SA-β-gal, in reoxygenated BM progenitor cells closely correlated with the extent of DNA damage and phosphorylation of ATM at Ser-1981 and p53 at Ser-15; and 4) inhibition of ATM signaling reversed reoxygenation-induced senescence, tested as staining for SA-β-gal, but increased apoptosis in BM progenitor cells. Thus, these results suggest that reoxygenation induces senescence in hematopoietic progenitor cells.
cells through the ATM/p53/p21 pathway. These findings are especially relevant to the survival and maintenance of hematopoietic progenitor cells that are often exposed to transient hypoxia in vivo, and since hematopoietic stem/progenitor cell depletion is the major cause of BM failure occurred in aplastic anemia including FA, to the molecular etiology of BM diseases.

We demonstrated that the ATM kinase played a major role in transducing the reoxygenation-induced DNA damage signal in BM progenitor cells. Reoxygenation can generate oxidative stress, which can damage DNA. It is known that ATM transmits the signal of DNA damage induced by oxidative stress. For instance, oncogenic insults promote the accumulation of reactive oxygen species, resulting in DNA damage and apoptosis by a p53-dependent pathway (21–23). More recently, ATM has been shown to play an essential role in transmitting DNA damage signals generated by reoxygenation, through phosphorylation of p53Ser-15 (4, 5). We used siRNA targeting ATM and the protein kinase inhibitor 2-AP to investigate the involvement of ATM in DNA damage response of reoxygenated BM progenitor cells. Inhibition of ATM signaling resulted in reduction of ATMSer-181, p53Ser-15, and p21 expression and reversal of senescence, tested as staining for SA-β-gal, in reoxygenated BM progenitor cells but sensitized these BM cells to apoptosis (Fig. 3). Our results thus indicate for the first time that senescence, tested as staining for SA-β-gal, induction in reoxygenated BM progenitor cells is regulated by the ATM/p53/p21 pathway. We propose the existence of distinct mechanisms for WT and Fancc−/− BM cells with regard to cell cycle arrest in response to DNA damage induced by reoxygenation-generated oxidative stress. Upon experiencing DNA damage, WT BM cells initially arrest cell cycle progression by ATM-dependent activation of the G1 checkpoint to gain time for DNA repair. The major pathway responsible for triggering the G1 checkpoint involves the activation of p53 by ATM, the p53-mediated induction of p21, and a reduction in the level of RB phosphorylation. If the DNA damage cannot be repaired in a timely manner, the cells undergo reversal senescence to prevent accumulation of genetic mutations until the damage has been repaired. In Fancc−/− BM progenitor cells, however, excessive DNA damage causes overactivation of the ATM kinase, resulting in a hyperactive G1 checkpoint. The arrested Fancc−/− BM cells enter senescence, while the DNA damage remains unrepaired. Inhibition of ATM in these FA cells thus likely results in bypass of G1 checkpoint and undergoing apoptosis.

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